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FAMILY RIVALRY IN THE TESTIS: RETINOBLASTOMA PROTEIN AND E2F TRANSCRIPTION FACTORS IN TESTICULAR DEVELOPMENT AND ADULT SPERMATOGENESIS

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Family rivalry in the testis: Retinoblastoma protein and E2F transcription factors in testicular development and adult spermatogenesis

ABSTRACT

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Disorders of male reproductive health are becoming increasingly prevalent globally. These defects, ranging from decreasing sperm counts to an increasing rate of infertility and testicular cancer, have a common origin in the early phases of testicular development, but the exact mechanisms that cause them remain unknown.

Testicular development and adult spermatogenesis are complex processes in which different cell types undergo mitosis, meiosis, differentiation and apoptosis. The retinoblastoma protein family and its associated E2F transcription factors are key regulators of these cellular events. In the present study, the functions of these factors in postnatal testicular development and adult spermatogenesis were explored using different animal models. In addition, a new application of flow cytometry to study testicular cell dynamics was developed.

An ablation of retinoblastoma protein in mouse Sertoli cells resulted in their cell cycle re-entry in adult testes, dedifferentiation and a severe spermatogenic defect. We showed that deregulated E2F3 contributed to these changes. Our results indicated that the E2F1 transcription factor is critical for the control of apoptosis in the developing postnatal testis. In the adult testis, E2F1 controls the maintenance of the spermatogonial stem cell pool, in addition to inhibiting apoptosis of spermatocytes.

In summary, this study elucidated the complex interdependencies of the RB and E2F transcription factor families in the control of postnatal testicular development and adult spermatogenesis. Furthermore, this study provided a new methodology for the analysis of testicular cells.

Keywords: testis, cell cycle, RB, E2F, flow cytometry

Emmi Rotgers

Retinoblastooma- ja E2F-proteiiniperheet kiveksen kehityksen ja spermatogeneesin säätelyssä

Fysiologian oppiaine ja Turun molekyylilääketieteen tohtoriohjelma (TUDMM), Biolääketieteen laitos, Lääketieteellinen tiedekunta, Turun yliopisto, Kiinamyllynkatu 10, 20520 Turku. Annales Universitatis Turkuensis, Medica-Odontologica.

TIIVISTELMÄ

Kivessyövän ja hedelmättömyyden lisääntyminen sekä miesten ehkäisypillerin puute ovat suuria haasteita miesten lisääntymisterveydelle maailmanlaajuisesti. Näiden ratkaisemiseksi tarvitaan enemmän tietoa kiveksen kehityksen ja siittiöntuotannon perusmekanismeista.

Kiveksen kehitys on monimutkainen prosessi, jonka aikana solun jakaantuminen, erilaistuminen ja solukuolema ovat tarkoin säädeltyjä sekä itusoluissa että kiveksen somaattisissa tukisoluissa. Retinoblastooma- ja E2F-proteiiniperheet ovat keskeisiä solusyklin säätelijöitä ja vaikuttavat näihin solun toimintoihin. Tutkimme retinoblastoomaproteiinin ja E2F- transkriptiotekijöiden toimintaa hiiren kiveksessä erilaisia poistogeenisiä hiirimalleja käyttäen ja kehitimme uusia menetelmiä kivestutkimusta varten.

Retinoblastoomaproteiini (RB) osoittautui välttämättömäksi Sertolin solujen toiminnalle aikuisessa kiveksessä. Ilman toimivaa RB:a Sertolin solut ensin erilaistuivat normaalisti, mutta nuorella aikuisiällä ne alkoivat jakaantua uudelleen, dedifferentoituivat ja ajautuivat vähitellen solukuolemaan. Samanaikaisesti ne eivät enää pystyneet tukemaan siittiönkehitystä ja hiiristä tuli hedelmättömiä. E2F3-transkriptiotekijän säätelyhäiriö aiheutti Sertolin solujen toimintahäiriön. Osoitimme, että E2F1-transkriptiotekijällä on monta roolia siittiönkehityksessä. Se sekä edisti, että esti apoptoosia ja sääteli ituradan kantasolupopulaatiota. Tässä työssä kehitimme lisäksi virtaussytometriamenetelmän, jolla voitiin tutkia kiveksen eri solutyyppien toimintaa kehityksen aikana nykyisiä menetelmiä tehokkaammin.

Tutkimuksemme on tuottanut uutta tietoa retinoblastooma- ja E2F-proteiiniperheiden välisistä monimutkaisista riippuvuussuhteista kiveksen kehityksessä ja aikuisen spermatogeneesissä.

Avainsanat: kives, solusykli, RB, E2F, virtaussytometria

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ABBREVIATIONS

ALC	adult Leydig cell
AMH	anti-Müllerian hormone
AR	androgen receptor
ATM	ataxia-telangiectasia mutated
BTB	blood-testis barrier
CCN	cyclin
CDK	cyclin-dependent kinase
CIS	carcinoma *in situ*
CNS	central nervous system
CXCL12	chemokine (C-X-C motif) ligand 12
CXCR4	chemokine (C-X-C motif) receptor 4
DAZL	deleted in azoospermia
DHEA	dehydroepiandrosterone
DHFR	dihydrofolate reductase
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
DSB	double strand break
DSD	disorders of sex development
E	embryonic day
E2F	E2 promoter targeting factor
ETV5	ets variant 5
FLC	fetal Leydig cell
FSC	forward scatter
FSH	follicle stimulating hormone
GATA4	GATA binding protein 4
GDNF	glial cell line derived neurotrophic factor
GFRα1	glial cell line derived neurotrophic factor family receptor alpha 1
GNIS	germinal neoplastic cells *in situ*
GnRH	gonadotrophin releasing hormone
hCG	human chorionic gonadotrophin
HDAC	histone deacetylase
HPG	hypothalamo-pituitary-gonadal
hpg	hypogonadal
HRP	horseradish peroxidase
Inha	inhibin alpha
Kit	kit oncogene
LH	luteinizing hormone
MAGE-B4	melanoma antigen family B4
MEF	mouse embryonic fibroblast
NGN3	neurogenin 3
NR5A1	nuclear receptor subfamily 5, group A, member 1, steroidogenic factor 1
OCT4	octamer-binding transcription factor 4
P	postnatal day
p107	retinoblastoma-like 1

p130	retinoblastoma-like 2
$p27^{Kip1}$	cyclin-dependent kinase inhibitor 1B
PGC	primordial gonocyte
pH2AX	S139-phosphorylated H2A histone family, member X
PI	propidium iodide
PLZF	promyelocytic leukaemia zinc finger protein
PTM	peritubular myoid cell
PPARγ	peroxisome proliferator activated receptor gamma
RA	retinoic acid
RB	retinoblastoma protein
RHOX5	reproductive homeobox 5
RUNX2	runt related transcription factor 2
s.cyte	spermatocyte
SCO	Sertoli cell-only
SC-RbKO	Sertoli cell-specific knockout of Rb
shRNA	short hairpin RNA
SMA	smooth muscle actin
SMAD4	SMAD family Member 4
SOX2	SRY (sex determining region Y)-box 2
SOX9	SRY (sex determining region Y)-box 9
spg	spermatogonium
SRY	sex determining region of chromosome Y
SSC	spermatogonial stem cell
SSC	side scatter
STAR	steroidogenic acute regulatory protein
STRA8	stimulated by retinoic acid gene 8
TDS	testicular dysgenesis syndrome
TGCC	testicular germ cell cancer
TGFβ	transforming growth factor beta
TRα1	thyroid hormone receptor alpha 1
WT1	Wilm's tumor 1

LIST OF ORIGINAL PUBLICATIONS

The study is based on the following publications, which are referred to in the text by Roman numerals (I-III). The original communications have been reproduced with the permission of the copyright holders.

I. **Emmi Rotgers**, Adolfo Rivero-Müller, Mirja Nurmio, Martti Parvinen, Florian Guillou, Ilpo Huhtaniemi, Noora Kotaja, Sonia Bourguiba-Hachemi, Jorma Toppari (2014). Retinoblastoma protein (RB) interacts with E2F3 to control terminal differentiation of Sertoli cells. *Cell Death Dis* 5:e1274.

II. **Emmi Rotgers**, Mirja Nurmio, Elina Pietilä, Sheyla Cisneros-Montalvo, Jorma Toppari (2015). E2F1 controls germ-cell apoptosis during the first wave of spermatogenesis. *Andrology*, 3(5):1000-14, 2015.

III. **Emmi Rotgers***, Sheyla Cisneros-Montalvo*, Kirsi Jahnukainen, Jouko Sandholm, Jorma Toppari, Mirja Nurmio (2015). A detailed protocol for a rapid analysis of testicular cell populations using flow cytometry. *Andrology*, 3(5):947-55, 2015. *Equal contribution.

1. INTRODUCTION

Infertility affects 15-20% of couples in developed countries and approximately half of the cases are caused by defects in the male (Louis et al. 2013, Schmidt 2006, Schmidt, Munster & Helm 1995). Male factor infertility as well as other male reproduction disorders are becoming more prevalent globally (Le Cornet et al. 2014, Jorgensen et al. 2011, Toppari et al. 2010). These defects range from congenital malformations, such as cryptorchidism, to declining sperm counts and an increased incidence of testis cancer in adults. Despite their seemingly different nature, these defects have a common origin in the fetal development of the testis (Juul et al. 2014). Various genetic and environmental factors can cause defective Sertoli and Leydig cell differentiation during fetal life, resulting in symptoms of androgen insufficiency and defects in germ cell differentiation (Juul et al. 2014).

Cell cycle control during postnatal testis development is a multilayered process. In juvenile testes both the various somatic cell types and the undifferentiated germ cells are mitotically amplified. In the maturing testis, the somatic cells gradually enter quiescence, while the active mitotic cycling of spermatogonia continues and meiosis of the more advanced germ cells begins. The retinoblastoma protein (RB) is a critical controller of the cell cycle progression in the mitotically dividing cells. In addition to the gatekeeper function it performs during the cell cycle, RB is also connected to the control of apoptosis, DNA damage responses, and differentiation. A key function of RB in all of these processes is to control the E2F transcription factors. Complex feedback loops and extensive functional redundancy operate between the different RB and E2F families *in vivo*. The role of RB in all of the facets of cell cycle control in the postnatal testis remains elusive.

In this study we set out to study how the dynamics of the RB and E2F protein families function to control postnatal testis development. As the RB/E2F pathway is commonly altered in different cancers, it also poses an attractive target for the development of new specific cancer drugs. Knowledge of the physiological function of these factors in the testis can be helpful in predicting possible adverse effects from new medications on the reproductive system. In a broader perspective, an understanding of the basic developmental mechanisms of the organ is necessary to elucidate the molecular mechanisms contributing to the increased incidence of testicular malfunctions.

2. REVIEW OF THE LITERATURE

2.1. Testis

The testis has two tasks: first, to produce androgens and second, to produce sperm for sexual reproduction. Spermatogenesis (i.e. the production of sperm) is a highly complex process where mitosis, meiosis and haploid cell differentiation occurs in succession. Despite this complexity, the mouse testis is a powerful sperm production factory, where the calculated efficiency is 4096 sperm produced from one spermatogonium: first through amplification of the spermatogonial population through consecutive mitotic divisions, and second by meiosis (Russell et al. 1990). In addition to the germ cells, several somatic cell types contribute to the formation and function of the testis (Fig. 1).

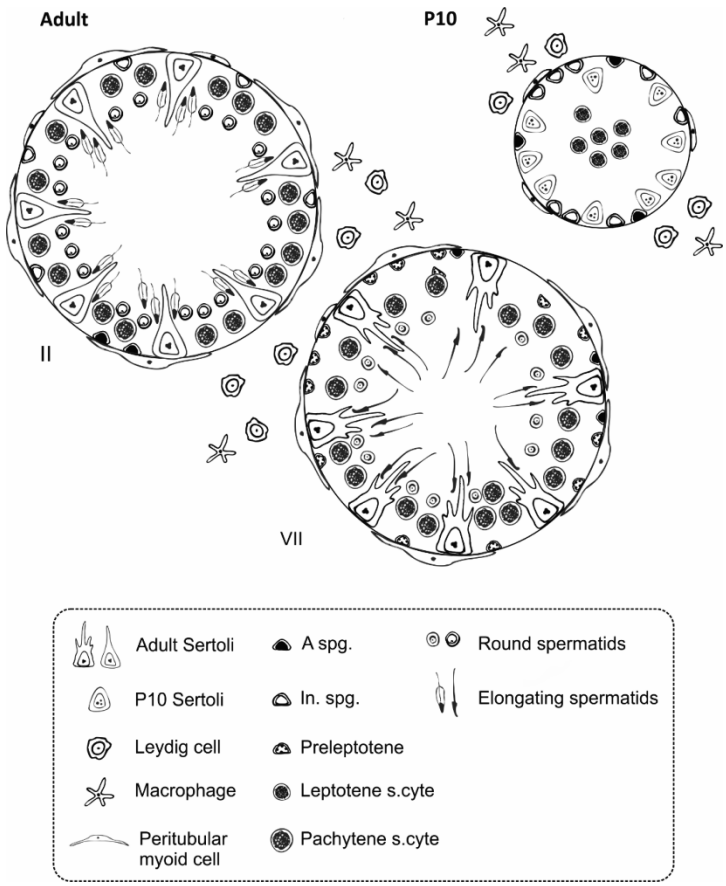

Figure 1. The structure and major cell types of the testis. The cell composition of the testes changes drastically during development. The seminiferous epithelial stages emerge at puberty as a result of the initiation of spermatogenesis. The cell composition of P10 mouse testes and adult seminiferous tubules from stages II and VII are depicted here.

Germ cell development is cyclic: after a generation of undifferentiated A spermatogonia has matured to differentiated spermatogonia, the next pool of undifferentiated A

spermatogonia begin the same process in the same position on the seminiferous epithelium (de Rooij 1998, Oakberg 1956a). In the mouse testis, one wave of spermatogenesis (i.e. the development of an A spermatogonium to mature spermatozoa) is completed in 34.5 days (Oakberg 1956b). The germ cells in different phases of differentiation form layers within the seminiferous tubules, and specific types of germ cells associate with each other at a given place on the epithelium. These associations form the basis for the morphological classification of the seminiferous tubules according to the stage of the seminiferous epithelial cycle. The identification of the different stages of the seminiferous epithelial cycle in rodents is based on spermatid morphology during differentiation (Leblond, Clermont 1952, Oakberg 1956a). In the mouse testis, the seminiferous epithelial cycle can be divided into 12 different stages during which spermiogenesis occurs in 16 differentiation steps (Oakberg 1956a). In the rat there are 19 steps of spermiogenesis and 14 stages of the seminiferous epithelial cycle (Leblond, Clermont 1952). Depending on the associated cellular processes, the stages can vary in duration, which leads to a varied abundance of each stage in the testes. For example, the longest stage I is more prevalent in the mouse testis than the shortest stage III (Oakberg 1956b). A systematic identification of the stages of the seminiferous epithelial cycle facilitates the precise identification of germ cell subtypes and dissection of different cellular events within the histologically complex testicular tissue.

2.1.1. Germ cells

2.1.1.1. Fetal germ cell development

Primordial gonocytes (PGC) are the precursors of all germ cells. The first signs of PGC differentiation can be seen at E7.25 in the mouse proximal epiblast (Ginsburg, Snow & McLaren 1990, Lawson, Hage 1994). The PGC commitment is initiated by a suppression of somatic gene expression and an upregulation of pluripotency markers such as OCT4 and NANOG (Chambers et al. 2007). The PGCs migrate to the genital ridge guided by a cascade of chemokine signaling [for a recent review see (Richardson, Lehmann 2010)]. The most characterized of these chemokine signaling pathways is the CXC12/CCR4 pathway, where somatic cells of the genital ridges secrete the CXCL12 chemoattractant and PGCs sense with the CXCR4 receptor and begin to migrate towards the somatic cells in the genital ridge (Molyneaux et al. 2003, Ara et al. 2003). The PGCs' motility and survival is also dependent on many other factors, such as the Kit/Steel system (Runyan et al. 2006, Mahakali Zama, Hudson & Bedell 2005). Once the PGCs reach the genital ridges, they become enclosed by the pre-Sertoli cells and testis cords, which are the fetal equivalent to seminiferous tubules, are formed. The fetal germ cells, now called gonocytes, undergo proliferation in the male gonad until E16 in the mouse, when all the germ cells have entered mitotic arrest, a hallmark of male development (Hilscher et al. 1974, Vergouwen et al. 1991a). The mitotic arrest is not solely an intrinsic function of the germ cells, but controlled by the somatic cells, for instance, a constitutive activation of the Notch signaling pathway in the Sertoli cells resulted in a premature proliferation and differentiation of gonocytes (Garcia et al. 2013).

The fetal mitotic arrest and subsequent prevention of meiotic entry are key events in the development of male germ cells. Complex signaling cascades operate to prevent the male

germ cells from entering meiosis prematurely. Retinoic acid (RA) promotes meiotic entry and expression of STRA8, a major gatekeeper of meiosis (Bowles et al. 2006, Baltus et al. 2006, Anderson et al. 2008). During fetal life, RA, produced by mesonephroi, is degraded in Sertoli cells by CYP26b1, preventing premature meiotic entry of gonocytes in the fetal mouse testis (Bowles et al. 2006). RA can also induce the expression of Kit, a hallmark of spermatogonial differentiation (Rossi et al. 1993). Deleted in azoospermia (DAZL) is a germ-cell intrinsic factor controlling the commitment of the PGCs to gametogenesis and their ability to respond to the cues from the somatic cells. Dazl-*null* germ cells remain in a PCG-like state and fail to respond to RA signaling from the somatic cells (Lin et al. 2008, Gill et al. 2011).

2.1.1.2. Postnatal spermatogonia

Postnatal germ cell development in rodents begins with gonocyte migration to the basement membrane of the seminiferous tubules. A reactivation of gonocyte proliferation at birth marks the initiation of the first wave of spermatogenesis (Vergouwen et al. 1991a). The first A spermatogonia emerge at postnatal day (P) 3 and gonocytes are simultaneously present with spermatogonia until P5 in the mouse (Vergouwen et al. 1991a). It has been suggested that directly differentiating gonocytes are the precursors of germ cells in the first wave of spermatogenesis (Yoshida et al. 2006).

The spermatogonia are divided into two subgroups: undifferentiated spermatogonia which form the self-renewing cell pool, and the differentiating spermatogonia which are committed to proceeding in development. The historical classification of spermatogonia included only three types of cells based on their heterochromatin content: A spermatogonia have no heterochromatin in the nucleus while B spermatogonia exhibit large areas of heterochromatin, and amount of heterochromatin in the intermediate spermatogonia is somewhere in between the two on this continuum (de Rooij, Russell 2000). The A spermatogonia population contains both undifferentiated and committed spermatogonia. The undifferentiated spermatogonia are further subdivided into A_{single}, A_{paired} and $A_{aligned}$, based on the number of cell clones that are connected by intercellular bridges. The differentiating proportion of A spermatogonia is called $A_{1\text{-}4}$. [For reviews see (Kanatsu-Shinohara, Shinohara 2013, de Rooij, Russell 2000)]. These traditional classifications of A spermatogonia have been subject to change in recent years, based on a new body of evidence which was acquired using various marker genes instead of morphology to identify the different subpopulations (Hara et al. 2014). Unlike all of the more advanced germ cell types, A spermatogonia disperse in a seemingly random manner through all the stages of the seminiferous epithelium, but their behavior is tightly connected to the cycling of the epithelium as a whole. Stage VII, where the differentiation of a new pool of A_{undiff} spermatogonia to A_1 spermatogonia is induced, marks the beginning of a new cycle of seminiferous epithelium (de Rooij 1998).

During the past decades, a debate concerning the dynamics of the stem cell pool and the identity of the true spermatogonial stem cells (SSC) has divided the field. The first A_0/A_1-theory, suggested by Clermont and Bustos-Obregon, stated that dormant spermatogonial cells, called the A_0 cells, are the bona fide SSCs which retain the capacity

to repopulate the testis after loss of the more differentiated A spermatogonia (Clermont, Bustos-Obregon 1968). However, upon steady-state spermatogenesis, the A_0 spermatogonia are quiescent and the continuous maintenance of the spermatogonial cell pool is achieved by transit-amplifying cells, namely the A_1-A_4 spermatogonia. In steady-state spermatogenesis a chain of clones of the more advanced A_4 spermatogonia breaks and reverts to the more undifferentiated A_1 cells to maintain the spermatogonial cell pool (Clermont, Bustos-Obregon 1968).

In 1978 Huckins and Oakberg suggested an alternative model (the A_s model), where only a subpopulation of the A_{single} cells are the self-renewing SSCs and they solely give rise to the more advanced A_{paired}, $A_{aligned}$ and A_{1-4} spermatogonia (Huckins, Oakberg 1978). This model implied that indeed, SSCs are not completely quiescent during steady-state spermatogenesis but that they regularly undergo mitosis to generate daughter cells for differentiation.

More recently, the Yoshida group has explored the SSC population identity and behavior using elegant pulse-chase and live-cell imaging techniques *in vivo*. They introduced the concept of actual and potential stem cells, following the footsteps of Clermont (Nakagawa, Nabeshima & Yoshida 2007). During steady-state spermatogenesis a subpopulation of the A_s spermatogonia act as stem cells, supplying spermatogenesis with germ cells destined for differentiation and self-renewing their own population. Upon loss of the actual stem cells in, for example, germ cell transplantation experiments, the more differentiated potential stem cells (comprised of A_{paired}, $A_{aligned4-16}$) revert in differentiation to behave like actual stem cells and repopulate the testis (Nakagawa, Nabeshima & Yoshida 2007). This reversion in commitment to SSCs also occurs in steady-state spermatogenesis, albeit at a lower rate (Nakagawa et al. 2010). The transition from a potential to an actual stem cell within the undifferentiated spermatogonial population is coupled with dissociation of the aligned chain of cells and a change in the gene expression program from the more committed Gfrα1$^+$/Ngn3$^+$ expression pattern to the more primitive Gfrα1$^+$/Ngn3$^-$ pattern (Nakagawa et al. 2010). Contrary to the previous dogma, also the most undifferentiated Gfrα1$^+$/Ngn3$^-$ spermatogonia form intercellular bridges and they are under continuous formation and break-down (Hara et al. 2014) (Fig. 2).

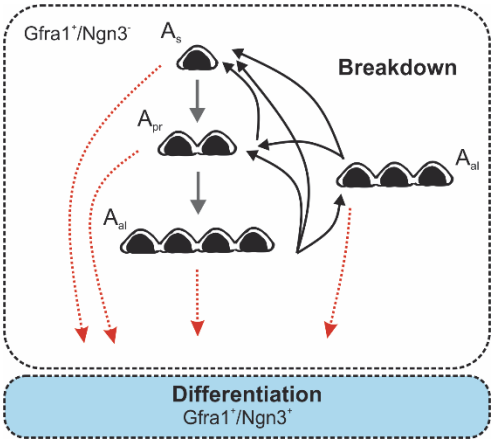

Figure 2. Dynamics of the Gfrα1$^+$ SSC population in the mouse testis. The Gfrα1$^+$/Ngn3- A_s, A_{pr} and A_{al} spermatogonia undergo mitosis (solid grey arrows) and yield daughter cells which are connected by intercellular bridges. All these cells can commit to differentiation and acquire Ngn3 expression (broken red arrows). The intercellular bridges between the A_{pr} and A_{al} spermatogonia can break and these cells can contribute to the A_s population (solid black arrows). (Modified from (Hara et al. 2014).

In light of the work of the Yoshida group, it appears that, in fact, both the A_0/A_1 and the A_{single} theory were partially correct. The actual stem cells exist as A_{single} cells but also as short chains. Even the more differentiated spermatogonia can revert to a more undifferentiated SSC-like state. In summary, the identification of the true SSCs remains difficult, as a clear-cut functional separation of the undifferentiated spermatogonial subpopulations does not exist and the morphological groups (A_{single}-$A_{aligned}$) do not delineate the behavior of these cells.

Each spermatogonium undergoes eight to nine rounds of mitosis prior to becoming a spermatocyte (de Rooij, Russell 2000). Three rounds of mitosis occurring at seminiferous epithelial stages IX, XI and I-II amplify the A spermatogonia (Oakberg 1956b, Tegelenbosch, de Rooij 1993). During stage III, the A spermatogonia differentiate to intermediate spermatogonia which enter mitosis in stage IV. The newly formed B spermatogonia enter mitosis in stage VI. After the extensive mitotic amplification the B spermatogonia differentiate to preleptotene spermatocytes (present in stage VII-VIII) in the mouse.

2.1.1.3. Meiosis

Meiosis is designed to generate gametes with half of the chromosome complement for sexual reproduction and create genetic diversity via meiotic recombination. (Figure 3). This is accomplished by having two successive rounds of cell divisions after one replication round of DNA. The nomenclature of the meiotic cells in the testis is based on the different phases of meiosis. The differentiation of the preleptotene spermatocytes on PND10 (in stage VII in adult spermatogenesis) marks the initiation of meiosis. STRA8 expression in germ cells is a major hallmark of commitment to proceed to meiosis (Baltus et al. 2006, Anderson et al. 2008). Premeiotic DNA replication takes place in the preleptotene spermatocytes. The maternal and paternal chromosomes (homologs) are replicated, which generates two sister chromatids for each homolog. DNA double strand breaks (DSB), which are necessary for the homolog pairing and meiotic crossovers, are induced by the SPO11-endonuclease in leptotene spermatocytes as the chromosomes begin to pair (Baudat, Manova, Yuen, Jasin, & Keeney, 2000; Romanienko & Camerini-Otero, 2000).The pairing facilitates the formation of the meiotic crossing-overs, where genetic material is exchanged between the maternal and paternal homologs to induce genetic variation.

The chromosomes condense and pair in *leptonema* [for a review, see (Cohen, Pollack & Pollard 2006)]. They will be held together during meiosis by a tripartite protein structure called the synaptonemal complex. The synaptonemal complex begins to assemble in *zygonema*, when the pairing has been completed.

Meiotic recombination with DSB repair and meiotic crossing-over takes place in the pachytene phase. Many main events of meiosis occur in *pachynema*: genetic material is exchanged between homologous chromosomes and the DNA is subsequently repaired. During the diplotene phase, the synaptonemal complex is dissolved and the chromosomes are held together by only the chiasmata. As the DNA has been repaired at the crossing-overs, the cells divide and diakinesis occurs. The second meiotic division

occurs rapidly after the first one and it divides the two sister chromatids into different haploid cells in a similar manner as takes place in mitosis. Due to the rapid nature of the division, the secondary spermatocytes are present for a very short time and are hard to identify in tissue sections (Russell et al. 1990). In the postnatal mouse testis, the first haploid cells emerge at P20 after the completion of the first round of meiosis.

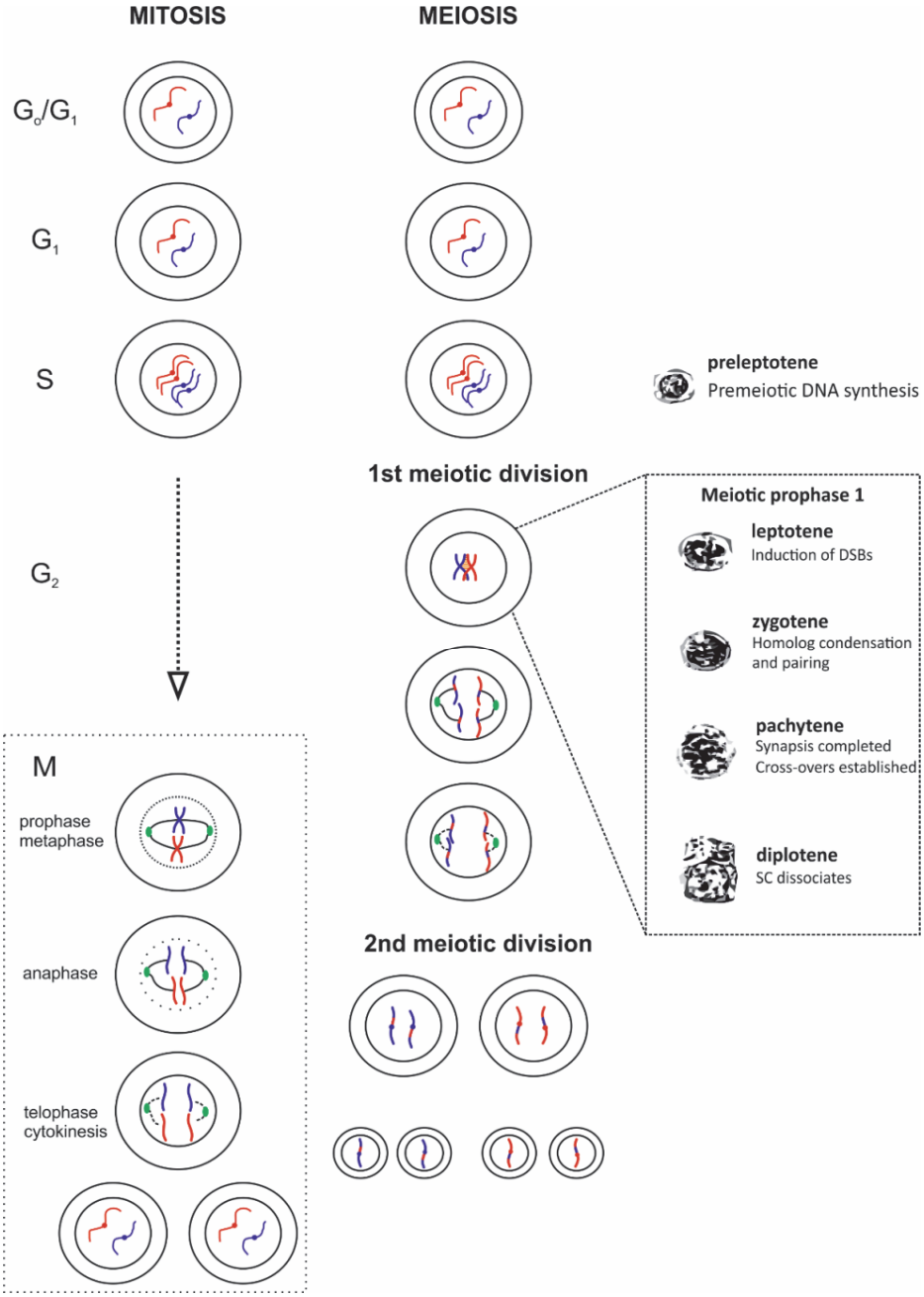

Figure 3. Overview of mitotic and meiotic cell cycles. In a mitotic cell cycle, the cells first grow in size during the G_1 phase. If cell cycle progression is permitted at the G_1/S checkpoint, the DNA is replicated in the S phase. The faithfulness of DNA replication is checked at the G_2/M checkpoint, after which the cell divides in mitosis (the M phase) to produce two identical daughter cells. Similar phases of the cell cycle occur in meiosis, with the exception that the DNA synthesis is followed by a special G_2 phase (meiotic prophase 1), where genetic material is exchanged between the maternal and paternal homologs and followed by a cell division in which the number of chromosomes is halved. In the second meiotic division, sister chromatids of the haploid secondary spermatocytes are separated (similarly to mitosis), yielding four haploid spermatids with unique genetic compositions. DSB: double strand break, SC: synaptonemal complex.

2.1.1.4. Physiological apoptosis in the testis

Apoptosis of germ cells is a common physiological phenomenon in the testis, both during development and in adult steady-state spermatogenesis [for a review, see (Print, Loveland 2000)]. Up to 75% of the type A spermatogonia degenerate in the rat testis and this loss primarily affects A_2 and A_3 spermatogonia in the early G_2 phase (Huckins 1978). In the mouse, only 33% of the expected number of preleptotene spermatocytes can be observed in the testis (Huckins, Oakberg 1978).

The purpose of the spermatogonial apoptosis is thought to be to adjust the Sertoli/germ cell ratio to an optimal one. The number of A_1 spermatogonia per Sertoli cell varies more between animals than the B spermatogonia/Sertoli cell ratio, and Huckins postulated in 1978 that the loss of A_1 spermatogonia adjusts the ratio to an optimal one in each animal (Huckins 1978). The degeneration of A spermatogonia is associated with the mitosis of these cells and typically the complete chain of A spermatogonia originating from a single clone degenerate simultaneously (Oakberg 1956a). There is extensive apoptosis of spermatogonia during the first wave of spermatogenesis, with a peak of apoptosis at P13 (Wang, Nakane & Koji 1998).

A considerable loss of germ cells occurs during the meiotic divisions as only 87% of the expected spermatids are formed (Oakberg 1956a, Huckins, Oakberg 1978). Another major contributor to physiological apoptosis is the meiotic checkpoint at mid-pachytene (seminiferous epithelial stage IV). At the meiotic checkpoint, spermatocytes with defective DNA repair and meiotic recombination are cleared from the testis through apoptosis (Hamer et al. 2008). It is activated especially in cases with a defective synapsis of meiotic chromosomes (Hamer et al. 2008).

2.1.2. Sertoli cells

2.1.2.1. Sertoli cells in the fetal and early postnatal life

Sertoli cells are the most important somatic cells in the testis, as their precursors are the first cell type to trigger a male-specific gene expression program. Sertoli cell differentiation is controlled by a myriad of different signals from the endocrine, paracrine and autocrine factors [reviewed in (Tarulli, Stanton & Meachem 2012)]. Cells that migrate to the gonad from the coelomic epithelium give rise to Sertoli cells and interstitial cells (Karl, Capel 1998). Activation of the sex-determining region of Chr Y (*Sry*) expression in a subset of somatic cells in the genital ridge marks the initiation of Sertoli cell differentiation and testis development (Koopman et al. 1990). The transient presence of SRY induces the expression of SOX9, which maintains the Sertoli cell differentiation by a feed-forward loop promoting testis development and suppressing the ovarian pathway (Sekido et al. 2004, Kim et al. 2006). The newly developed Sertoli cells begin to secrete anti-Müllerian hormone (AMH), which induces the regression of the Müllerian ducts and inhibits the formation of the female internal genitalia (Munsterberg, Lovell-Badge 1991, Behringer, Finegold & Cate 1994). In the XX fetal gonad, AMH can induce testis-type vasculature patterning, but it is not sufficient to induce sex reversal (Ross et al. 2003). Germ cells will follow the sex determination triggered in somatic

cells, irrespective of their sex chromosome content (Adams, McLaren 2002, Byskov, Saxen 1976).

The emergence of Sertoli cells triggers testicular organogenesis, which is associated with a drastic reorganization of the organ and extensive Sertoli cell proliferation during E11.5 and 12.5 [for recent reviews see (Ungewitter, Yao 2013, Svingen, Koopman 2013)]. As a result, the formerly uniform tissue becomes organized as testis cords, where germ cell clusters are enclosed by Sertoli cells, a basement membrane and peritubular myoid cells (Tung, Skinner & Fritz 1984, Skinner, Tung & Fritz 1985). A second wave of rapid Sertoli cell proliferation occurs after E15.5, when adult type seminiferous epithelium forms as a result of cord elongation and coiling. This is under paracrine control by the fetal Leydig cells, which secrete activin A to induce a SMAD4-dependent proliferation of the Sertoli cells (Archambeault, Yao 2010).

After birth the Sertoli cell proliferation begins to decline gradually as the maturation process is initiated at P10 (Vergouwen et al. 1991b, Zimmermann et al. 2015). The Sertoli cells are equally crucial for both normal adult spermatogenesis and fetal testis differentiation. Ablation of Sertoli cells in fetal and perinatal life led to a complete collapse of the tubular structures as a result of germ cell and peritubular myoid cell loss (Rebourcet et al. 2014). The reproductive capacity of an adult male depends on the number of Sertoli cells and the level of Sertoli cell proliferation in the juvenile phase (Orth, Gunsalus & Lamperti 1988). Defective Sertoli cell maturation, function or development causes disorders in spermatogenesis [reviewed in (Sharpe et al. 2003)] and is associated with an increased risk of germ cell cancer (Skakkebaek, Rajpert-De Meyts & Main 2001). The long-term survival of Sertoli cells is crucial for the maintenance of spermatogenesis through adult life, but there is limited knowledge on the mechanisms that sustain this cell type.

2.1.2.2. Sertoli cell differentiation at puberty

Sertoli cell differentiation is a long process which is initiated by the gradual induction of cell cycle exit from P10 onwards. By P35 in the rat, the Sertoli cells exhibit full structural maturity, which coincides with the emergence of the first fully differentiated germ cells (Ramos, Dym 1979, Clermont, Perey 1957). Some major hallmarks of Sertoli cell differentiation and the related molecular mechanisms are summarized in (Fig.4).

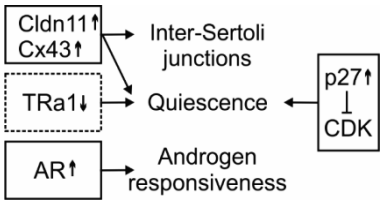

Figure 4. **Control of Sertoli cell differentiation.** Quiescence, formation of the inter-Sertoli cell junctions, and androgen responsiveness are major hallmarks of Sertoli cell differentiation. Induction of the CDK-inhibitor p27 and the junction proteins Cldn11 and Cx43 contribute to the cessation of Sertoli cell proliferation. During maturation, the thyroid hormone responsiveness of Sertoli cells is down-regulated and androgen responsiveness is up-regulated.

As Sertoli cells prepare to exit the cell cycle, they upregulate the expression of the cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$) (Beumer et al. 1999). $p27^{Kip1}$ inhibits cyclin-dependent kinases (CDK) that inactivate RB by phosphorylation (Polyak et al. 1994).The loss of $p27^{Kip1}$ leads to gigantism, multiorgan hyperplasia and hypertrophy of

the testes due to enhanced proliferation of Sertoli cells, consistent with its role as a cell cycle inhibitor (Fero et al. 1996, Holsberger et al. 2005, Kiyokawa et al. 1996, Nakayama et al. 1996).

There is evidence that, rather than being senescent permanently non-proliferative cells, adult Sertoli cells are rather in a state of constant active repression of cell cycle entry. For instance, in seasonal breeders, Sertoli cell numbers in testis increase during the reproductive season (Tarulli et al. 2006, Johnson et al. 1991). Adult mouse Sertoli cells begin to proliferate when they are removed from their natural environment and put into cell culture conditions (Ahmed et al. 2009). Irradiation induced DNA damage in Sertoli cells leads to an accumulation of phosphorylated ataxia-telangiectasia mutated (ATM) which is a kinase that is associated with active DNA repair (Hamer et al. 2004). Treatment with gonadotropin inhibitors can induce Sertoli cell proliferation as a result of germ cell loss in humans (Tarulli et al. 2013).

Cell cycle control and the differentiation state are closely connected to the function of the inter-Sertoli junctions A loss of connexin 43 (Cx43), a gap-junction protein, in Sertoli cells leads to continued Sertoli cell proliferation, immaturity and failure to support spermatogenesis (Sridharan et al. 2007). In mice deficient for claudin 11 (Cldn11), a tight-junction protein, Sertoli cells continue to proliferate in adulthood and form papilloma-like clusters within the seminiferous tubules (Mazaud-Guittot et al. 2010).

The internal organization of the testicular tissue into seminiferous tubules is mediated by the Sertoli cells from the fetal period onwards. A functional blood-testis barrier (BTB) begins to form at P16 in the rat, which coincides with the emergence of zygotene and early pachytene spermatocytes (Vitale, Fawcett & Dym 1973, Clermont, Perey 1957). The BTB is a highly complex structure formed by an intricate interplay between tight junctions (e.g. claudins 1-24, ZO-1, JAM1-3), ectoplasmic specialization (e.g. α1β6 integrin, espin), gap (e.g. connexin 43) and desmosome-like junctions (e.g. desmoglein, desmocollin, vimentin) (Mruk, Cheng 2004). The purpose of BTB is to seclude the meiotic and haploid germ cells from the surrounding body to create an immune-privileged environment and to allow them to be in contact only with substances that passed the control of the Sertoli cells [reviewed in (Mruk, Cheng 2004)]. BTB undergoes considerable remodeling during the spermatogenic wave, especially during the late stage VIII-early stage IX, to allow the passage of the preleptotene spermatocytes to the ad luminal compartment for further differentiation (Russell 1977a).

Emerging androgen responsiveness is another hallmark of functional differentiation of Sertoli cells. During the past years, work on different transgenic animal models has revealed the complex nature of androgen signaling in Sertoli cells [recently reviewed in (Smith, Walker 2014)]. Despite acting in an endocrine manner on the rest of the body, androgens can be seen as paracrine controllers of spermatogenesis. In rats and mice, the androgen receptor (AR) is expressed in Sertoli cells from early postnatal life (P5 in rats) onwards, and as the animals reach adulthood the expression levels increase and become stage-specific (Bremner et al. 1994, Zhou et al. 1996). The highest expression of AR is observed in stages II-VII in the rat testis (Parvinen, Vihko & Toppari 1986, Bremner et al. 1994), which coincides with the highest intratubular testosterone concentrations

(Parvinen, Ruokonen 1982). AR-signaling in Sertoli cells is crucial for supporting spermatogenesis beyond the pachytene phase (De Gendt et al. 2004). Testosterone appears to control Sertoli cell proliferation indirectly via peritubular myoid cells, since testosterone administration promotes the recovery Sertoli cell number in gonadotropin-deficient hpg mice (Haywood et al. 2003). However, the SCARKO mice with *Ar* ablation in specifically Sertoli cells had essentially normal Sertoli cell numbers (De Gendt et al. 2004).

Thyroid hormone action provides an additional layer of endocrine control on the differentiation of Sertoli cells. An excess of thyroid hormone in juvenile rodents causes a premature cessation of Sertoli cell proliferation, while hypothyroidism has the opposite effect (De Franca et al. 1995, Van Haaster et al. 1992, van Haaster et al. 1993). Induction of neonatal hypothyroidism by goitrogens in mice results not only in delayed maturation and extended proliferation of Sertoli cells, but also in consequently larger testes and a higher sperm output (Joyce, Porcelli & Cooke 1993). The thyroid hormone receptor alpha (TRα1) is the main mediator of the thyroid hormone actions of Sertoli cells and it is a marker for Sertoli cell immaturity (Joyce, Porcelli & Cooke 1993, Holsberger, Kiesewetter & Cooke 2005).

2.1.2.3. Sertoli-germ cell interactions

Sertoli cell function within the seminiferous epithelium is very dynamic (Elftman 1950). Sertoli cell function changes cyclically according to the stages of the seminiferous epithelial cycle. Sertoli cell filament organization and nuclear localization, as well as the gene expression profile vary between the stages (Leblond, Clermont 1952, Sugimoto, Nabeshima & Yoshida 2012). Sertoli cells appear to have an intrinsic cycling behavior even in the absence of germ cells (Timmons, Rigby & Poirier 2002). However, if rat spermatogonia are transplanted to mouse testis, the timing of the seminiferous epithelial cycle follows that of the spermatogonia of the donor, not the recipient (Franca et al. 1998). Thus, the germ cells have an intrinsic clock which determines the timing of the seminiferous epithelial cycle and modulates Sertoli cell function (Franca et al. 1998). It has been shown that reciprocal modulations in retinoic acid signaling between Sertoli cells and germ cells contribute to the establishment and maintenance of the seminiferous epithelial cycle (Sugimoto, Nabeshima & Yoshida 2012).

Extensive physical contacts exist between Sertoli cells and germ cells [for review see (Kopera et al. 2010)]. Germ cells, from undifferentiated spermatogonia through the round spermatid stage, are connected to the Sertoli cells with desmosome-like junctions. The BTB-associated basal ectoplasmic specializations contribute to the adhesion of the immature germ cells to Sertoli cells, and they are actively cycled within the Sertoli cells as the connected germ cells mature (Russell 1977b). As the spermatids reach the elongation phase, they become connected to the Sertoli cells with apical ectoplasmic specializations. When spermiation approaches the apical ES is gradually replaced by the tubulobulbar complexes, which are formed by invaginations of mature spermatid cytoplasm towards the Sertoli cell cytoplasm and endoplasmic reticulum and supported by actin-filaments (Russell 1979).

2.1.3. Leydig cells

In addition to sperm production, the second pivotal role of the testes is to produce androgens. Androgens promote the formation of the male genitalia during fetal life, trigger the appearance of the secondary male characteristics during puberty, and maintain overall well-being and sperm production in adulthood. The main cell type responsible for androgen production is the Leydig cell and castration results in a 97.4% reduction of serum testosterone levels in men (Labrie et al. 2009). The adrenal cortex contributes to the circulating total androgens by producing a testosterone precursor, dehydroepiandrosterone (DHEA), which can be converted to more potent androgens peripherally, for example in the prostate (Labrie 2011).

There are two different populations of Leydig cells during development: the fetal Leydig cells and the adult Leydig cells (ALC). The origin of the fetal Leydig cells and the adult Leydig cells is still debated [for a recent review, see (Svingen, Koopman 2013)]. The fetal Leydig cells can be identified in ED12 in the mouse testis interstitium when the emerging fetal androgen production peak can be observed (Merchant-Larios, Moreno-Mendoza & Buehr 1993). Recent evidence has suggested that the Nr5a1-positive cells in the interstitium of the bipotential gonad are the fetal Leydig cell precursors (Barsoum et al. 2013). The fetal Leydig cell population expands rapidly during E12.5-15.5 through recruitment and differentiation of precursors, not by proliferation (Orth 1982, Migrenne et al. 2001). The fetal androgen production peak is essential for the differentiation of the male genitalia from the Wolffian duct. Adult Leydig cells begin to emerge at puberty from their stem-like progenitors and they become the main cell type responsible for testosterone production (Habert, Lejeune & Saez 2001). The adult mouse Leydig cells acquire a full androgen biosynthetic capacity at P24, evidenced by a rapid increase in the androstenedione and testosterone levels in serum (Wu et al. 2010). The testosterone levels reach a peak at P45 (Wu et al. 2010). The adult Leydig cells proliferate rapidly until P28, after which they gradually become quiescent (Rijntjes et al. 2009). Sertoli cells are necessary for the formation of the adult Leydig cell population, but ablation of Sertoli cells in the prenatal mouse testis does not disturb fetal Leydig cell function (Rebourcet et al. 2014). Unlike Sertoli cells, adult Leydig cells can resume active proliferation in adult life after damage, for example, after ethane-1,2-dimethyl sulfonate (EDS) administration (Molenaar et al. 1985, Kerr, Donachie & Rommerts 1985).

The role of AR in the fetal and adult Leydig cells has remained a mystery for decades. In fetal life, the testicular somatic cells do not express AR and the fetal androgens have been thought to be responsible only for the masculinization of the fetus, not for testicular development. Nevertheless, there is mounting evidence indicating the presence of an androgen-sensitive period, which determines adult reproductive functions, during fetal testicular development (Kilcoyne et al. 2014, Juul et al. 2014). A recent study established a link between the androgen action on fetal Leydig cells and the adult Leydig cell population (Kaftanovskaya et al. 2015). Ablation of AR in the precursors of the fetal Leydig cells resulted in an incomplete differentiation of the adult Leydig cells, infertility, and increased survival of the fetal Leydig cells in the adult testis, even though these two cell types do not share a common origin (Kaftanovskaya et al. 2015). It appears that AR expression is required to restrict the fetal Leydig cell population in the adult testis and

the fetal Leydig cells control the adult Leydig cell differentiation in a paracrine manner (Kaftanovskaya et al. 2015). Androgens also play a role in the control of the adult Leydig cell progenitors in fetal life. Anti-androgenic treatment of the fetus modulates the epigenetic regulation of the steroidogenic acute regulatory protein (*Star*) and influences adult Leydig cell function (Kilcoyne et al. 2014). AR in the adult Leydig cell progenitors is essential for their final maturation, but not essential for the attainment of the final Leydig cell number (O'Hara et al. 2015).

2.1.4. Peritubular myoid cells

Peritubular myoid cells (PTM) make up the outer layer of the seminiferous tubules and produce the basement membrane which lines the seminiferous tubules together with Sertoli cells (Tung, Skinner & Fritz 1984). The origin of the PTMs remains unknown, but the first evidence of them can be observed in the mouse testis at E13 (Jeanes et al. 2005). PTMs play an important role in the postnatal development of the testis, as disrupted PTM proliferation in juvenile rats leads to decreased testis size (Nurmio et al. 2007, Nurmio et al. 2012). Sertoli cells control the differentiation of PTMs, and ablation of Sertoli cells in the prenatal testis causes a drastic disorganization of the seminiferous tubules and PTM dedifferentiation (Rebourcet et al. 2014). Androgen action on spermatogenesis is partially mediated by the PTMs (Welsh et al. 2009). *Ar* ablation in PTMs results in azoospermia and infertility (Welsh et al. 2009).

2.1.5. Other somatic cell types

Testicular macrophages are required for the establishment of the male-specific vasculature during testicular organogenesis in the fetal gonad (DeFalco et al. 2014). In the adult testis, macrophages form the second largest cell population within the interstitium after the Leydig cells [for a recent review see (Winnall, Hedger 2013)]. Endothelial cells play a central role in fetal testis development. Correct patterning of the testicular vasculature is essential for testicular organogenesis (Combes et al. 2009, Cool et al. 2008). Endothelial cells from the adjacent mesonephros infiltrate the developing testis and carve the way to the development of the testis-specific vasculature. Cooperation of all the different testicular cell types is crucial during development and in the adult testis. A good example of this was provided by the ablation of Smad4, which acts downstream of the canonical TGFβ-signaling pathway, in both Sertoli and Leydig cells. Loss of Smad4 resulted in a testis cord dysgenesis in the fetal life and hemorrhagic tumors in adult testis as a result of defective testicular vasculature (Archambeault, Yao 2014).

2.1.6. Endocrine control of spermatogenesis

Endocrine control of the testis occurs via the hypothalamo-pituitary-gonadal (HPG) axis where complex feedback and feedforward loops operate. Kisspeptins are one of the major upstream regulators of the HPG-axis [for review see (Roa et al. 2008)]. Gonadotropin-releasing hormone (GnRH) is secreted from the GnRH neurons in the hypothalamus in response to the activation of the Kiss1-receptor (also known as GPR54) by the kisspeptins (Castellano et al. 2006). Loss of Kiss1-receptor in mice and humans results in a hypogonadotropic hypogonadism and absence of pubertal development

(d'Anglemont de Tassigny et al. 2007, Seminara et al. 2003, de Roux et al. 2003). GnRH in turn triggers the secretion of the gonadotropins from the anterior pituitary. There are two gonadotropins in both females and males: follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH acts primarily on Sertoli cells to support their function, while LH stimulates Leydig cells to produce androgens. Human chorionic gonadotropin (hCG), which is secreted from the placenta, activates LH receptors and stimulates the development of the fetal Leydig cells. The secretion of inhibin B from Sertoli cells and testosterone from Leydig cells creates a negative feedback loop for gonadotropin secretion by the pituitary.

The establishment of the Sertoli cell population is not gonadotropin-dependent, since the Sertoli cells of the hpg mouse model lacking both FSH and LH differentiate normally (Myers et al. 2005, Cattanach et al. 1977). However, Sertoli cell proliferation is dependent on FSH, as the population is considerably smaller in hpg mice than in the controls (Myers et al. 2005, Cattanach et al. 1977). Furthermore, the increase in the serum FSH levels induced by unilateral castration of juvenile rats results in an FSH-dependent increase in Sertoli cell proliferation and testicular hypertrophy in the remaining testis (Orth, Higginbotham & Salisbury 1984, Nurmio et al. 2012).

2.1.7. Somatic cells in the control of the spermatogonial stem cell niche

A complex network of paracrine signaling controls spermatogenesis and the SSC niche in particular. The Sertoli cells secrete several growth factors and signaling molecules, which act on the SSCs to control self-renewal, colony expansion and meiotic entry.

FSH stimulates Sertoli cells to secrete glial cell-derived neurotrophic factor (GDNF) and Kit ligand (also known as stem cell factor) (Rossi et al. 1993, Yan et al. 1999, Tadokoro et al. 2002). GDNF promotes SSC maintenance by activating the GFRα1 receptor and RET tyrosine kinase (Meng et al. 2000, Tadokoro et al. 2002, Naughton et al. 2006, Jing et al. 1996). Insufficient GDNF secretion leads to an exhaustion of the SSC population in mice, while GDNF overexpression results in their accumulation (Meng et al. 2000). Kit ligand promotes both the proliferation of spermatogonia and the meiotic entry (Manova et al. 1990, Yoshinaga et al. 1991, Schrans-Stassen et al. 1999). The expression of the receptor for Kit ligand, Kit, marks the commitment of spermatogonia to differentiate (Schrans-Stassen et al. 1999) and it is subject to direct negative regulation by PLZF in undifferentiated spermatogonia (Filipponi et al. 2007).

The CXCL12/CXCR4 system, which mediates the migration of PGCs, has also been shown to be involved in the maintenance of the SSC population (Yang et al. 2013, Molyneaux et al. 2003). A decrease in the expression of the various Sertoli cell-secreted chemokines compromises the SSC maintenance. ETV5 is a transcription factor that has been shown to control the expression of several chemokines in the Sertoli cells (*Cxcl12, Cxcl5, Ccl7, Ccl9*) (Simon et al. 2010, Chen et al. 2005). Loss of ETV5 led to a progressive loss of SSCs and a Sertoli-cell-only phenotype (Chen et al. 2005). A decrease in the *Cxcl12*/*Cxcr4* transcript levels was associated with the deletion of SIN3a, a transcriptional co-repressor, and it resulted in the loss of the PLZF-positive spermatogonia in the postnatal testis (Payne et al. 2010).

The Sertoli cells play a central role in controlling the meiotic entry. Retinoic acid (RA) is a potent inducer of meiosis in the juvenile mouse testis and it is produced by Sertoli cells from retinol (Vernet et al. 2008). RA induces the expression of Kit ligand and GDNF in Sertoli cells (Pellegrini et al. 2008). In addition, FSH and RA act on the Sertoli cells to induce the expression of Neuregulin 1 and 3, which promote STRA8 expression and meiotic progression in differentiating spermatogonia (Zhang et al. 2011). Conversely, the Sertoli cell-secreted fibroblast growth factor 9 (FGF9) has been shown to decrease the sensitivity of germ cells to STRA8 (Bowles et al. 2010). In summary, a tight-knit balance of endocrine and paracrine factors with opposing actions is required for the control of the meiotic entry.

PTMs participate in forming the SSC niche together with the Sertoli cells (Spinnler et al. 2010). Testosterone promotes the secretion of GDNF by PTMs *in vitro,* and spermatogonia co-cultured with the testosterone-treated PTMs were more potent in colonizing testes in transplantation assays than non-treated PTMs (Chen et al. 2014). PTMs and Leydig cells produce colonystimulating factor 1 (CSF1), which controls SSC survival (Oatley et al. 2009).

All the somatic cells contribute to the germ cell microenvironment. In the normal testis, they ensure the maintenance of the SSCs and promote a timely meiotic entry and differentiation. A compromised function of the somatic cell niche can lead to the depletion of the SSCs, infertility or even malignant transformation of the germ cells.

2.1.8. The clinical connection - testicular dysgenesis syndrome

All the testicular cell types, their functions and paracrine effects intersect in a clinical context when testicular dysgenesis syndrome (TDS) is considered. The prevalence of several disorders of male reproduction has been increasing during the past decades [recently reviewed in (Juul et al. 2014)]. These disorders range from congenital malformations such as cryptorchidism and hypospadias to an increasing incidence of germinal neoplastic cells *in situ* (GNIS), testicular germ cell cancer (TGCC) and male factor infertility. These defects often present together and being affected with one is associated with an increased risk for the others (Juul et al. 2014). Consequently, it has been hypothesized that these defects have an origin in the fetal testicular development and that, despite their different natures, they share a similar etiology (Fig. 5).

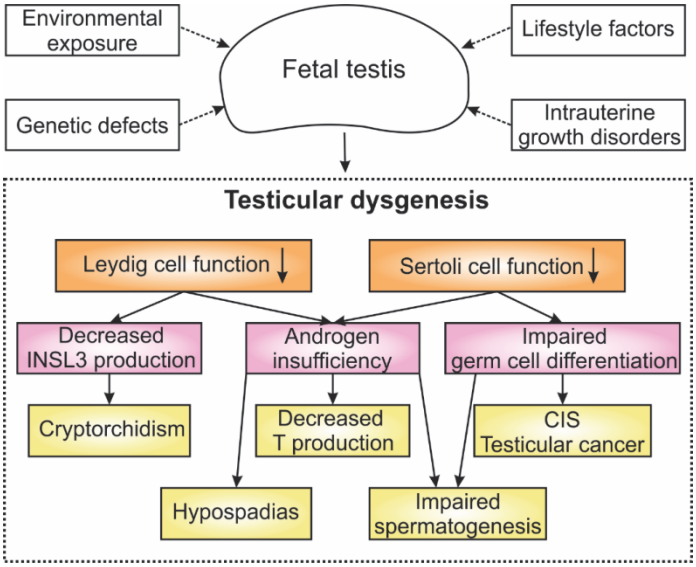

Figure 5. Pathogenesis of testicular dysgenesis syndrome. Multiple environmental and genetic factors can disturb the fetal testis development and result in aberrant Leydig cell and Sertoli cell function. These defects can present in postnatal life and adulthood as cryptorchidism, hypospadias, hypogonadism, infertility and/or testicular cancer. Modified from Juul et al. 2014.

Several causes for the TDS spectrum disorders have been identified. Gonadal dysgenesis is a more severe manifestation of the TDS spectrum. It is observed in patients with disorders of sex development (DSD) and is caused by genetic factors (sex chromosome alterations or mutations in single genes). Many other factors have been connected to the more subtle phenotypes of TDS. Disorders of fetal growth are associated with increased risk of cryptorchidism, hypospadias and TGCC (Moller, Skakkebaek 1997, Francois et al. 1997, Main et al. 2006). Maternal factors such as pre-eclampsia and diabetes have been linked to genital malformations (Damgaard et al. 2008, Virtanen et al. 2006).

It has been proposed that the observed increase in the prevalence of TDS could be caused by environmental pollutants with endocrine-disrupting potential. Obviously, it is very difficult to demonstrate a direct causal link between environmental exposure and TDS in humans. However, in rodents, a TDS-like syndrome can be induced by exposing the animals to di(n-butyl) phthalate (DBP) during fetal life. In addition, genetic or chemical blocking of androgen action impairs Sertoli cell proliferation and fertility (Scott et al. 2007, Tan et al. 2005, Atanassova et al. 2005).

TGCC can be considered the most severe outcome of TDS. Non-seminoma and seminoma both derive from the similar precursor cells called GNIS (Skakkebaek 1972). TGCC is often surrounded by seminiferous tubules with GNIS cells and these premalignant lesions can even be found in the contralateral testis in 5% of cases (Berthelsen et al. 1982, Dieckmann, Loy 1996). Based on these data, it has been proposed that TGCC is the result of a widespread defect in the testes, rather than a manifestation of a malignant development in one germ cell clone. The GNIS cell morphology and protein expression pattern resemble gonocytes. These premalignant cells are thought to arise as a result of a defective somatic cell niche function: an insufficient signaling to downregulate pluripotency markers and a dysregulation of meiosis signaling (Jorgensen et al. 2015, Jorgensen et al. 2013).

2.1.9. How should testicular cell dynamics be studied?

The complex dynamics of the testicular cell types during development have been traditionally studied using morphometric methods. Identification of the different stages of the seminiferous epithelium in histological sections enables dissecting the different germ cell subtypes to a certain level of accuracy. More specific information can be obtained by immunohistochemical methods to study the cells based on different marker expression. A common pitfall in both of these approaches is that often the analysis has to be done manually, which makes it laborious and time-consuming. During the past decades several advances have been made applying flow cytometry in the study of testicular cell types to overcome the problems associated with morphometry (Toppari, Eerola & Parvinen 1985, Toppari et al. 1988, Ferguson, How & Agoulnik 2013, Elzeinova et al. 2013, Wakeling, Miles & Western 2013).

2.1.9.1. Flow cytometry to quantify testicular cells

In flow cytometry, cells in suspension are injected into a stream of fluid, which forces the cells into a single file as they pass through the detection system. The different characteristics of the cells (number, size, granularity, fluorescence) are detected by subjecting them to a laser beam and detecting the characteristics of the transmitted light. Forward scatter (FSC) gives information about the size of the cell, while side scatter (SSC) is a parameter for cell granularity. The golden standard of testicular cell flow cytometry since the late 1970's has been coupling the detection of the scatter properties to a stoichiometric DNA stain such as propidium iodide to allow detection of the different testicular cell types based on their DNA amount (Toppari, Eerola & Parvinen 1985, Toppari et al. 1988, Pfitzer et al. 1982, Meistrich et al. 1978, Clausen, Purvis & Hansson 1977). The adult testicular cell types fall into four different categories based on their DNA staining characteristics. The quiescent diploid somatic cells and spermatogonia in the G_0/G_1 phase form the 2C cell population (Toppari, Eerola & Parvinen 1985). Mitotically dividing spermatogonia in the G_2/M phase of the cell cycle and the meiotic spermatocytes from leptotene onwards have doubled their DNA amount and form the 4C cell population. Cells undergoing DNA synthesis in the S phase cluster between the 2C and 4C populations. The haploid cells form two distinct populations. The 1C population with half of the DNA staining intensity of the 2C cell is comprised of the round and early elongated spermatids. As the nucleus becomes more compacted in the later stages of spermatid development, the DNA binding characteristics change and the second population of hypohaploid cells, with a 0.6-0.7C peak in the DNA histogram, emerges (Toppari, Eerola & Parvinen 1985).

Despite its wide usability, the DNA stain-based flow cytometry has significant limitations in studying the testis. First, the 2C population is very heterogeneous in the adult testis as all the somatic cell types and the different spermatogonia subtypes cluster within this population. Second, further limitations arise when the DNA flow cytometry is applied in studying testicular development, since both the somatic and the germ cells are rapidly proliferating. This results in the somatic cell subtypes clustering within 2C, proliferating, and 4C populations. To overcome these issues, flow cytometry has also been coupled with fluorescently labeled antibodies against specific cell types (Hou et al.

2011, Valli et al. 2014). The most common application has been to use antibodies targeted against cell surface markers on fresh cell preparations. However, the availability of suitable antibodies is limited, since most intense antibody development has been targeted for the needs of stem cell research and immunology. In addition, this approach renders the study of intracellular signaling pathways impossible.

A more recent application of flow cytometry has been to permanently fix and permeabilize the analyzed cell prior to staining procedures to allow for the detection of intracellular antigens (Strobl, Knapp 2004, Turac et al. 2013). The principle is similar to that in immunofluorescent microscopy, with the exception that the sample preparation is performed on cells in solution and the signal is detected with the flow cytometer instead of a microscope. Since the spatial information about the cells within the tissue is lost in flow cytometry, a more rigorous sample analysis and antibody validation is required than in analysis of homogenous cell lines by flow cytometry or when using immunohistochemistry on testis tissue. However, despite recent advances in applying intracellular flow cytometry in the study of testis, there is no agreement within the field on how to validate antibodies for flow cytometry experiments or for data normalization (Elzeinova et al. 2013, Ferguson, How & Agoulnik 2013, Wakeling, Miles & Western 2013).

2.2. The retinoblastoma and E2F protein families

2.2.1. Mitotic and meiotic cell cycle

One of the most fundamental functions of a cell is to produce a progeny by cell division. Extensive cell cycle control mechanisms exist to ensure that replication of DNA and cell division occur without the genetic integrity being compromised. During the cell cycle, a cell first grows and prepares for the upcoming division in the G_1 phase. The decision on whether to proceed to division is made in the G_1/S phase checkpoint. If the intracellular and extracellular conditions are favorable and cell division is permitted, the cell enters S phase where the DNA is replicated. A second checkpoint occurs after DNA replication, when the accuracy of the DNA replication is checked and possible errors are repaired. After this the cell divides by mitosis, where the genetic material is separated into two daughter cells. The driving force of cell cycle progression is the activation of different types of cyclins in all the phases of the cell cycle. The control of the whole cell cycle machinery is highly centered on controlling the cyclins and cyclin-dependent kinases and their upstream and downstream effectors.

2.2.2. The retinoblastoma protein family

The retinoblastoma protein was the first identified tumor suppressor (Lee et al. 1987a, Lee et al. 1987b). It was discovered that inactivating germline mutations of RB in humans cause malignant retinoblastoma tumors of the retina in children (Friend et al. 1986). Two other proteins (p107 and p130) with structures similar to that in RB were identified later and these three form the retinoblastoma protein family (Ewen et al. 1991, Zhu et al. 1993, Mayol et al. 1993, Hannon, Demetrick & Beach 1993, Li et al. 1993). The RB protein family can also be called the pocket protein family, according to the shared pocket domain which dictates most of the functions of the protein family.

RB family members have distinct roles during cell cycle regulation. RB is active during the G_0/G_1 phase and is inactivated by phosphorylation during the S phase transition. Conversely, p107 is activated as the cells enter the cell cycle, while p130 is active in quiescent cells (Classon, Harlow 2002). p107 and p130 share the most structural homology and functional redundancy. Mice *null* for either *p107* or *p130* are viable, but a compound deletion induces a severe phenotype (Cobrinik et al. 1996, Lee et al. 1996). In the absence of one pocket protein family member, the other ones may take over binding E2Fs (Lee et al. 2002). The RB proteins have different preferences for partnering with the E2F transcription factors. RB binds E2Fs 1-4 (Moberg, Starz & Lees 1996, Lees et al. 1993), while p107 and p130 associate preferably with the repressive E2Fs 4 and 5 (Fig. 6) (Hijmans et al. 1995, Moberg, Starz & Lees 1996).

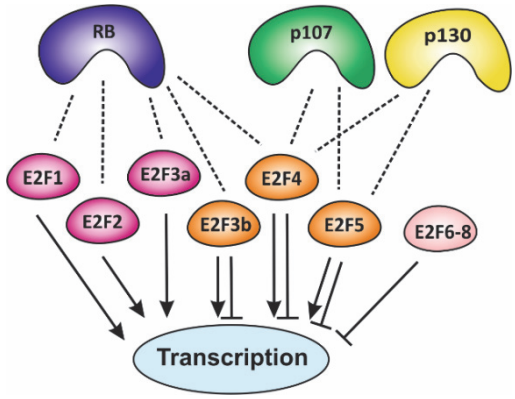

Figure 6. RB and E2F family interactions. RB binds preferentially E2Fs 1-4, p107 and p130 with E2Fs 4-5. E2Fs 6-8 do not interact with the RB family.

2.2.2.1. RB family in the cell cycle

Loss of RB function in mammalian cells is frequently associated with unrestrained and aberrant proliferation. RB-E2F complexes provide a mechanism to control the temporal expression of genes that are needed for cell proliferation, development, apoptosis and cell survival [reviewed in (Classon, Harlow 2002, Burkhart, Sage 2008)]. According to the classical view of the cell cycle control, RB is phosphorylated and deactivated by cyclin D/CDK4/6 complexes during G_1/S transition of the cell cycle, which results in freeing E2F to activate genes required for S-phase progression such as *Dhfr* and *Tk*, *Cdk2*, *Ccn A* and *E* and E2F (Fig. 7) (Wolgemuth, Manterola & Vasileva 2013). Overexpression of RB induces cell cycle arrest at G_1 (Qin et al. 1992, Huang et al. 1988), while loss of RB results in an accelerated transit through the G_1 phase and extended S phase (Herrera et al. 1996, Classon et al. 2000). RB also controls cell cycle progression independently of the E2Fs via stabilizing the CDK-inhibitor $p27^{Kip1}$ through different mechanisms (Ji et al. 2004, Binne et al. 2007).

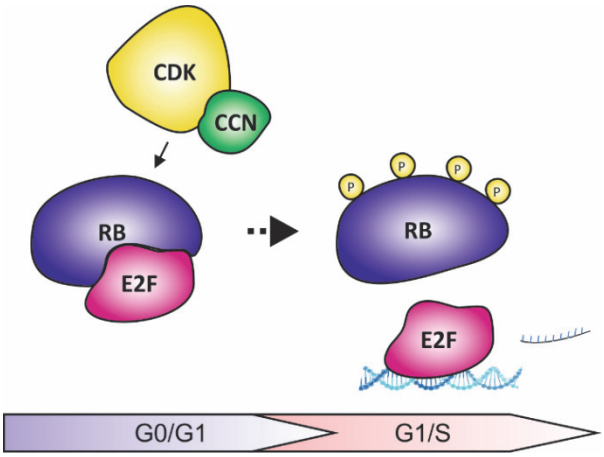

Figure 7. Classical view of RB and E2Fs in cell cycle control. In the G0/G1 phase the active underphosphorylated RB inhibits E2F by binding with the pocket domain. Upon G1/S phase transition cyclin (CCN)/cyclin-dependent-kinase (CDK) complexes inactivate RB by phosphorylation and E2F is free to activate genes required for the S phase.

2.2.2.2. Post-translational modifications of RB

Control of RB function during the cell cycle is achieved through extensive reversible post-translational modifications, unlike most cell cycle regulatory proteins which undergo cyclic degradation during cell cycle (Munro, Carr & La Thangue 2012).

RB is phosphorylated by cyclin D/Cdk4/6 complexes on S249, T356, S807, S811 and T826 in early G_1 leading to dissociation of the partners binding the LXCXE motif on RB. As the cell cycle progresses, other CDKs take over RB phosphorylation: cyclin E/CDK2 phosphorylate RB on S612 and T821 in the late G1 and early S phase and RB inactivation is maintained for the remaining cell cycle by cyclin A/CDK2 (Zarkowska, Mittnacht 1997). Phosphorylation of RB on S567, which is located in the core of the pocket region, disrupts binding with E2Fs (Harbour et al. 1999). As cells exit the mitotic cell cycle, protein phosphatase 1 (PP1) reactivates RB by dephosphorylation (Ludlow et al. 1993). RB is also phosphorylated by checkpoint kinases Chk1 and 2 during DNA damage, which leads to RB-mediated inactivation of E2Fs (Inoue, Kitagawa & Taya 2007).

In addition to phosphorylation, RB is also regulated by other post-translational modifications. Acetylation of RB inhibits CDK-mediated phosphorylation and is associated with differentiation of multiple different cell types (Chan et al. 2001, Nguyen et al. 2004, Pickard, Wong & McCance 2010). RB methylation during DNA damage responses and permanent cell cycle arrest of differentiating cells induces gene silencing via chromatin remodeling (Munro et al. 2010, Carr et al. 2011). Moreover, ubiquitination and SUMOylation have been shown to play a role in the post-translational control of RB (Uchida et al. 2005, Ledl, Schmidt & Muller 2005).

2.2.2.3. RB in apoptosis

RB can be both pro- and anti-apoptotic depending on the cellular context. RB can inhibit apoptosis through the negative control of the E2F transcription factors and by inducing senescence after DNA damage. The pro-apoptotic function of RB is mediated by a

specific interaction of RB with E2F1 via an alternative domain other than the pocket, and it is not connected to the other E2Fs (Dick, Rubin 2013). This interaction can resist inactivation by CDKs during the S phase and lead to recruitment of RB to E2F target genes (Wells et al. 2003, Cecchini, Dick 2011, Dick, Dyson 2003). DNA damage results in extensive post-translational modifications on both RB and E2F1, leading to the stabilization of the RB/E2F1complexes, activation of pro-apoptotic genes and repression of cell cycle related genes (Carnevale et al. 2012, Ianari et al. 2009).

2.2.2.4. RB in differentiation

Cell cycle exit precedes terminal differentiation in several cell types [for a recent review, see (Julian, Blais 2015)]. RB loss in quiescent terminally differentiated cell leads to different phenotypic outcomes than a developmental loss of RB (Sage et al. 2003, Andrusiak et al. 2012, Lorz et al. 2010).

Rb-null mice die *in utero* already at E14.5 due to defects in the central nervous system (CNS) and erythropoiesis (Jacks et al. 1992, Clarke et al. 1992, Lee et al. 1992). If the *Rb*-status is restored to normal in the extraembryonic cell lineages of an *Rb*-null embryo, many of the erythroid and neural defects can be rescued, but the animals die at birth due to abnormalities in the skeletal muscles (de Bruin et al. 2003, Wu et al. 2003).

RB cooperates with the E2F transcription factors to control differentiation. For example, in the osteoblast precursors, RB and E2F1 are both recruited to promoters to activate expression of osteocalcin and alkaline phosphatase (major marker of osteogenesis) during differentiation (Flowers, Xu & Moran 2013). However, *in vitro* and *in vivo* studies on differentiation can provide contradictory results. A study on immortalized *Rb*-deficient cell lines suggested that RB promotes osteoblast differentiation by directly interacting with RUNX2 (Thomas et al. 2001). Conversely, a conditional knockout of Rb in osteoblasts *in vivo* resulted in increased osteoblast differentiation by an E2F-dependent cell cycle defect (Berman et al. 2008).

RB has been shown to be critical, not only for the induction of differentiation but also for the survival of terminally differentiated cells. When RB is depleted from post-mitotic neurons, their cell cycle machinery is reactivated and the cells are lost by an E2F-independent mechanism (Andrusiak et al. 2012).

2.2.3. The E2F transcription factors

The E2 promoter targeting factors (E2Fs) were first discovered through their ability to bind RB (Helin et al. 1992). The E2Fs can function as oncogenes and tumor suppressors, depending on the context. Eight E2F genes coding for nine different proteins have been described in the mouse and human (Chen, Tsai & Leone 2009). Two different isoforms of *E2f3* are transcribed from the *E2f3* gene locus through the use of an alternative promoter within the locus (Leone et al. 2000, He et al. 2000). The longer *E2f3a* isoform was shown to be expressed only in actively proliferating cells and to activated genes in G_1/S, while the shorter b isoform is expressed in both quiescent and proliferating cells and represses gene expression in G_0, but also acts in concert with E2f3a in G_1/S to control gene expression (He et al. 2000, Leone et al. 2000, Chong et al. 2009a).The different

E2Fs have been traditionally classified as transcriptional activators (E2F1-3a) and repressors (E2F3b-8). Recent studies have, however, challenged this traditional grouping. Activator E2Fs in progenitor cells transformed to active transcriptional repressors upon differentiation *in vivo* by forming complexes with RB (Chong et al. 2009b). Loss of RB prevents the formation of the repressive RB/E2Fcomplexes and result in a strong activation of cell cycle genes and subsequent ectopic cell divisions (Chong et al. 2009b). Furthermore, E2F4 has been shown to both activate and repress gene expression *in vitro* and it can bind DNA on both long-range repressive complexes and distant enhancers of genes (Lee, Bhinge & Iyer 2011).

All the E2Fs share the same winged-helix DNA-binding motif (Zheng et al. 1999) and the same consensus DNA binding site (Lees et al. 1993, Buck et al. 1995). Consequently, there is considerable redundancy within the E2F family, especially between the activator E2Fs. In combined knockouts of all the activator E2Fs (E2F1-3), restoration of E2F3a expression was sufficient to support normal mouse development in the absence of the other activator E2Fs (Tsai et al. 2008). Surprisingly, expression of *E2f1* or *E2f3b* from the *E2f3a* locus was able to induce the same rescue effect, showing that temporal and spatial control of the expression of the different activator E2Fs may be more crucial than the presence of different family members (Tsai et al. 2008). The repressive E2Fs can compensate for each other as well, since loss of E2f3b led to a compensatory loading of the repressive E2F4 on E2F target genes in G_0 (Chong et al. 2009a).

Despite these examples of functional redundancy, the different E2Fs also have specific roles, which are mediated at large by their association with different cofactors. E2Fs bind DNA rather promiscuously, meaning that they do not require a consensus binding site for interaction. Instead, they may function as cofactors for other transcription factors and thus control the expression of a number of genes through an indirect interaction with DNA (Rabinovich et al. 2008). E2Fs can also associate with enhancer and repressor complexes that act from a long distance (Lee, Bhinge & Iyer 2011). E2Fs are important cofactors in mediating the transcriptional actions of the RB family members. In Drosophila larvae, dE2F1 and 2 occupied the vast majority of the sites where RBF (Drosophila RB homolog) bound and loss of dE2Fs abrogated RBF binding to chromatin, suggesting that most of the RBF-mediated transcriptional control is dE2F-dependent (Korenjak et al. 2012). E2F1 has been shown to mediate the recruitment of RB to CpG islands in all phases of the cell cycle in human cells (Wells et al. 2003).

The E2Fs are further divided into the classical (E2F1-6) and atypical (E2F7-8) subclasses based on their sequence homology. The classical E2F6 and the atypical E2Fs 7 and 8 all lack the carboxy-terminal activation domain, which is required for binding the RB protein family (Morkel et al. 1997, Lammens et al. 2009). They act as transcriptional repressors, independently of binding to the pocket proteins by recruiting chromatin remodeling enzymes to facilitate gene silencing (Lammens et al. 2009). E2F6 has been shown to be critical for restricting the expression of male germ cell-specific transcripts in somatic cells (Pohlers et al. 2005). E2F7 and E2F8 resemble each other very closely and they both are involved in the negative regulation of gene expression and the inhibition of proliferation (Maiti et al. 2005, Christensen et al. 2005). Ablation of both E2F7 and 8 leads to activation, ectopic E2F1 expression and massive E2F1- and

p53-dependent apoptosis in the embryo, which is a prime example of the interconnectivity of the E2F family members (Li et al. 2008)

2.2.3.1. Function of the E2Fs *in vivo*

The initial in vitro studies using mouse embryonic fibroblasts (MEFs) suggested that the activator E2Fs are required for cell proliferation. Loss of all the activator E2Fs (1-3) in MEFs abrogated proliferation and led to a cell cycle exit due to RB/p130-mediated transcriptional repression (Wu et al. 2001, Timmers et al. 2007). The proliferative ability of the triple-E2F-deficient (TKO) MEFs was partially rescued *in vivo*, as they were able to form teratomas in nude mice and embryo development proceeded until E9.5 even in the absence of all the activating E2Fs (Chong et al. 2009b). Nevertheless, there are many examples of affected proliferation, apoptosis and cell survival in different cell types in the absence of the different activator E2Fs, implying context-specific roles for the different family members. For instance, ablation of E2Fs 1-3 in retinal progenitors does not block proliferation, but induces apoptosis (Chen et al. 2009). A similar phenomenon occurs in the triple-E2F-deficient lens, where initial cell cycle entry of the lens cells is not affected, but upon differentiation they fail to exit the cell cycle and undergoes massive apoptosis (Wenzel et al. 2011). Furthermore, $E2F3^{-/-}$ animals suffer from heart failure due to decreased proliferation of cardiac myocytes (King et al. 2008). In the bone marrow, the proliferation of the myeloid progenitors does not depend on the activator E2Fs but they support the survival of these cells by acting as transcriptional repressors (Trikha et al. 2011). As the myeloid progenitors differentiate to bone marrow macrophages, the activator E2Fs switch back to transcriptional activators and promote proliferation (Trikha et al. 2011).

E2F1 is the primary mediator of apoptosis and induction of E2F1 is often responsible for apoptosis observed as a result of alteration in the other E2F family members (Martinez et al. 2010). However, E2F2 and E2F3a can induce apoptosis independently, albeit not as efficiently as E2F1 (Vigo et al. 1999, DeGregori et al. 1997).

2.2.3.2. E2F transcription factors control the differentiation of several tissues

An intricate interplay between the different E2F transcription factors is required for the differentiation of multiple different cell lineages. So far the most studies have focused on the contribution of E2F3 to neural and myoid cell differentiation. E2F3b attenuates the expression of genes required for myogenic differentiation in an RB-independent manner in an *in vitro* model for myocyte differentiation (Asp et al. 2009). Moreover, E2F3a and E2F3b counterbalance each other in the control of SOX2 expression in neural progenitors (Julian et al. 2013). Loss of E2F3b led to an increased expansion and decreased differentiation of neural progenitors and loss of E2F3a resulted in the opposite (Julian et al. 2013). E2F1 stimulated proliferation of adipogenic progenitors via activation of PPARγ expression, while E2F4 suppressed PPARγ-expression and promoted differentiation to adipocytes (Fajas et al. 2002). In the endocrine progenitors, E2F1 directly induced the expression of neurogenin 3 (NGN3), which drives pancreatic cell differentiation (Kim, Rane 2011). E2Fs appear to have a conserved function in multiple different cell lineages to regulate cell cycle-related genes, but upon

differentiation they assume a very tissue-specific target gene pattern, which suggests a new inter-linkage between cell cycle regulation and differentiation via a switch in the recruitment sites of the E2F transcription factors (Julian et al. 2015).

In summary, the different E2Fs have critical roles in proliferation, apoptosis, cell survival and differentiation *in vivo*, but the functional redundancy between the family members and the extensive context-dependency of their function make it impossible to create a conclusive description of their function within the whole organism.

2.2.4. The RB/E2F-pathway in the testis

The RB family proteins and the E2F transcription factors show distinct expression and post-translational modification profiles in the testes, suggesting a dynamic role for them in spermatogenesis (Fig. 8) (Yan et al. 2001, Novotny et al. 2007, Bartkova et al. 2003, El-Darwish, Parvinen & Toppari 2006). There is mounting evidence of an essential role for these factors in the testis from both mouse and human studies (Hu, de Rooij & Page 2013, Bartkova et al. 2003, Nalam et al. 2009).

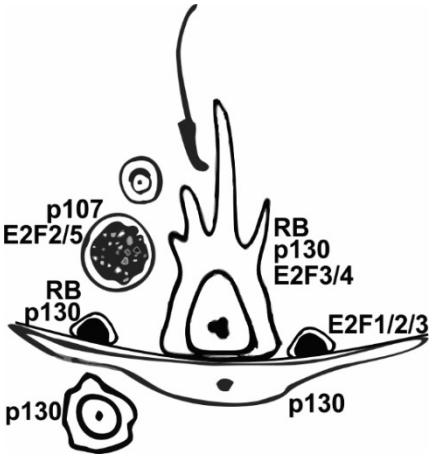

Figure 8. The expression patterns of the RB and E2F protein families in the rodent testis. The Sertoli cells express RB, p130, E2F3 and E2F4. RB, p107 and E2F1-3 are expressed in spermatogonia. During meiosis, p107 and E2F2 and 5 are present. (Yan et al. 2001, El-Darwish, Parvinen & Toppari 2006).

A major hallmark differentiating the fetal testis development from the ovarian pathway is the induction of mitotic arrest in the male germ cells at E12.5 (Hilscher et al. 1974). Not surprisingly, RB is a critical controller of the mitotic arrest in the male germs cells. Fetal *Rb*-null testis *in vitro* shows a delay in mitotic arrest, but compensatory $p27^{Kip1}$ and $p15^{INKB}$ activation resulted in cessation of germ cell proliferation at E16.5 (Spiller, Wilhelm & Koopman 2010). In the postnatal rat testis, all the RB family members are expressed in spermatogonia: RB in A spermatogonia, p130 in the Kit negative undifferentiated spermatogonia, and p107 in all developmental stages of germ cells from spermatogonia to late pachytene spermatocytes (Yan et al. 2001). SCF-induced spermatogonial proliferation in vitro was associated with RB phosphorylation, as would be expected from increased cell cycle entry (Yan et al. 2001). Conversely, spermatogonial apoptosis coincided with RB hypophosphorylation and spermatocyte apoptosis with decreased p107 expression (Yan et al. 2001).

The role of RB in the germ cells was further explored using an *Mvh*-cre mediated ablation of RB in the gonocytes at E15.5 (Hu, de Rooij & Page 2013). As a result, the proliferation of PLZF-positive spermatogonia increased but the transit amplifying spermatogonial stem cell pool was lost, leading to gradual exhaustion of spermatogenesis. This did not, however, affect the differentiation of the germ cells, as normal sperm was produced even in the absence of RB. RB loss in cultured SSCs induced cell cycle arrest at G_2/M, p53-dependent apoptosis and E2F1-dependent activation of DNA damage responses, while a constitutive activation of RB impaired self-renewal and colonization in transplantation assays (Tanaka, Kanatsu-Shinohara & Shinohara 2015). RB loss in murine spermatogonia does not induce the development of germinal neoplastic cells *in situ*, (GNIS; formerly referred to as carcinoma *in situ*, CIS) or testicular germ cell tumors, even though RB loss is associated with germ cell tumors and GNIS cells in the human testis (Bartkova et al. 2003). The tumor-suppressive function of RB may have been compensated by $p27^{Kip1}$ or the other RB family members in these germ cell-specific RB knockout models. Interestingly, the role of RB in the female germ cells, oocytes, appears to be very different from the male. Oocyte-specific ablation of *Rb* results in both aberrant proliferation and differentiation, since the *Rb*-deficient oocytes formed ovarian teratomas (Yang et al. 2015).

E2F1 protein was expressed in germ cells from A spermatogonia to zygotene spermatocytes in the adult mouse testis (El-Darwish, Parvinen & Toppari 2006). In the human testis, *E2f1* mRNA expression was posttranscriptionally downregulated in GNIS cells, which may have contributed to the survival of this premalignant cell type (Novotny et al. 2007). Both loss and overexpression of *E2f1* led to disruption of spermatogenesis in the mouse (Agger et al. 2005, Yamasaki et al. 1996). In the first reports on the E2F1 knockout phenotype, testicular atrophy was observed from the age of 3 months onwards (Yamasaki et al. 1996, Field et al. 1996). The activator E2Fs appeared to have a dose-dependent role in mouse spermatogonia, since $E2F1^{-/-}E2F3^{+/-}$ knockout mice exhibited an exacerbated testicular atrophy in comparison with the $E2F1^{-/-}$ mice (Cloud et al. 2002). Transgenic overexpression of E2F1 in the adult testis led to a rapid increase in apoptosis of spermatocytes and accumulation of *carcinoma in situ*-like cells after a long exposure to the transgene and loss of more mature germ cells (Agger et al. 2005). This transgene-induced apoptosis in the testis was p53-independent (Holmberg et al. 1998). E2F1 has also been suggested to play a role in Sertoli cell function and cause Sertoli cell apoptosis in the absence of retinoblastoma protein (Nalam et al. 2009).

E2F2 was expressed in a subpopulation of spermatogonia and in pachytene spermatocytes in the mouse testis (El-Darwish, Parvinen & Toppari 2006). The repressive E2F5 was expressed in germ cells from B spermatogonia to late pachytene spermatocytes in the rat testis (El-Darwish, Parvinen & Toppari 2006). E2F6 has been shown to repress the expression of germ-line specific genes in mice (Pohlers et al. 2005).

The RB family has been shown to localize also to the somatic cell of the testis. All the RB family members are expressed in the juvenile (P5) Sertoli cells in the rat testis (Yan et al. 2001). p107 and p130 showed surprisingly different expression patterns in the adult rat testis, with p130 expression in the terminally differentiated somatic cells (Sertoli, Leydig and PTM) and p107 in spermatogonia and spermatocytes (Yan et al. 2001).

Mouse Sertoli cells have been shown to express both E2F3 and E2F4 (El-Darwish, Parvinen & Toppari 2006). Concomitant expression of p130, RB and E2F4 in Sertoli cells may imply that the transcriptional repression in these terminally differentiated cells is governed by an E2F-dependent mechanism.

Despite these studies, the roles of the RB and E2F protein families in the mouse testis remain largely unknown.

3. AIMS OF THE STUDY

The main objective of this study was to dissect the role of the retinoblastoma protein and the E2F transcription factors in the complex nature of cell cycle control during testicular development and in the adult testis. In addition, this study aimed to develop new methods to study testicular development using flow cytometry.

The specific aims were:

1. To study how RB influences Sertoli cell development using a Sertoli cell specific RB knockout mouse model.
2. To show the developmental expression pattern of E2F1 in the mouse testis and to study the mechanisms of testicular atrophy in E2F1$^{-/-}$ testis.
3. To develop a novel rapid method for analysis of the complex testicular tissue by flow cytometry coupled with intracellular staining of antigens.

4. MATERIALS AND METHODS

Complete information on the manufacturers of the reagents are given in the original publications (I-III).

4.1. Experimental animals (I-III)

Amh-cre (Plekha5$^{Tg(AMH-cre)1Flor}$) (Lecureuil et al. 2002) in C57-B6/SJL genetic background and Rb$^{flox/flox}$ (FVB;129-*Rb1*^{tm2Brn}/Nci) in a mixed FVB:129 genetic background (Marino et al. 2000) mouse strains were cross-bred to obtain Amh-cre^{+}/Rb$^{flox/flox}$ (SC-RbKO) mice with conditional knockout of *Rb* specifically in Sertoli cells. Both cre-/Rb$^{flox/flox}$ and cre+ Rb$^{wt/wt}$ were used as controls in the experiments. (I) Commercially available E2F1$^{-/-}$ mice (B6;129S4-E2f1tm1Meg/J) (Field et al. 1996, Yamasaki et al. 1996) were cross-bred with C57Bl/6 mice for eight consecutive generations to obtain a uniform genetic background. Age-matched controls were used throughout the studies and the animals were also litter-matched if possible. (II) Spraque-Dawley rats of different ages were obtained from the University of Turku Central Animal Laboratory's breeding stocks. (III)

All animals were housed under environmentally controlled conditions (12h light/12h darkness; temperature, 21±1 °C) in the animal facilities of the University of Turku. The mice were fed the mouse chow SDS RM-3 (Special Diet Service, E, Soy-free, Whitman) and tap water ad libitum. All procedures were carried out according to the institutional and ethical policies of the University of Turku and approved by the local ethics committee on animal experimentation.

4.2. Fertility test and sperm counts (I, II)

The epididymides of adult mice were collected, rinsed with saline solution, and weighed. The sperm were released from cauda epididymides into KSOM-AA medium (cat. no. MR-121, specialty media, Millipore) by applying pressure with forceps. After removal, the sperm were incubated at 37°C for 15 minutes. The number of spermatozoa was counted under a microscope (Olympus CK 2, Olympus) using a Bürker chamber. Sperm smears were stained with the Papanicolaou stain for morphological analysis. (I)

Male mice (n=3-4) were bred with four different C57Bl/6J females. The animals were mated and on the following day the presence of a copulative plug was observed. In the absence of a copulative plug, the animals were allowed to mate two more times within the week and the females were checked for copulative plugs. The litter size and the sexes of the resulting pups were recorded and the results of the breeding were presented as an average litter size/male. (I, II)

4.3. Hormone measurements (I, II)

Serum LH and FSH levels were measured by immunofluorometric assay (IFMA, Delfia, Wallac Oy, Turku, Finland) as described previously (van Casteren, Schoonen & Kloosterboer 2000, Haavisto et al. 1993). (I, II) The sensitivity of the LH and FSH assays

was 0.03µg/L in 25µl and 0.1µg/L respectively. For serum testosterone determination, 25µl aliquots were used. The sera was extracted twice with 2 ml diethyl ether and evaporated to dryness. The residues were reconstituted in PBS with 0.1% BSA and measured using standard radio-immuno-assay, as described previously (Huhtaniemi, Nikula & Rannikko 1985). The sensitivity of the assay was 1nmol/L. All serum samples were assayed at the same time using specific antiserum and radiolabeled hormones diluted the same day. (I, II)

4.4. Histology and histomorphometry (I, II)

Testes for histological analysis were fixed in Bouin's fixative and stained with hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS). For histology, samples were cut from three different levels of the paraffin-embedded testes, to ensure the analysis of different cells in each section. The samples were imaged with a Leica DMRBE microscope (I, II) or a Pannoramic Slidescanner (3D Histech) (II). Seminiferous tubule diameters were measured from 30 randomly selected round seminiferous tubule cross-sections using Leica IM500 software (n=3-4 animals). In the gross morphological analysis, seminiferous tubules from a given sample were grouped into three groups according to their histological appearance: normal (all stage-appropriate layers of germ cells present in the seminiferous epithelium), germ-cell-loss (apparent loss of germ cells) and Sertoli-cell-only (complete absence of germ cell in the seminiferous tubules cross-section) (II).

4.5. Flow cytometry of mouse testes (II)

The development of the flow cytometry protocol is described in the Results section and the protocol itself is shown in (III). In (II) four $E2F1^{+/+}$ and $E2F1^{-/-}$ animals at P20 and P40 were included in the assay. Briefly, whole testis tissue was cut into 10 mg pieces and disrupted both mechanically and enzymatically to obtain a single cell suspension. The cells were fixed using 4% paraformaldehyde, followed by a permeabilization with 90% methanol. To assess the germ cell populations in the testes, all the cells were stained with propidium iodide (Sigma-Aldrich) to analyze the DNA content of the cells. A fixed volume of samples was run with a BD LSRII flow cytometer (Becton Dickinson). The testicular cell populations were gated based on their DNA amounts: 2C cells (spermatogonia and somatic cells), 4C cells (primary spermatocytes and proliferating cells in the G2/M phase of the cell cycle), proliferating cells in the S phase with a DNA amount of between 2C and 4C populations, and 1C cells (spermatids). The number of cells in each population was represented as cells per milligram of tissue. (II)

4.6. Immunohistochemistry

4.6.1. Standard immunohistochemistry (I, II)

4% paraformaldehyde (PFA) fixed paraffin-embedded testicular tissue sections were dewaxed using serial incubations in xylene and ethanol. Antigen retrieval was performed in a pressure cooker in 10mM sodium citrate buffer, pH 6 or in TE buffer, pH 9. Endogenous peroxidase activity was quenched with 3% H_2O_2. The samples were blocked with suitable sera depending on the secondary antibody. The primary antibodies, their

concentrations, and their downstream detection systems are listed in Table 1. The primary antibody was detected with different HRP-linked secondary antibodies and DAB. The following kits were used: the Vectastain Elite kit (Vector laboratories, Inc), the Envision anti-mouse or anti-rabbit kit (Dako), the Goat-on-Rodent-HRP-polymer kit (Biocare Medical) and the Novocastra Novolink Polymer Detection Systems (Leica Biosystems).

4.6.2. Double label immunohistochemistry (I)

PFA-fixed paraffin-embedded adult SC-RbKO and control testes samples were processed for immunohistochemical staining of p130 or p107 (Table 1.) using an HRP-based detection system (Goat-on-Rodent-HRP-polymer kit for p130 and Envision anti-rabbit for p107). After the DAB reaction, the samples were washed with a buffer and blocked with a 5% normal donkey serum. Next, they were incubated overnight with the anti-vimentin antibody. The following day the vimentin signal was detected using the Vectastain ABC-AP Standard kit (cat. # AK-5000, Vector Laboratories) and Vector Blue alkaline phosphatase substrate (cat. # SK-5300, Vector Laboratories) and the samples were mounted aqueously using Vectastain Hard-set mounting medium (cat. # H-1400, Vector Laboratories).

4.7. Immunofluorescence

4.7.1. Standard immunofluorescence (I-III)

The samples were processed until antigen retrieval the same way as for immunohistochemistry. Thereafter, autofluorescence was quenched with 100mM NH_4Cl. After washing in buffer (PBS or TBS), the unspecific binding was blocked with a suitable serum- Depending on the secondary antibody normal goat serum, normal rabbit serum or normal donkey serum was used. Next the samples were incubated with the primary antibody diluted in the blocking solution. AlexaFluor goat or donkey secondary antibodies (Life Technologies) were used to detect the primary antibodies. DAPI was used as a nuclear counterstain and the slides were mounted with Mowiol (I) or Vectashield Hard-set mounting medium (cat. # H-1400, Vector Laboratories). The samples were imaged using a Zeiss Axiovert microscope.

4.7.2. Immunofluorescent detection of BrdU and cell markers (I, II)

Cell proliferation and DNA synthesis in specific testicular cell populations were evaluated using 5-bromo-2-deoxyuridine (BrdU) incorporation. Male mice at different postnatal time points were injected with BrdU (200 mg/kg body weight; Sigma) in NaCl (0.9%). After two hours, the animals were sacrificed, then the testes and small intestines collected and fixed in 4% PFA (van de Kant, de Rooij 1992). Paraffin-embedded sections were processed as described previously in (Nurmio et al. 2009) for detection of BrdU and different cell markers (I: Wilm's tumor 1 (WT1) for Sertoli cells; II: MAGE-B4 for spermatogonia and WT1 for Sertoli cells). The sections were examined with fluorescence microscopy (Zeiss AxioVert 200M). Cells positive for BrdU and cell markers were counted from 20 round cross-sections in each testis. Primary antibody suppliers and catalogue numbers are listed in Table 1.

4.7.3. Immunofluorescent detection of apoptosis with TUNEL and cell markers (I,II)

Evaluation of apoptotic germ cells and Sertoli cells was performed by fluorescent dual TUNEL (TdT-mediated dUTP-nick end labeling) and immunofluorescent detection of cell type markers (I: WT1 for Sertoli cells; II: MAGE-B4 for spermatogonia). Commercially available terminal transferase and biotin-16-dUTP (Roche/Boehringer Mannheim) were used. Briefly, the 4% PFA fixed sections were deparaffinized and rehydrated. The permeabilization step was carried out in a pressure cooker in a 0.1 M citrate buffer, pH 6. Slides were rinsed with TBS, and immersed in 100mM NH_4Cl to block autofluorescence. Sections were incubated with the TUNEL reaction mixture for 60 min at 37°C and the reaction was stopped using 300mM NaCl, 30mM NaCitrate. Unspecific binding of the primary antibody was blocked with 3% BSA in TBS and the primary antibody, diluted in blocking solution, was incubated overnight at 4°C. Next a sequential incubation of AvidinTexasRed (Vector labs) and AlexaFluor488 anti-rabbit antibody was performed to detect the TUNEL reaction and the primary antibodies. DAPI was used as a nucleic counterstain. A positive control for the TUNEL reaction was prepared by adding 1U/μL DNAse for 30 minutes at 37°C and in the negative control the terminal deoxynucleotidyl transferase (TdT) was omitted. The number of apoptotic cells per section was determined by counting intratubular TUNEL-positive cells. In the SC-RbKO, round seminiferous tubule cross-sections were chosen randomly, the positive cells were counted, and the results were presented as the percentage of apoptotic Sertoli cells. In the E2F1KO study, the number of MageB4 or WT1 and TUNEL or BrdU-positive and double positive cells was determined as an intratubular cell count per analyzed round seminiferous tubule cross-section. In the samples from the P10 testes, the fifty round cross-sections analyzed were chosen randomly. For the P40 animals, only tubule cross-sections in stage VII-VIII of the seminiferous epithelial cycle were chosen for the analysis and the minimum number of analyzed tubules was five per animal. The stage-specific analysis was done to decrease the variation caused by the stage-specific pattern of apoptosis and to increase the sensitivity of the assay as there is almost no apoptosis in these stages in the normal testis.

4.8. RNA *in situ* hybridization (II)

RNAscope® 2.0 Assay (cat. ACD-310035, Advanced Cell Diagnostics) was used to detect *E2f1* transcripts on PFA-fixed, paraffin embedded section from 6, 10, 20, and 40-day-old wild-type testis (Wang et al. 2012). The probe against mmE2f1 was custom-made and the standard positive control (Mm-PPIB, cat. ACD-313902) and negative control (DapB, cat. ACD-310043) probes were used. The assay was performed according to the manufacturer's instructions. The negative signal threshold was set according to the manufacturer's instructions to no staining or less than 1 dot to every 10 cells per cell-type. The samples were imaged using Pannoramic Slidescanner (3D Histech).

For a more precise identification of different germ cell types, the RNAscope assay was coupled to the immunohistochemistry of PLZF and γH2AX-S139 (antibody information in Supplementary Table S1). The RNAscope assay was performed according to the manufacturer's instructions until the DAB reaction. After washing the slides with dH2O, the samples were blocked with 5% horse serum in PBS for 1h at RT. The primary

antibodies were diluted 1/200 in the blocking solution and they were incubated overnight at +4°C. After washing off the primary antibody, the slides were incubated with the biotinylated horse anti-mouse (cat. # BA-2000, Vector laboratories) or anti-goat (cat. # BA-9500, Vector laboratories) secondary antibodies diluted 1/400 in blocking solution for 30 minutes at RT. The Vectastain ABC-AP Standard kit (cat. # AK-5000,Vector Laboratories) was used to visualize the reaction according to the manufacturer's instructions. Vector Blue alkaline phosphatase substrate (cat. # SK-5300, Vector Laboratories), supplemented with a drop of Levamisole (cat. # SP-5000, Vector Laboratories), was used to visualize the signal. The slides were aqueously mounted using Vectashield Hard-set mounting medium (cat. # H-1400, Vector Laboratories).

4.9. Western blot and co-immunoprecipitation (I)

Fresh whole testes were homogenized in a non-denaturing lysis buffer (50mM Tris-HCl, 170mM NaCl, 5mM EDTA, 1mM DTT, 1% NP-40) containing the protease inhibitor cocktail cOmplete, Mini (Roche). The lysates were sonicated using Bioruptor (Diagenode) at 2 x 30s. After incubation on ice, the lysates were centrifuged at 10000 x g for 25 minutes. 1mg of protein was pre-cleared using 2µg of normal rabbit IgG and 20µl of Dynabeads Protein G (Invitrogen). The lysate was then incubated with 2µg of E2F3 C-18 antibody (sc-878, Santa Cruz) overnight at 4°C. A control sample was prepared by adding 8µg of E2F3 blocking peptide (sc-878P, Santa Cruz) to the reaction. 50µl of Dynabead Protein G was used to pull down the complexes. The immunoprecipitate together with the input lysate was loaded to Mini-Protean TGX 4-20% SDS-PAGE gel (Bio-Rad). RB was detected from the upper part of the blot with the mouse monoclonal anti-RB (MAB3186, Chemicon) and E2F3 was detected from the lower part of the blot with the E2F3 C-18 (sc-878, Santa Cruz).

Table 1. Antibodies used in the studies.

Name	Manufacturer	cat. code.	conc.
WT-1	Santa Cruz	180	1/200
PLZF	RD systems	AF2944	1/200
MAGE-B4	non-commercial rabbit antisera (Österlund et al.)		1/200
BrdU	Roche	11170376001	1/50
γH2A.X-Ser139	Millipore	05-636	1/200
RB	BD Biosciences	G3-245	1/200
RB	Chemicon	MAB3186	1/500
CRE	Covance	MMS-106P	1/200
p130	Santa Cruz	C-20, sc-633	1/400
p107	Santa Cruz	C-18, sc-318	1/400
E2F3	Santa Cruz	C-18, sc-878	4µg/ml
E2F3 blocking peptide	Santa Cruz	sc-878P	12µg/ml
Claudin 11	Santa Cruz	H-107, sc-25711	1/200
Keratin 18	Santa Cruz	C-04, sc-51582	1/200
Espin	BD Biosciences	611656	1/50
ATM-S1981	Rockland	200-301-400	1/100
β-actin	Sigma	AC-15, A1978	1/1000

4.10. *In vivo* shRNA (I)

For silencing the expression of *E2f3* (*E2f3a*, *E2f3b*), an expression vector containing shRNA that inhibits both isoforms was constructed using a previously published sequence (Smith et al. 1996). The annealed oligos were inserted into the BglII/HindIII sites of the pSUPER.gfp/neo vector (OligoEngine, Seattle, WA) following the manufacturer's instructions. Electro-competent *E.coli* cells were transformed by the shRNA-E2F3 vector or the pSUPER.gfp/neo control empty vector. All vectors were sequenced by The Finnish Microarray and Sequencing Centre (CBT sequencing service, Turku, Finland). Plasmids were propagated in the bacteria by standard procedures and purified using a Maxiprep kit (Qiagen).

The plasmid DNA was diluted with 5% glucose to the chosen concentration (2 µg/ in 10µl per testis), and complexed with *in vivo*-jetPEI (cationic polymer transfection reagent, PolyPlus Transfection, Illkirch, France) according to the manufacturer's instructions. SC-RbKO male mice at PND15 were anesthetized with isoflurane. The testes were pulled out from the abdominal cavity, and approximately 10 µl of plasmid DNA solution was injected into the rete testis using glass capillaries under a binocular microscope as previously described (Sofikitis et al. 2008). The testes were then returned to the abdominal cavity, and the abdominal wall and skin were closed with sutures. Injected animals were sacrificed at the age of 10–12 weeks; testes were fixed in 4% paraformaldehyde.

4.11. Gene expression profiling with microarray (I, II)

Total RNA was isolated with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). P10 SC-RbKO, SC-Rb+/- and control testes (I) and P20 $E2F1^{+/+}$ and $E2F1^{-/-}$ (II) snap-frozen testis tissue was used, n=4/per group. The RNA was precleaned using RNeasy MinElute Cleanup Kit (Qiagen). The gene expression was analyzed using Sentrix MouseWG-6 v2.0 Expression Bead Chip-microchip (Illumina Inc) by the Finnish Microarray and Sequencing Centre at the Turku Centre for Biotechnology according to the manufacturer's instructions. The arrays were scanned with Illumina Bead Array Reader and the data was obtained using GenomeStudio v. 2011.1; Gene Expression Module v. 1.9.0-software. Normalization and statistical analyses of the microarray data were performed using the statistical software R package limma (http://www.R-project.org) or Sigma Stat 3.1 (SPSS Inc.).

4.12. Downstream functional data analysis workflow (II)

The differentially expressed transcripts in the $E2F1^{-/-}$ microarray were ranked according to their likely expression site. Information on the relative expression levels of the transcripts in each testicular cell type was derived from the RNA-seq data published by Soumillon et al. in 2013 (GEO accession code GSE43717) (Soumillon et al. 2013). Data on Sertoli cells, spermatogonia, spermatocytes and spermatids were chosen for the purposes of this study. The RNA-seq data analysis was performed using the Chipster software (Kallio et al. 2011). The raw data was aligned to reference using TopHat2 and statistically significant differential expression over the cell types was analyzed using the

DESeq^2 tool (Love, Huber & Anders 2014). The transcripts were ranked according to their primary expression site by ranking the pair-wise comparisons (for example., a Sertoli cell enriched transcript should have high expression levels when compared to the other germ cell data sets and vice versa) and the differentially expressed transcripts from the E2F1$^{-/-}$ array were compared against these lists. The resulting transcript lists were referred to as the cell-type weighed transcript lists. Enriched biological process GO terms were analyzed using the online tool Amigo by Gene Ontology Consortium (geneontology.org) (Ashburner et al. 2000). The cell-type weighed gene ID lists were submitted to the tool using *Mus musculus* as the reference and Bonferroni correction for multiple hypothesis testing. The obtained GO term lists and adjusted p-values were visualized using the ReviGO software to assess general trends and to filter redundant terms (Supek et al. 2011). The enrichment of putative E2F1 target genes in the lists of altered transcripts was analyzed using the PASTAA tool with stringent settings (-200 to + 200bp, sites conserved between *Mus musculus* and *Homo sapiens*, maximum affinity) (Roider et al. 2009).

4.13. Quantitative RT-PCR (I,II)

The total RNA was isolated from the testis tissue of SC-RbKO, E2F1$^{-/-}$ and control animals at different time points in the same way as was done for the microarray analysis. cDNA synthesis and quantitative RT-PCR were performed using the DyNAmo HS two step SYBR Green qRT-PCR kit (Finnzymes Oy, Espoo, Finland). The data were normalized by relating gene expression to the mouse ribosomal protein L19 (*L19*) (I, II), peptidyl-prolyl isomerase A (*Ppia*, cyclophilin A) (I, II) and hypoxanthine guanine phosphoribosyl transferase (*Hprt*) using the Pfaffl method (Pfaffl 2001).

4.14. Statistical analyses (I-III)

SigmaStat software (SigmaStat 3.5 for windows; SPSS Inc) (I) and GraphPad Prism 5 (II, III) were used for conducting the statistical analyses and to plot the data. The data was first inspected for normal distribution by plotting the data and Kolmogorov-Smirnov normality test, if it was applicable. Data from two experimental groups was analyzed using an unpaired t-test. Data with three or more experimental groups was analyzed using one-way ANOVA followed by the Holm–Sidak test (I) or Tukey's test (II, III). The threshold for statistical significance was set at $p<0.05$.

5. RESULTS

Main results of studies I-III

I. Maintenance of terminal differentiation in Sertoli cells is dependent on an RB-mediated inhibition of E2F3.
II. E2F1 has a differential role in the control of SSC fate and germ cell apoptosis in the adult and juvenile testis.
III. Testicular cell populations can be accurately studied using flow cytometry to detect intracellular antigens.

5.1. RB interacts with E2F3 to control terminal differentiation of Sertoli cells (I)

5.1.1. Testicular phenotype of the SC-RbKO (I)

Amh-cre and $Rb^{flox/flox}$ mouse strains were cross-bred to obtain a conditional knockout of *Rb* in Sertoli cells (SC-RbKO) from E14.5 onwards (Lecureuil et al. 2002). Despite the developmental loss of RB, the testis differentiation was normal and the 1-month-old SC-RbKO testes differed from the controls only subtly in size or morphology (I: Fig. S2). However, the SC-RbKO testes atrophied gradually by the age of 10 weeks, the SC-RbKO testes were significantly smaller than the control testes, and the seminiferous tubule architecture was severely disrupted (I: Fig. 1b-c). SC-RbKO mice were initially sub-fertile with sperm counts that were only a fraction of normal (I: Fig. 1d) and the mice became infertile at the age of 6 months as the phenotype progressed. At the age of 6 weeks, the testicular architecture of the SC-RbKO was still normal, but increased germ cell apoptosis was observed as a sign of Sertoli cell dysfunction (I: Fig. 2a, S3). In the older SC-RbKO mice, the Sertoli-germ cell contacts became severely disrupted and the immature germ cells sloughed to the seminiferous tubule lumen and the cauda epididymis (I: Fig. 1e). This coincided with an abnormal accumulation of ectoplasmic specialization and BTB proteins (espin and claudin11, respectively) (I: Fig. S3).

5.1.2. Sertoli cell proliferation and apoptosis in the adult SC-RbKO (I)

Sertoli cell proliferation during the normal proliferative period (at P7 and P15) was unaltered in the SC-RbKO, but at P30 a significant proportion of the *Rb*-deficient Sertoli cells re-entered the cell cycle, consistent with the role of RB as a gatekeeper for maintaining quiescence (I: Fig. 2b-c). As the Sertoli cell phenotype progressed, the Sertoli cells began to express Keratin 18 (a marker for immaturity) as a sign of dedifferentiation (I: Fig. 2b). Despite the aberrant proliferation, no Sertoli cell tumors formed in the aging SC-RbKO (I: Fig. S2). The increased apoptosis of Sertoli cells in the adult SC-RbKO likely contributed to the inhibition of tumorigenesis (I: Fig. 2a). The ectopic cell cycle activation was associated with a nuclear localization of phosphorylated ATM (a marker for DNA double strand break repair) indicating a possible accumulation of DNA damage in the adult SC-RbKO Sertoli cells (I: Fig. 2b).

5.1.3. E2F3 function in the SC-RbKO testis (I)

E2F transcription factors are the main downstream targets of RB in the cell cycle control. E2F3 and E2F4 have been shown to be expressed in the mouse Sertoli cells (El-Darwish, Parvinen & Toppari 2006). A strong E2F3 signal was detected in spermatogonia and Sertoli cells in the adult SC-RbKO and control testes (I: Fig. 3a-c).

Since E2F3 has been shown to induce ectopic proliferation and apoptosis in the $Rb^{-/-}$ lens and CNS (Ziebold et al. 2001), we decided to further explore the possible contribution of E2F3 to the SC-RbKO phenotype. Co-immunoprecipitation showed that RB and E2F3 interacted in the juvenile (P10) wild-type testis (I: Fig. 3d). No clear interaction was observed in the adult testis (I: Fig. 3d), but this was likely due to insensitivity of the immunoblotting. However, the p107 and p130 began to bind E2F3 in the adult SC-RbKO testis, implying a role for the RB family in the control of E2F3 in Sertoli cells (I: Fig. 3d). The interaction of p107 with E2F3 was unexpected, as p107 and E2F3 do not colocalize in the same cell types in wild-type mouse testes. However, ectopic expression of p107 was activated in the SC-RbKO Sertoli cells, which explained the p107/E2F3-interaction (Fig. 9., I: Fig. 4a,).

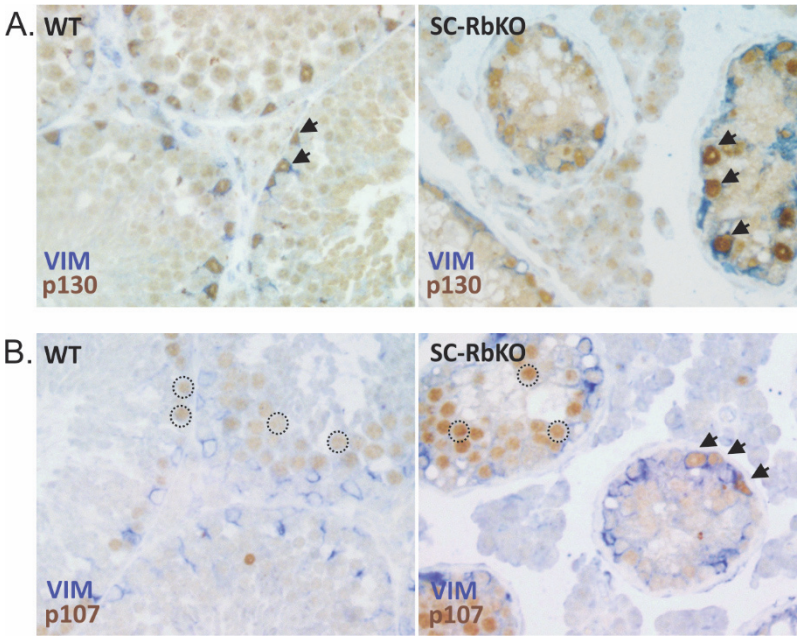

Figure 9. Ectopic expression of p107 is induced in the adult SC-RbKO Sertoli cells. Double-label immunohistochemistry was used to detect Vimentin (VIM, Sertoli cell marker) and RB protein family members A. p130 and B. p107 in 10-week-old control and SC-RbKO testis. Dashed circles = pachytene spermatocytes, black arrows = Sertoli cells.

To test whether deregulated E2F3 is the main downstream mediator of the SC-RbKO phenotype, *E2f3* was knocked down *in vivo* using a specific shRNA against both *E2f3* isoforms under the hypothesis that the *E2f3* knockdown would rescue the SC-RbKO phenotype. The shRNA was introduced by microinjection to the rete testes of P15 SC-RbKO mice and the mice were allowed to age until they were 10 weeks old, the age at

which the severe testicular phenotype is clearly seen in the SC-RbKO. Each animal served as its own control, as one testis was treated with the *E2f3* shRNA and the other with a mock plasmid. The testes treated with *E2f3* shRNA were significantly heavier than the mock-treated testes at the time of sacrifice (I: Fig. 5a). There was also a recovery of histological tissue architecture of the testis in the rescued SC-RbKO (I: Fig. 5c). The *E2f3* shRNA treatment restored the claudin 11 and espin to normal in the SC-RbKO testes (I: Fig. 5d). However, the rescue was only partial since the shRNA-treated testes recovered to only 60% weight of control (untreated $Rb^{flox/flox}$) testes and the histological improvement of the tissue architecture was patchy (I: Fig. 5a, c).

5.2. E2F1 function in the postnatal mouse testis (II)

5.2.1. Expression pattern of *E2f1* in postnatal mouse testis (II)

The role of E2F1 in the adult testis has previously been addressed by different studies (Yamasaki et al. 1996, Holmberg et al. 1998, Hoja et al. 2004, Agger et al. 2005, Novotny et al. 2007). However, the significance of E2F1 in the developing testis and the possible contribution of a developmental phenotype to the E2F1-/- testicular phenotype have remained elusive.

We set out to study this developmental role of E2F1 by first showing the ontogenic expression pattern of *E2f1* mRNA. E2F1 protein has previously been shown to be expressed in type B spermatogonia, preleptotene and leptotene and zygotene spermatocytes (El-Darwish, Parvinen & Toppari 2006). We opted for RNA *in situ* hybridization (ISH) to localize *E2f1* in the developing testis. We were unfortunately unable to correlate the mRNA expression pattern with protein expression, because the thirteen tested antibodies performed inconsistently and the production of the antibody used by El-Darwish et al. (El-Darwish, Parvinen & Toppari 2006) had been discontinued. The RNA ISH assay was performed on PFA-fixed paraffin-embedded testis samples from P10, P20 and P40 wild-type animals in C57Bl/6J genetic background. To aid the identification of the different *E2f1* expressing cell types, the RNA ISH was coupled with an immunohistochemical detection of different marker antibodies: PLZF for undifferentiated spermatogonia and phosphorylated γH2AX for distinction between leptotene and pachytene spermatocytes. The *E2f1* expression pattern in the P40 testis with full spermatogenesis was similar to the previously published protein expression pattern (El-Darwish, Parvinen & Toppari 2006) with a high level of E2f1 mRNA in the preleptotene, leptotene and zygotene spermatocytes and a decreasing amount of E2f1 mRNA transcript in the pachytene spermatocytes (II: Fig. 1C). E2f1 expression in the germ cell of the juvenile (P10 and P20) testes was observed in similar cell types as in the adult testes, but somatic cells showed a differential expression pattern (II: Fig1A-B). In the P10 testes, E2f1 mRNA was observed not only in the germ cells but also in some peritubular myoid cells and interstitial cells (II: Fig. 1A). In the P20 testis, *E2f1* was detected in spermatogonia and meiotic cells with a similar expression pattern as in the P40 testis. There were some E2f1-positive interstitial cells observed as well (II: Fig. 1B).

5.2.2. A differential role for E2F1 in SSCs, spermatogonia and spermatocytes (II)

We took advantage of the commercially available E2F1$^{-/-}$ mouse line to study the function of E2F1 in the postnatal testis. To reduce variability between the animals, we first crossed them into the C57Bl/6J genetic background over eight generations. This led to an exacerbation of the testicular phenotype with a significant decrease in relative testis weights at the age of 20 days already, considerably earlier than in the hybrid animals where atrophy was observed at the age of 3 months (Yamasaki et al. 1996) (II: Fig. 2B, 3).

Apoptosis of germ cells was affected during the first wave of spermatogenesis in the E2F1$^{-/-}$. The number of spermatogonia at P10 was decreased (II: Fig. 5B), but surprisingly their apoptosis was simultaneously significantly decreased (II: Fig. 4B). Spermatogonial proliferation was not significantly altered (II: Fig. 5A). Contrary to the P10 testis, significantly increased apoptosis of germ cells was observed in the adult and P20 testis. Meiotic cells were lost from the P20 testis (II: Fig. 3, Table 1) and this was further exacerbated in the P40 E2F1$^{-/-}$ testis (II: Fig. 3, 4A, Table 1) The progressive nature of the phenotype with emerging SCO tubules as the animals aged (II: Fig. 2F) could be attributed mostly to the loss of the undifferentiated spermatogonia and especially the SSC subpopulation, which was supported by the decreased *Plzf* and *Oct4* mRNA levels in the adult E2F1$^{-/-}$ testis (II: Fig. 5A).

5.2.3. E2F1 was redundant in somatic cells (II)

E2f1 mRNA was also expressed in somatic cells during development (II: Fig. 1A-B). Both peritubular myoid cells and interstitial cells expressed *E2f1* in the P10 testes (II: Fig. 1A), and *E2f1* persisted in a small subset of interstitial cells in the P20 testes (II: Fig. 1B). Nevertheless, *E2f1* appeared to be redundant in these cell types. Serum LH, FSH and testosterone levels were comparable in E2F1$^{-/-}$ males and controls in adulthood, reflecting unaltered androgen production (II: Fig. 7A). In addition, *Ar* expression and levels of *Eppin* and *Rhox5* (known androgen responsive genes) were not altered in the E2F1$^{-/-}$ animals in comparison with the controls (II: Fig. 7B). Smooth muscle actin (*Sma*) mRNA levels, which were used as a functional marker for peritubular myoid cells, were not significantly altered either (II: Fig. 6A).

The Sertoli cell number and proliferation rate were not affected by the loss of E2F1 (II: Fig. 6B). Claudin 11(*Cldn11*), which is a BTB component, was used as a functional marker for Sertoli cells and its expression was unaltered in the absence of E2F1 (II: Fig. 6A).

5.2.4. Gene expression analysis (II)

Global gene expression profiling was performed using the Illumina Mouse-WG-6 v. 2 Bead Chip to assess the transcriptional consequences of E2F1 loss in the testis. P20 was chosen as the time point for the experiment to avoid confounding effects due to radically different cell compositions of the testis between E2F1$^{-/-}$ and controls, as observed in the older mice. Indeed, the samples clustered very closely to each other in the principal component and hierarchical clustering analyses, showing that the time point choice was

successful (data not shown). The expression of 164 genes was up-regulated and 56 genes were down-regulated in the absence of E2F1, and the fold changes (FC) of the differentially expressed genes were relatively modest (FC -4.4 to 2.6).

When the gene expression profiling has been performed on whole tissue with multiple different cell types present, interpreting the significance of the results in each different cell type is difficult. To overcome this issue, a comparative analysis of the E2F1-array data and previously published data on the transcriptomes of the different testicular cell types was performed (Soumillon et al. 2013). The rationale of the analysis was to determine the principal expression sites of the transcripts which were in the $E2F1^{-/-}$ testes, in other words, to determine which cell type was responsible for the observed change in expression. The relative abundance of each transcript in different cell types was determined by the analysis of previously published data on transcriptomes of different testicular cell populations (Soumillon et al. 2013) (II: Fig. 8A). Soumillon and co-workers had extracted primary Sertoli cells, spermatogonia, spermatocytes, spermatids and spermatozoa from mice at different time points and analyzed cell-type specific transcriptomes using RNA-seq. In the present study, the altered transcripts from the E2F1-array were compared against the Soumillon data, resulting in the cell-type weighed transcript lists where the E2F1-array transcripts were clustered according to the most likely expression site of the genes. The general trends were that transcripts of Sertoli cell- and spermatogonia-associated transcripts were up-regulated, while transcripts associated with the more advanced germ cell types were generally down-regulated (II: Fig. 8C).

The cell-type-weighed transcript lists were submitted to the Gene Ontology Consortium search tool to identify affected biological processes based on gene enrichment. In the spermatogonia-weighed transcript list, the top enriched GO terms were the regulation of granulocyte chemotaxis (GO:0071622), the homocysteine biosynthetic process (GO:0071268), the uridine metabolic process (GO:0046108), the S-adenosylhomocysteine catabolic process (GO:0019510) and the negative regulation of transcription involved in G1/S transition of the mitotic cell cycle (GO:0071930) (II:Table 2). Among the most significantly enriched cellular processes in the E2F1-/- Sertoli cells were the response to stimulus (GO:0042981), the immune system process (GO:0006950), the response to stress (GO:0050896), the regulation of apoptotic process (GO:0008152) and the metabolic process (GO:0002376) (II: Table 2).

Furthermore, the enrichment of E2F consensus binding sites in the promoter regions of each of the altered transcripts clustering in each cell type was assessed *in silico* using the PASTAA tool (Roider et al. 2009). Enrichment of putative E2F target genes was observed only within the spermatogonia-weighed transcript list (II: Table S4).

5.3. Flow cytometry to study testicular cell populations (III)

5.3.1. Development of the protocol (III)

We wanted to develop a new fast, accurate, and reproducible application of flow cytometry to study the dynamics of the testicular cell populations. To broaden the range of suitable cell type markers and to enable studying signaling cascades, we opted for detection of intracellular antigens in fixed and permeabilized cells, instead of the more commonly used cell surface markers.

The protocol for the tissue dissociation and subsequent fixation and permeabilization was first optimized using adult rat testes (III: Fig. 1). The goal was to obtain representative amounts of the different testicular cell populations from the tissue and minimize the amount of cell debris. Spermatocytes, especially in the pachytene phase, are the most sensitive cell for damage during the tissue dissociation procedures. The critical points during the tissue dissociation appeared to be the DNAse concentration and the amount of mechanical disruption of the tissue. As shown by the analysis of the testicular cell populations by propidium iodide staining (PI), representative cell populations were obtained in flow samples prepared from the rat testes of different ages using the protocol (III: Fig. 3). The cell populations in the juvenile (P5 and P10) testes represented the expected ratios of 2C and 4C cells, which corresponded to the active mitotic proliferation of both somatic and germ cells. At P16, the 4C population began to expand as meiosis emerged. At P24 the first meiotic division had been completed and the first haploid cells 1C began to emerge. In the adult testis the relative proportion of 2C and 4C cells decreased, while the relative abundance of haploid 1C cells increased. The sample preparation was reproducible at all time points, as intra- and inter-animal variation was relatively low despite very different tissue composition during development (III: Fig. 6) .

5.3.2. Analysis of somatic cells using vimentin (III)

Rat testis ontogenesis was chosen as an experimental model to validate the performance different antibodies used for flow cytometry. Vimentin was used as a somatic cell marker to dissect the identity of the 2C population, because it is expressed in both Sertoli cells and interstitial cells in the rat testis (Fig. 10, III: Fig: 4A) and allows identification of the somatic cell component within the different DNA-stain-based cell populations (III: Fig. 2). The clustering of the vimentin-positive cells within the cell populations representing the different phases of the cell cycle followed the expected trends of rat testicular ontogenesis (III: Fig. 4B-D). In the juvenile testes, a large proportion of the vimentin-positive cells clustered within the proliferating and 4C population, corresponding to Sertoli cell proliferation (III: Fig. 4B-D). In addition, the Sertoli/germ cell-ratio was high, as expected, before the onset of spermatogenesis (III: Fig. 4B, D). As the animals aged, the proportion of the proliferating vimentin-positive cells gradually diminished and their relative abundance decreased (III: Fig. 4B-D).

5.3.3. Phosphorylated γ-H2AX as a germ cell marker (III)

S139-phosphorylated γH2AX (pH2AX) was used as a marker for the different germ cell populations. It is expressed primarily in the meiotic spermatocytes in the testis from leptotene to pachytene spermatocytes and in step 11-14 spermatids, which are engaged in the histone-protamine exchange (Fig. 10, III: Fig. 5A) (Hamer et al. 2003, Meyer-Ficca et al. 2005). This expression pattern could be detected by flow cytometry in the rat testis ontogenesis (III: Fig. 5B-F) with an increasing number of pH2AX-positive cells in the 4C population in puberty as the germ cells enter meiosis. The 4C population begins to represent solely germ cells as somatic cell proliferation ceases (III: Fig. 5E). In the adult testis a subpopulation of haploid cell expressed pH2AX corresponding to the step 11-14 spermatids (III: Fig. 5F).

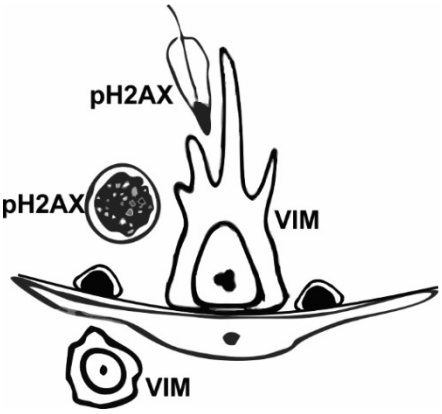

Figure 10. The cell types detected by the antibodies used in the flow cytometry study. Sertoli cells and interstitial cells are positive for Vimentin. pH2AX is a germ cell marker and it is detected in spermatocytes and elongating spermatids.

6. DISCUSSION

6.1. RB family as a gatekeeper of terminal differentiation in Sertoli cells (I)

We set out to study the role of RB in Sertoli cells by creating a conditional knockout mouse model for RB (SC-RbKO). Sertoli cells are highly differentiated cells which depend on active cell cycle repression in the adult life (Tarulli, Stanton & Meachem 2012). RB was chosen as a primary target to study the cell cycle regulation in Sertoli cells, since it is a key gatekeeper of the cell cycle in many differentiated cell types (Yang et al. 2015, Andrusiak et al. 2012). In the testis, the RB family members have been shown to have tightly regulated expression and phosphorylation patterns, indicating a dynamic role in the control of spermatogenesis (Yan et al. 2001, Yan et al. 1997).

RB was conditionally deleted in Sertoli cells already in the fetal life (Lecureuil et al. 2002). However, the initial Sertoli cell proliferation and differentiation during the first wave of spermatogenesis occurred without disturbance in the SC-RbKO, which indicates a redundant role for RB in the juvenile testis. The other RB family members possibly compensate RB fully as all the RB family members are expressed in the juvenile Sertoli cells (Yan et al. 2001). The first signs of Sertoli cell dysfunction were observed in 1-month-old SC-RbKO mice when the Sertoli cells had terminally differentiated. First, the SC-RbKO Sertoli cells re-entered the cell cycle and lost their ability to support the meiotic and post-meiotic germ cells, evidenced by the increased germ cell apoptosis in the 4-6-week-old animals. The phenotype exacerbated rapidly and at the age of 8-12 weeks, the Sertoli cells dedifferentiated, the BTB disintegrated, the DNA damage repair system was activated, and finally some Sertoli cells underwent apoptosis. A somewhat similar age-dependent testicular degeneration was observed in the mice with conditional deletion of Gata4 in Sertoli cells (Gata4 cKO) (Kyrönlahti et al. 2011). As the Gata4 cKO animals aged, the BTB barrier disintegrated and germ cell apoptosis was increased. The age-dependent testicular degeneration was apparent at a later time point in the Gata4 cKO testis than in the SC-RbKO, possibly due to a delayed penetration of the cre-expression in the Gata4 cKO. BTB defects and a block in post-meiotic germ cell development are also common in different Sertoli cell-specific *Ar* ablation models, implying that these are major hallmarks in Sertoli cell dedifferentiation phenotypes (De Gendt et al. 2004, Meng et al. 2005).

All of these studies suggest that post-natal Sertoli cell function during the first wave of spermatogenesis is governed by different mechanisms than the maintenance of the differentiated phenotype and function of Sertoli cells in the adult animals. Even though Sertoli cells have formed the BTB, gained AR and $p27^{Kip1}$ expression and lost the expression of AMH and other markers of immaturity before puberty, there is an additional level of control, ensuring the maintenance of these features in the adult testis. In fact, it has been suggested that the adult Sertoli cells are not in a state of a permanent cell cycle exit and terminal differentiation but exhibit more plasticity than previously thought (Tarulli, Stanton & Meachem 2012). The Sertoli cells of men treated with testosterone and progesterone to inhibit gonadotropin secretion began to proliferate in adulthood (Tarulli et al. 2013). Moreover, adult mouse Sertoli cells continue to express marker for DNA damage repair and cell cycle repression even after terminal

differentiation and they can resume proliferation *in vitro* (Ahmed et al. 2009). However, the major changes in gene expression that occur in mouse Sertoli cells during this time window between P18 and P35 are metabolic, including lipid and lactate metabolism, and not related to cell cycle control (Zimmermann et al. 2015). It is possible that multiple levels of cell cycle control operate in a redundant fashion during the initial maturation of Sertoli cells (Yan et al. 2001) and in adult testis these systems are partially eliminated and Sertoli cell cycle control depends on fewer factors. RB appears to be critical in controlling this time point. Adult Sertoli cells may also require RB to induce a senescence-like state. In non-transformed human fibroblasts, RB promotes senescence in a non-redundant manner by suppressing cyclinE1 (Chicas et al. 2010).

In the adult rat testis the phosphorylation patterns and therefore the activity levels of RB and p130 have been shown to vary stage-specifically: RB activity peaked at the androgen-dependent stages VII-VIII (Yan et al. 1997) and p130 at stages I-IV. Androgen ablation using EDS-treatment (Leydig cell ablation) or flutamide (anti-androgen) altered the stage-specific expression and phosphorylation pattern of RB (Yan et al. 2001, Tan et al. 2005). As the adult Sertoli cells do not proliferate, the stage-specific fluctuations in RB activity in normal testes are more likely to reflect a dynamic functional role for the RB proteins in the control of transcription in adult Sertoli cells than a strictly cell cycle-related role. For instance, RB may participate in the control of BTB remodeling during spermatogenesis. Recently Wu et al. showed that ARID4A and ARID4B (previously known as RB-binding protein 1 (RBBP1) and RBBP1-like protein) promote the transcription of Claudin3 (an integral part of the BTB) by interacting with RB and AR as enhancers (Wu et al. 2013). The observed accumulation of ectoplasmic specialization (espin) and BTB (claudin 11) proteins in the adult SC-RbKO testes thus reflected the functional role of RB in the control of the Sertoli-germ cell associations during spermatogenesis.

We hypothesized that deregulated E2F3 was the main downstream effector responsible for the SC-RbKO phenotype. In other tissues a loss of E2F3 has been shown to rescue the defects of the *Rb*-null animals and both E2F3a and b isoforms interact with RB. In lens and CNS cells of $Rb^{-/-}$ embryos, a compound loss of E2F3 rescued the defects in proliferation and apoptosis (Ziebold et al. 2001), and E2F3a was responsible for this effect in the CNS (Chong et al. 2009a). In addition, E2F3a suppresses the embryonic lethality of $Rb^{-/-}$ through suppression of the placental phenotype (Chong et al. 2009a). We conducted a rescue experiment by knocking down E2F3 *in vivo* to test this hypothesis. Indeed, the shRNA-mediated E2F3 knock-down resulted in a partial rescue of the SC-RbKO phenotype, which was seen in increased testis weight and improved morphology. Low transfection efficiency *in vivo* and mechanical damage caused by the microinjections were likely causes for the incomplete recovery.

Rb loss is associated with the induction of pituitary tumors in the mouse (Jacks et al. 1992). In human tissues, loss of function mutations of *Rb* have been shown in multiple different malignancies, ranging from retinoblastomas to osteosarcoma and gliomas (Di Fiore et al. 2013). Contrary to these reports, *Rb* loss in mouse Sertoli cells did not result in the formation of Sertoli cell tumors, despite the induction of Sertoli cell proliferation in the adult SC-RbKO and RB cKO animals (Nalam et al. 2009). The aberrant

proliferation was counterbalanced by the Sertoli cell apoptosis. Induction of ectopic E2F1 expression in the Rb cKO Sertoli cells may have driven the Sertoli cell to apoptosis (Nalam et al. 2009). Compensation of RB by the other RB family members in the control of E2F3 possibly contributed to the absence of Sertoli cell tumors in the SC-RbKO testes, as the simultaneous inactivation of all the RB family members and p53 in mouse Sertoli cells by transgenic expression of SV40-oncogene can induce the formation of mixed Sertoli-Leydig cell tumors (Quintana et al. 2010). Moreover, a combined loss of RB and inhibinα (INHA) in mouse Sertoli cells did not lead to an exacerbation of the $Inha^{-/-}$ Sertoli cell tumors but an accelerated Sertoli cell dysfunction in comparison with the Rb cKO (Nalam, Andreu-Vieyra & Matzuk 2010). These results can be interpreted in two ways: first, INHA appears to have a role in Sertoli cell function beyond being merely a tumor suppressor, and second, RB is not the only tumor suppressor nor the most critical tumor suppressor in Sertoli cells but rather plays a role in the maintenance of terminal differentiation, possibly by mechanisms separate from cell cycle control.

There is evidence of a direct role of RB in germ cell tumors in humans and a loss of RB alters SSC dynamics, even though it is not sufficient to induce germ cell cancer in the mouse (Hu, de Rooij & Page 2013, Bartkova et al. 2003). RB may also play an extrinsic role in the development of germ cell cancer via its action in Sertoli cells. According to the testicular dysgenesis syndrome (TDS) theory, the defects in fertility and the increase in testicular cancer incidence, phenomena that appear germ cell-dependent, are in fact a consequence of a defective Sertoli and Leydig cell differentiation and function (Juul et al. 2014). Patients with testicular germ cell cancers how a widespread array of pathological alterations (e.g. hypospermatogenesis, Leydig cell hyperplasia, Sertoli cell dedifferentiation and involution), even in testis tissue that is not affected by the cancer, suggesting a more disseminated testicular pathology than merely the cancerous development in one cell clone (Nistal et al. 2006). Sertoli cells which are adjacent to tubules with seminoma resume proliferation as a sign of a severe functional defect in the adult Sertoli cells (Tarulli et al. 2013). Disorganization of tight-junctions in tubules with GNIS and seminoma is another hallmark of Sertoli cell dedifferentiation in TDS (Tarulli et al. 2013). Decreased AR expression is also present in TDS (Tarulli et al. 2013). Thus, defective Sertoli cell function and differentiation is closely connected to testicular germ cell cancer development and, if we want to pinpoint the mechanisms that cause the increased incidence of male reproductive disorders, we need more systematic studies focused on the somatic-germ cell connections.

Despite all the advances in studying the cell cycle regulators in developing testes, the definitive roles of many of the factors operating in this process remain unknown. There is considerable functional redundancy between the RB and E2F family members, which makes dissecting the *in vivo* roles of individual factors challenging. They not only compensate for each other in cell types where they are known to be co-expressed, but non-physiological expression of the proteins can also be triggered as a compensatory action. p130 and p107 attempted to compensate RB action in adult Sertoli cells but failed to do so completely. It would be interesting to study the significance of p107 and p130 in Sertoli cells by generating double knockout animal models with SC-RbKO. The contribution of E2F1 expression in the SC-RbKO Sertoli cell remains elusive and $E2F1^{-/-}$/SC-RbKO double knockout mice could resolve this issue. Compound mutants of the

RB family proteins in spermatogonia could further elucidate their roles in the control of spermatogonial proliferation and possible mechanisms of testicular germ cell cancer development. Furthermore, this study showed that RB interacts physically with E2F3 to control Sertoli cell function, but RB may have also other, still unknown, binding partners controlling Sertoli cell function. Discovery of these novel RB partners by co-immunoprecipitation and mass spectrometry analysis would further elucidate the dynamics of RB action in Sertoli cells.

6.2. E2F1 has a multifaceted role in the control of germ cell apoptosis and survival (II)

The role of E2F1 in postnatal testis development was studied using the E2F1$^{-/-}$ mouse model. The E2F1$^{-/-}$ mice were back-crossed to a uniform C57Bl/6 genetic background, which revealed that the genetic background had an impact on the testicular phenotype. Macroscopic testicular atrophy, which had been previously described to occur at the age of 3 months in the hybrid background (Yamasaki et al. 1996), was apparent already at P20 during the first wave of spermatogenesis. This was not surprising, as the genetic background has also been shown to have a clear impact on the phenotype of the *E2f3-null* mice (Cloud et al. 2002). In the Sv129 genetic background, the loss of *E2f3* is embryonic lethal, while in a mixed genetic background some pups survive to postnatal life (Cloud et al. 2002, King et al. 2008).

The testicular atrophy in the young animals was caused by increased apoptosis of meiotic cells during the first wave of meiosis, indicating an anti-apoptotic role for E2F1 during meiosis, contrary to previous suggestions in the literature (Novotny et al. 2007). Apoptosis of spermatocytes persisted during the adult spermatogenesis in a similar fashion as has been previously reported (Hoja et al. 2004). On the other hand, E2F1 appeared have a pro-apoptotic role in spermatogonia in the first wave of spermatogenesis, as loss of E2F1 led to a decrease in spermatogonial apoptosis in the first wave of spermatogenesis. Physiological apoptosis of spermatogonia occurs at a high rate at this time point, possibly to adjust the germ cell numbers to the Sertoli cells (Print, Loveland 2000). The pro-apoptotic Bax and anti-apoptotic Bcl-w, Bcl-X_L and Bcl-2 control this surge of apoptosis (Furuchi et al. 1996, Jahnukainen et al. 2004, Knudson et al. 1995, Rodriguez et al. 1997, Yan et al. 2003). E2F1 has been shown to regulate these factors either directly or via p53 (Polager, Ginsberg 2009). Loss of E2F1-mediated activation of Bax in the developing E2F1-/- testis could be the mechanism contributing to the decreased spermatogonial apoptosis.

E2f1 was expressed in all the analyzed PLZF-positive undifferentiated spermatogonia. In the adult E2F1$^{-/-}$ testis, a major contributor to the testicular atrophy was the loss of the PLZF- and OCT4-positive spermatogonia and the subsequent exhaustion of spermatogenesis. Even though PLZF is also expressed in the more committed Ngn3+ spermatogonia, the emergence of SCO tubules in the absence of E2F1 implies that E2F1 is necessary for the maintenance of the more primitive GFRα1+ spermatogonial population (Nakagawa et al. 2010, Nakagawa, Nabeshima & Yoshida 2007). Loss of PLZF in mouse testis led to a very similar SSC phenotype as observed in the E2F1$^{-/-}$ (Costoya et al. 2004). E2F1 loss thus also impairs the capacity of the potential SSCs (Ngn3+ spermatogonia) for

sustaining spermatogenesis. It is interesting to note, that a similar phenomenon was observed in the germ cell-specific RB knockout mouse model (Hu, de Rooij & Page 2013). RB and E2Fs are known to act as complexes to control gene expression (Chong et al. 2009b) and E2F1 recruits RB to the majority of the RB-regulated gene promoters (Korenjak et al. 2012, Chicas et al. 2010, Wells et al. 2003). Consequently, the $E2F1^{-/-}$ spermatogonia may have a defect in the RB/E2F-mediated repression of the expression of genes related to the maintenance of pluripotency, which leads to the gradual loss of the SSCs. It would be interesting to further assess whether such an RB/E2F1-mediated mechanism controls spermatogonial stem cell fate by studying the binding patterns of each of the factors on gene promoters in RB or E2F1-deficient spermatogonia using ChIP-seq. The *in silico* analysis of E2F consensus binding sequences on the promoters of the genes altered in the E2F1 gene expression array showed enrichment of putative E2F1binding sites only in the spermatogonia-enriched gene list. This further supported the role of spermatogonia as the primary E2F1 target cell type.

E2F1 has been identified as a master regulator of genes involved in cell cycle control in P5 Sertoli cell which are at the height of their proliferative activity (Zimmermann et al. 2015). Unfortunately, our data covered *E2f1* expression pattern only from P10 onwards in the mouse testis. Nevertheless, E2F1 appears to be redundant for Sertoli cell proliferation during this early time- window, since the number or proliferation of Sertoli cells was not affected at P10 in $E2F1^{-/-}$ testes. This may be a result of compensation of E2F1 action by E2F3, which is expressed in the mouse Sertoli cells (El-Darwish, Parvinen & Toppari 2006). *E2f1* mRNA was also detected in some juvenile interstitial cells, but this had no functional consequences. The serum LH and testosterone levels were unaffected in the adult animals and the expression of androgen target genes was normal throughout development. *E2f1* expression in the somatic cells of the testis may well be dependent on their cell cycle phase, as only a proportion of the peritubular myoid cells and interstitial cells expressed *E2f1*. However, E2F1in these cell types was likely to be compensated by other E2Fs as the somatic cells did not contribute to the $E2F1^{-/-}$ phenotype.

In the human testis, alterations in E2F1 expression levels have been linked to both testicular carcinoma *in situ* and infertility (Novotny et al. 2007, Jorgez et al. 2015). In human testis, post-transcriptional regulation by the mir-17-92 cluster during the transition to the pachytene phase of meiosis possibly down-regulates E2F1 (Novotny et al. 2007). A similar phenomenon was observed in GNIS cells, where absence of the E2F1 protein coincided with expression of E2f1 mRNA and mir-17-92 (Novotny et al. 2007). The interpretation was that E2F1 is a pro-apoptotic factor in meiotic cells and its down-regulation is crucial during meiosis and for the survival of the GNIS cells. Recently, E2F1 was identified as a candidate gene in an array comparative genomic hybridization (aCGH) study of infertile men with non-obstructive azoospermia (Jorgez et al. 2015). E2F1 microduplications appeared to be connected to hypospermatogenesis, while E2F1 microdeletions led to a more severe phenotype with Sertoli-cell-only syndrome (Jorgez et al. 2015). In addition, one patient with hypospermatogenesis presented with an SNP affecting the cyclin A-binding domain of E2F1. The E2F1 microdeletion phenotype in humans resembled the $E2F1^{-/-}$ adult mice, suggesting that the mouse can serve as a useful model in studies of the role of E2F1 in spermatogenesis.

In the present study, we have elucidated the role of E2F1 in the developing testis. However, determining the definitive function of E2F1 in all of the different facets of the control of germ cell apoptosis and SSC survival will require further studies. One approach would be to uncover the genes that are controlled by E2F1 in the germ cell subpopulations at various stages of germ cell differentiation using ChIP-seq. Furthermore, studying the expression patterns of the other E2F family members in the $E2F1^{-/-}$ testis could elucidate the possible compensatory mechanisms. E2F1 has lately emerged as a possibly clinically relevant factor in the control of human spermatogenesis, and further studies on the mechanisms of E2F1 action, especially in SSC maintenance, are warranted.

6.3. Development of new applications of existing methods to study testis (II, III)

Developing and adult testes are particularly complex tissues and new methods are needed to accelerate the studies on testicular development and to obtain more accurate data on the dynamic nature of the different cell types within the testes.

6.3.1. A readily applicable workflow to study testicular tissue using flow cytometry (III)

Flow cytometry is increasingly used to study testicular cell populations, either using DNA content analysis or specific antibodies, but there has been a lack of consensus concerning the validation of different antibodies and interpretation and normalization of the flow cytometry data (Elzeinova et al. 2013, Ferguson, How & Agoulnik 2013, Wakeling, Miles & Western 2013). We demonstrated a readily applicable flow cytometry protocol with detailed instructions for the validation of different antibodies using testicular ontogenesis as a model (III). We have tested several antibodies against various intracellular antigens (Sox9, Ki67, and cleaved caspase 3, among others). It has proven challenging to find other applicable antibodies in addition to the vimentin and pH2AX described in (III). Neither the performance of an antibody in immunofluorescence on tissue sections nor the manufacturer's recommendations are necessarily predictors of the antibody's performance in flow cytometry. Instead, each antibody has to be rigorously validated specifically for this application. Naturally, the fixation and permeabilization procedure that is used can be incompatible with some of the antibodies, but having separate sample preparation protocols for different antibodies is not practical. There are also inter-species differences in the performance of the antibodies. It appears that the specificity of the antibodies is an issue more often when using mice than rats.

The sample preparation was optimized for both mouse and rat to yield the expected ratios of the different testicular cell types at different time points and to minimize cell damage. To our surprise, the same treatment conditions were applicable on rat testis tissue from early postnatal to adult testes, despite very different tissue morphology. However, in future applications of this method to study, for instance, the structurally challenging human testis and fibrotic samples from infertile men, careful optimization of the sample

preparation procedure is important to ensure that representative and comparable cell populations are obtained.

There are many advantages in fixing and subsequently freezing the flow cytometry samples. It enables the study of intracellular antigens, as opposed to only surface-antigens in live cells, which broadens the range of possible flow cytometry-optimized antibodies and enables studying intracellular signaling pathways related to the cell cycle and apoptosis.

This flow cytometry protocol is useful for collaboration, since the frozen samples can be sent from one laboratory to another. Samples can also be collected over a longer period of time, then stained and analyzed simultaneously to reduce variation between experiments in studies that are spread over a long period of time. Applying flow cytometry to study testis significantly accelerates research, since information of the testicular cell composition can be obtained within one working day, as opposed to days or weeks for histomorphometry. This is very useful, for example, in the first-line analysis of testicular phenotypes of new animal models, where there is the need to rapidly screen different time points to find the most suitable age for analysis. The fixation and permeabilization naturally render the cells non-viable, which limits the applicability of intracellular flow to the sorting of cells for live-cell experiments. The largest limitation is, however, the availability of suitable and trustworthy antibodies.

6.3.2. From tissue to cell level in array analysis? (II)

A common pitfall in analyzing gene expression microarray or RNA-seq data generated from whole tissue RNA is the inability to dissect the contribution of each different cell type into the data and the observed changes. We employed a novel data analysis workflow in the E2F1-project to overcome this limitation of the array data. The transcripts altered in the $E2F1^{-/-}$ array were compared against pre-existing data on the transcriptomes of the different testicular cell types. Naturally, this method of analyzing data is mainly supportive and hypothesis-generating due to many potential pitfalls. First, gene expression in the testis is highly dynamic and changes in the course of testicular differentiation. The repository data was acquired from the testes of animals at different ages, depending on the most suitable time point for the extraction of each cell type, and these did not fully match the data used in the E2F1 study. For instance, the primary spermatogonia were extracted from the 6-day-old mouse testis and the E2F1 array was performed on 20-day-old animals. Thus, the repository data presented the first wave spermatogonia, while the testes analyzed in the E2F1 array had adult-type spermatogonia. Second, the initial primary cell extraction is hardly 100% pure for the cell type of interest. Third, as was shown in the SC-RbKO with the induction of p107 expression in the adult Sertoli cells, the experimental intervention may alter the physiological gene expression patterns in the tissue and lead to erroneous conclusions. However, when used with care, taking all of these issues into account and analyzing more general patterns than the expression levels of individual genes, this analysis workflow may provide useful additional insight to transcriptomic data on whole tissues. Furthermore, this experiment demonstrated a way of utilizing the ever-growing publicly-available data.

7. SUMMARY AND CONCLUSIONS

Proliferation, apoptosis and differentiation are fundamental events governing the fate of individual cells, tissues and whole organisms, and complex layers of signaling cascades operate to control these events. In this study we explored the role of the cell cycle associated RB and E2F proteins in controlling these events in the developing testis. Novel methods to study the complex testis tissue were developed to achieve the goals.

The main findings of the study were:

1. The retinoblastoma protein is essential for the maintenance of Sertoli cell quiescence and terminal differentiation, but dispensable for the function of juvenile Sertoli cells.
2. Aberrant E2F3 activity is involved in the Sertoli cell dysfunction in the absence of RB. The other RB protein family members attempt to compensate RB in the control of E2F3.
3. E2F1 is required for the maintenance of the spermatogonial stem cells in the adult testis. In the juvenile testis it has a dual role: proapoptotic in spermatogonia and antiapoptotic in spermatocytes.
4. Testicular cell populations can be accurately studied using flow cytometry to detect intracellular antigens. Testis ontogenesis serves as a powerful tool to validate different antibodies and stains for flow cytometry.

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