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ABSTRACT

Janne Hakkarainen
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Estrogens and androgens are critical regulators of several physiological processes, including development and reproduction. In addition, these hormones play key roles in several physiological complications and diseases, such as infertility, endometriosis, breast cancer and prostate cancer. According to the classical view steroid hormone actions are thought to be regulated by systemic hormone levels, where endocrine glands produce the hormones and blood circulation delivers the hormones to target organs. However, accumulative evidence has shown that the control of steroid hormone actions is also regulated at the level of target cell metabolism. Due to the crucial roles of steroid hormones in physiological functions and in the etiology of several diseases, a better understanding of steroid hormone metabolism is needed.

In this study, we generated a conventional global knockout mouse model, *Hydroxysteroid (17β) dehydrogenase 1* (HSD17B1KO), to achieve a better understanding of enzymatic regulation of steroid hormone actions and of the role of the HSD17B1 in the development of reproductive organs and fertility in females and males. This study revealed that a lack of the enzyme does not have an impact on fetal or pubertal development in either males or females. However, steroid synthesis is affected in both females and males, and enzyme deficiency leads to severely impaired fertility in females and infertility in males. Thus, this study indicates that the HSD17B1 enzyme plays a critical role in regulating steroid hormone synthesis and actions, and that a lack of the enzyme has a profound influence on mouse gonadal function in both males and females.

In summary, the produced knockout mouse model provides a tool and knowledge to understand steroid hormone synthesis and actions in reproductive tissues.

Keywords: HSD17B1, reproduction, estrogen, androgen

TIIVISTELMÄ

Janne Hakkarainen
LISÄÄNTYMISELINTEN HORMONISYNTEESIN JA LISÄÄNTYMISEN SÄÄTELY: HYDROKSISTEROIDI (17β) DEHYDROGENAASI 1:N UUDET TEHTÄVÄT

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Estrogeenit ja androgeenit ovat tärkeitä säätelytekijöitä useissa erilaisissa fysiologisissa tapahtumissa, kuten kehityksessä ja lisääntymisessä. Lisäksi hormonit ovat avainasemassa monissa sairauksissa, kuten hedelmättömyydessä, endometrioosissa, rintasyövässä ja eturauhassyövässä. Hormonitasojen säätelyn on yleisesti ajateltu tapahtuvan systeemisesti siten, että umpieritysrauhaset tuottavat hormonit ja verenkierto välittää ne kohdekudoksiin. Yhä kasvava määrä tutkimuksia on kuitenkin osoittanut, että hormonitoimintaa säädellään myös kohdesolutasolla. Koska hormonit ovat tärkeitä monille fysiologisille toiminnoille ja useiden sairauksien etenemiselle, syvempää ymmärrystä hormoniaineenvaihdunnasta tarvitaan.

Tässä tutkimuksessa olemme tuottaneet hydroksisteroidi (17β) dehydrogenaasi 1-poistogeenisen hiirimallin. Tämän mallin avulla pyrimme ymmärtämään nykyistä paremmin hormonisynteesin entsymaattista säätelyä sekä tämän entsyymin vaikutusta kehitykseen ja lisääntymiseen. Tässä tutkimuksessa olemme osoittaneet, että HSD17B1-entsyymin puutteella ei ole vaikutusta sikiökautiseen tai puberteetin aikaiseen kehitykseen. Kuitenkin HSD17B1:n poisto on vaikuttanut kummankin sukupuolen hormonituotantoon, sekä lisäksi naarashiirten hedelmällisyys alentui huomattavasti ja uroshiiret olivat täysin hedelmättömiä. Tutkimustuloksemme osoittavat, että HSD17B1-entsyymillä on tärkeä merkitys hormonisynteesin säätelyssä ja hormonitoiminnassa, ja HSD17B1-entsyymin puutos vaikuttaa merkittävästi hiiren lisääntymiseen sekä uroksilla että naarailla.

Yhteenvetona voidaan todeta, että kehitetty poistogeeninen hiirimalli mahdollistaa yhden lisääntymiselimissä hormonisynteesiin ja hormonitoimintaan vaikuttavan entsyymin tutkimisen ja tiedon lisäämisen hormonisynteesin säätelystä.

Avainsanat: HSD17B1, lisääntyminen, estrogeeni, androgeeni

TABLE OF CONTENTS

ABBREVIATIONS

20α-HSD	20-alpha-Hydroxysteroid Dehydrogenase
αβERKO	alpha-beta Estrogen receptor knockout
A-dione	androstenedione
aES	apical ectoplasmic specialization
AKR1C3	aldo-keto reductase family 1 member C3
AKR1C6	aldo-keto reductase family 1 member C6
AIS	androgen insensitivity syndrome
AMH	anti-Müllerian hormone
AR	androgen receptor
ARE	androgen response element
ARKO	androgen receptor knockout
BAX	BCL2 associated X, apoptosis regulator
BCL-2	BCL-2, apoptosis regulator
Bid	Bh3 interacting domain death agonist
BMPR1A	bone morphogenetic protein 1 receptor
BRDT	Bromodomain Testis Associated
BTB	Blood-testis barrier
cAMP	cyclic adenosine monophosphate
CAH	congenital adrenal hyperplasia
CEBPβ	CCAAT/enhancer-binding protein β
CL	corpus luteum
CLIP170	cytoplasmic linker protein of 170 kDa
CNS	cental nervous system
CREM	cAMP response element modulator
CREB	cAMP response element binding
CYP11A1	cytochrome P450 family 11, subfamily A, polypeptide 1
CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide 1
CYP19A1	cytochrome P450, family 19, subfamily A, peptide 1
DA	Daltons
DAX1	dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome, gene 1
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DES	diethylstilbestrol
DMRT1	doublesex and mab-3 related transcription factor 1
E1	estrone
E2	estradiol
EGF	epidermal growth factor
ELMO1	engulfment and cell motility 1
ENERKI	estrogen-nonresponsive estrogen receptor α knockin
ERαKO	estrogen receptor α knockout
ERβKO	estrogen receptor β knockout
ES	ectoplasmic specialization
FGF	fibroblast growth factor
FOXL2	winged helix/forkhead transcription factor 2
FP-/-	prostaglandin F receptor knockout
FSH	follicle-stimulating hormone
FSHβKO	follicle-stimulating hormone β knockout
FSHR	folliscle-stimulating hormone receptor
FSHRKO	follicle-stimulating hormone receptor knockout
FZD4	frizzled class receptor 4
GCARKO	granulosa cell-specific androgen receptor knockout

GDF-9	growth differentiation factor 9
GnRH	gonadotropin-releasing hormone
hCG	human chorionic gonadotropin
HSD17B	hydroxysteroid (17β) dehydrogenase
HSD17B1KO	hydroxysteroid (17β) dehydrogenase 1 knockout
HSD17B1TG	hydroxysteroid (17β) dehydrogenase 1 transgene
HSD-3β	hydroxy-delta-5-steroid dehydrogenase, 3β- and δ-isomerase
HOOK1	hook microtubule tethering protein 1
hpg	hypogonadal
HPLC	high-performance liquid chromatography
iARKO	inducible AR knockout
IGF	insulin-like growth factor
Il	interleukin
JAM	junctional adhesion molecule
KIF3AKO	kinesin family member 3A knockout
KISS1	KISS-1 metastasis-suppressor
LH	luteinizing hormone
LHCGR	lutropin-choriogonadotropic hormone receptor
LuRKO	luteinizing hormone receptor knockout
NAD	β-nicotinamide adenine dinucleotide
NADPH	β-nicotinamide adenine dinucleotide 2'-phosphate
NEAT1	nuclear enriched abundant transcript 1
P4	progesterone
PBR	peripheral-type benzodiazepine receptor
PGF2A	prostaglandin F2alpha
PGC	primordial germ cells
PGR	progesterone receptor
PKA	protein kinase A
PKC	protein kinase C
PNA	rhodamine-conjugated peanut agglutinin
PRLR	prolactin receptor
RSPO1	R-spondin1
S-AR-/y	Sertoli cell-specific AR knockout
SC	Sertoli cell
SCARKO	Sertoli cell-specific AR knockout
SDR	short-chain dehydrogenase/reductase family
SF-1	steroidogenic factor 1
SIRH7LDOC1	sushi-ichi retrotransposon homolog 7/leucine zipper downregulated in cancer 1
SOX-9	sex-determining region Y-Box 9
SPAG4/SUN4KO	sperm associated antigen 4
SPEF2KO	sperm flagellar 2 knockout
SRD5A2	5α-reductase type 2
SRY	sex-determining region Y
T	testosterone
TESCO	testis-specific enhancer of Sox-9
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
WNT4	wingless-type MMTV integration site family, member 4
Wnt7a	Wnt family member 7a
WT	wild-type

LIST OF ORIGINAL PUBLICATIONS

The study is based in the following original publications, which are referred to in the text by Roman numeral I-II.

I. Hakkarainen, J., Jokela, H., Pakarinen, P., Heikelä, H., Kätkänaho, L., Vandenput, L., Ohlsson, C., Zhang, F.P., Poutanen, M., " Hydroxysteroid 17beta dehydrogenase 1 deficient female mice present with normal puberty onset but are severely subfertile due to a defect in luteinisation and progesterone production", FASEB J. 2015 Sep; 29(9):3806–16, doi: 10.1096/fj.14–269035

II. Hakkarainen, J., Zhang, F-P., Jokela, H., Mayerhofer, A., Behr, R., Cisneros-Montalvo, S., Nurmio, M., Toppari, J., Ohlsson, C., Kotaja, N., Sipilä, P., Poutanen, M., "Hydroxysteroid (17β) dehydrogenase 1 expressed by Sertoli cells contributes to steroid synthesis and is required for male fertility" Under revision

1 INTRODUCTION

It has been estimated that infertility affects approximately 15 % of couples globally (Agarwal et al., 2015; Sharlip et al., 2002). Several factors can cause infertility, such as genetic and environmental factors. Moreover, defects in hormonal balance and the steroidogenic pathways contribute to the infertility problems in females and males (Hotaling and Patel, 2014; Luciano et al., 2013).

In addition to reproduction, steroid hormones are essential for several biological functions, such as development, puberty, metabolism and overall homeostasis. Reproductive physiology, which includes ovarian development and growth, the menstrual cycle, folliculogenesis and pregnancy maintenance in females and testicular development and growth and spermatogenesis in males, requires steroid hormonal actions. Steroid hormones are produced by the gonads and adrenal glands. Gonadal steroid hormones comprise estrogens, androgens and progestins. One of the enzyme families involved in steroid hormone synthesis is the hydroxysteroid (17β) dehydrogenase (HSD17B) family. These enzymes are involved in the conversion between low potency 17-ketosteroids and high potency 17β-hydroxysteroids. The type 1 HSD17B (HSD17B1) converts estrone (E1) to estradiol (E2) and androstenedione (A-dione) to testosterone (T), and HSD17B1 has been shown to be expressed in the gonads and extragonadal tissues. In females, the main targets of gonadal steroid hormone actions include the ovary, uterus, mammary gland and central nervous system (CNS), while in males the main target organs are the testes, prostate and CNS. Steroid hormones act through nuclear receptors, particularly the estrogen, androgen and progesterone receptors, to control transcription of target genes (McKenna, 2014). In addition to direct regulation of target genes, steroid hormone actions are directed through receptors localized at the plasma membrane, where they induce cellular kinase cascades resulting in transcriptional and non-transcriptional actions (Levin, 2011).

In the present study, we generated a global knockout mouse model of hydroxysteroid (17β) dehydrogenase 1 (HSD17B1KO) to study the role of HSD17B1 in steroid hormone biosynthesis and reproduction. The results from the present study demonstrate that HSD17B1 is necessary for normal female and male reproductive function and for gonadal steroid hormone synthesis. Loss of HSD17B1 causes premature luteolysis of the *corpus luteum* (CL) in female HSD17B1KO mice and improper maturation of Sertoli cells in male HSD17B1KO mice, thus resulting in an inability to maintain pregnancy and a disruption of spermatogenesis in females and males, respectively.

With deeper knowledge of hormonal biosynthesis in the gonads, we can gain a better understanding of reproduction and determine how to treat infertility problems more precisely. In addition, with a better understanding of the regulation of steroid hormone actions, we will be able to develop better drugs for steroid hormone-dependent diseases.

2 REVIEW OF THE LITERATURE

2.1 HSD17B1

2.1.1 *HSD17B enzymes in reproductive tissues*

Functional reproduction requires estrogens and androgens. Steroid hormones are primarily produced in the ovaries and testes, and through the circulatory system, they reach their target organs and cells. In addition to the traditional idea of steroid hormone action, more precise regulation, known as intracrine regulation of steroid action, has been suggested to regulate steroid concentrations at the level of the target organs and cells (Saloniemi et al., 2012). In this form of regulation, steroid hormone concentrations are controlled by the steroidogenic enzymes in the target cells themselves.

Hydroxysteroid (17β) dehydrogenases comprise one of the enzyme families involved in steroid metabolism. These enzymes regulate the latter phases of the formation of androgens and estrogens by catalysing the conversion between 17-keto steroids and 17β-hydroxysteroids (Peltoketo et al., 1999). The enzyme family consists of 14 members in mammals, and these members compose a short-chain dehydrogenase/reductase family (SDR), with the exception of type 5 enzyme, which belongs to the aldoketo reductase gene family (AKR1C3 in humans and AKR1C6 in mice, Saloniemi et al., 2012). Several members of the HSD17B family, such as HSD17B1, HSD17B2, HSD17B3, HSD17B5, HSD17B7 and HSD17B12, have been shown to be expressed in reproductive organs (Table 1.). However, these enzymes are not expressed merely in reproductive tissues but are also expressed in several peripheral tissues. It has been demonstrated *in vitro* that HSD17B1, HSD17B2, HSD17B7 and HSD17B12 are associated with estradiol biosynthesis and that HSD17B3 and 5 are associated with testosterone biosynthesis (Geissler et al., 1994; Jokela et al., 2010; Lin et al., 1997; Liu et al., 2007; Luu-The et al., 2006; Nokelainen et al., 1998; O'Shaughnessy et al., 2000; Puranen et al., 1999; Wu et al., 1993).

Table 1. List of HSD17B enzymes expressed in reproductive organs.

	Expression		
HSD17B	Human	Mouse	Reference
Type 1	Ovary, placenta	Ovary, testis	(Fournet-Dulguerov et al., 1987; Ghersevich et al., 1994a; Nokelainen et al., 1996; O'Shaughnessy et al., 2000; Tremblay et al., 1989)
Type 2	Placenta, uterus	Placenta	(Moghrabi et al., 1997; Mustonen et al., 1997a, 1998a)
Type 3	Testis	Testis	(Geissler et al., 1994; Mustonen et al., 1997a; O'Shaughnessy et al., 2000)
Type 5	Testis, prostate	Testis	(Azzarello et al., 2008; Mustonen et al., 1997a; Penning et al., 2001)
Type 7	Ovary, testis, placenta, prostate	Pregnant ovary, placenta, testis	(Krazeisen et al., 1999; Nokelainen et al., 1998)
Type 12	Ovary, placenta, uterus, testis, prostate	Ovary, testis, prostate, uterus	(Blanchard and Luu-The, 2007; Kemiläinen et al., 2016; Luu-The et al., 2006; Sakurai et al., 2006)

The type 1, 7 and 12 of HSD17B enzymes are expressed in the ovaries (Duan et al., 1997; Kemiläinen et al., 2016; Luu-The et al., 2006; Nokelainen et al., 1996, 1998; Pelletier et al., 2005; Sawetawan et al., 1994). The expression of HSD17B7 is highest in the ovaries of pregnant mice, and HSD17B7 has been shown to localize to the *corpus luteum* (Nokelainen et al., 1998, 2000). Both HSD17B7 and HSD17B12 have been shown to convert E1 to E2, but human HSD17B7 has also been linked to cholesterol biosynthesis, which was also demonstrated in the HSD17B7KO mouse model (Jokela et al., 2010; Liu et al., 2007; Luu-The et al., 2006; Nokelainen et al., 1998). HSD17B12 is localized in theca cells, granulosa cells, oocytes and the surface epithelium in mouse ovaries (Kemiläinen et al., 2016). Luu-The *et al.* and Liu *et al.* demonstrated in *in vitro* that the HSD17B12 enzyme possesses an E1-to-E2 conversion ability (Liu et al., 2007; Luu-The et al., 2006). However, the main function of HSD17B12 appears to be in fatty acid synthesis, as has been shown with the HSD17B12KO mouse model (Kemiläinen et al., 2016). In contrast to the type 1, 7 and 12 HSD17B enzymes, HSD17B2 has been associated with dehydrogenase activity and shown to inactivate E2 to E1 and T to A-dione (Puranen et al., 1999; Wu et al., 1993). *Hsd17b2* is expressed in several tissues, such as the placenta, uterus, intestine, liver and kidney. (Moghrabi et al., 1997; Mustonen et al., 1997a, 1997b, 1998b).

HSD17B3 and 5 are mainly associated with T biosynthesis (Geissler et al., 1994; Lin et al., 1997; O'Shaughnessy et al., 2000). HSD17B3 is mostly expressed in the Leydig cells of the testis in both humans and mice; however, HSD17B3 has also been demonstrated to be present in human platelets, whereas HSD17B5 expression

is more widely distributed in tissues such as the kidney, prostate and testis in humans and mostly expressed in the liver with minimal expression in the testis in mice (Azzarello et al., 2008; Geissler et al., 1994; Gnatenko et al., 2005; Khanna et al., 1995; Labrie et al., 1997; Mustonen et al., 1997a; O'Shaughnessy et al., 2000).

2.1.2 *HSD17B1 gene and expression*

2.1.2.1 Human HSD17B1

Human HSD17B1 is coded by two genes (I and II) located in chromosome 17q12-21 (Luu-The et al., 1990; Peltoketo et al., 1988; Simard et al., 1993; Winqvist et al., 1990). The two genes are located in tandem on chromosome 17, and they exhibit an 89 % sequence similarity to each other (Luu-The et al., 1990; Peltoketo et al., 1992). HSD17B1 I contains a premature in-frame stop codon in the third exon, which has been identified as a pseudogene (Luu-The et al., 1990; Peltoketo et al., 1992). HSD17B1 II (referred hereafter as HSD17B1) includes six exons and five introns that produce two transcripts (1.3 kb and 5.0 kb, Yates et al., 2016, Ensembl release 89, human GRCh38.p10, Luu-The et al., 1989a, 1990). The shorter transcript starts 9 – 10 nucleotides upstream from the in-frame ATG initiating codon, whereas there are at least 814 noncoding nucleotides at the 5′ end of the longer transcript (Luu-The et al., 1989, 1990). The 1.3 kb transcript is expressed in the placenta, ovaries and breast tissue, while the longer transcript is expressed more widely in tissues such as the myometral, endometrial, prostate and abdominal fat tissues (Luu-The et al., 1989, 1990; Poutanen et al., 1992).

2.1.2.2 Mouse HSD17B1

In contrast to human *HSD17B1*, the mouse *Hsd17b1* gene encodes one transcript (Yates et al., 2016, Ensembl release 89, mouse GRCm38.p5). The transcript is 1339 bp long, and the *Hsd17b1* gene is located on chromosome 11 (Yates et al., 2016, Ensembl release 89, mouse GRCm38.p5). Mouse *Hsd17b1* in females is mainly expressed in the ovaries but also in uterus and adrenal glands. In males, *Hsd17b1* is expressed in the testis and liver (Nokelainen et al., 1996).

2.1.3 *HSD17B1 protein*

2.1.3.1 Human HSD17B1

HSD17B1 was discovered by Langer and Engel in 1958 and purified by Jarabak *et al.* from the human placenta in 1962 (Jarabak et al., 1962; Langer and Engel, 1958). Human HSD17B1 comprises of 327 amino acids with a calculated molecular weight of 34 853 Daltons (Da), and the protein exists as a homodimer (Lin et al., 1992; Luu-The et al., 1989; Peltoketo et al., 1988). The HSD17B1 protein core comprises seven-stranded aligned β-sheets (βA to βG) that are enclosed by six α-helices (αB to αG) (Ghosh et al., 1995). The βA to βF segment forms a Rossmann fold with a nicotinamide adenine dinucleotide (NAD) binding site (Ghosh et al., 1995). HSD17B1 utilizes NAD(H), NADP(H) and their reduced counterparts as cofactors but has been shown to prefer NADP(H) (Jarabak and Sack, 1969). The active site of human HSD17B1 contains three α-helices and a helix-turn-helix motif, which limits access to the active site and affects substrate specificity (Ghosh et al., 1995). These helices are not found in other short-chain dehydrogenases. Studies by Puranen *et al.* have indicated that the 148 – 268 amino acid region is responsible for different substrate specificities in the human and rat HSD17B1 enzymes (Puranen et al., 1997). The most prominent difference between human and rodent HSD17B1 is evident in the 148 – 191-amino-acid region. Compared to human HSD17B1, the differences in the amino acids in this region in the rodent enzyme alter the steroid-binding site of the enzyme to be more receptive to androgens (Puranen et al., 1997).

2.1.3.2 Mouse HSD17B1

The mouse HSD17B1 protein was cloned 1996 by Nokelainen *et al.* (Nokelainen et al., 1996). The mouse *Hsd17b1* gene codes a 344-amino-acid protein with a molecular weight of 37 055 Da (Nokelainen et al., 1996; Yates et al., 2016, Ensembl release 89, mouse GRCm38.p5). The overall similarity to human HSD17B1 is 63 %, with the highest similarity (81 %) occurring in the first 200 amino acid residues (Nokelainen et al., 1996). The mouse HSD17B1 contains typical SDR family residues in positions 10 and 156 (Gly-Xaa-Xaa-Xaa-Gly-Xaa-Gly and Tyr-Xaa-Xaa-Xaa-Lys, respectively), and the primary structure contains a cAMP-dependent (cyclic adenosine monophosphate) phosphorylation site that is also present in the human HSD17B1 sequences (basic-basic-Xaa-Ser, Nokelainen et al., 1996; Peltoketo et al., 1988).

2.1.4 *Function of HSD17B1*

HSD17B1 catalyses the conversion of E1 to E2 in both humans and mice (Nokelainen et al., 1996; Poutanen et al., 1993; Puranen et al., 1997). Sf9 insect cells expressing human or mouse HSD17B1 have been used to demonstrate that both the human and mouse proteins produce E2 with equal efficiencies. However, Sf9 cells with human HSD17B1 produced T from A-dione with a low capacity, whereas Sf9 cells with mouse HSD17B1 produced T with the same efficacy as E2, demonstrating the different substrate specificities of human and mouse HSD17B1 (Nokelainen et al., 1996). The different substrate specificity of mouse HSD17B1 was further analyzed by adding an excessive amount of A-dione into the E1-to-E2 conversion and E1 into the A-dione-to-T conversion. A ten-fold higher amount of A-dione did not affect the conversion rate of E1 to E2 of human HSD17B1, whereas the conversion rate of E1 to E2 of mouse HSD17B1 was reduced by 43 % with a five-fold higher amount of A-dione, and 59 % with a ten-fold higher amount of A-dione. Androgen conversion (A-dione to T) by mouse HSD17B1 was also observed to be affected by excessive amounts of E1, by 43 % when a five-fold higher amount of E1 was and 70 % when a ten-fold higher amount of E1 was used (Nokelainen et al., 1996).

2.1.5 *Regulation of HSD17B1*

Human *HSD17B1* expression is regulated by several different factors. cAMP regulates human HSD17B1 expression, but the findings are controversial. In cultured granulosa-luteal cells and choriocarcinoma cells, cAMP increases human *HSD17B1* expression, while in cytotrophoblast cells, the expression of *HSD17B1* is decreased (Tremblay et al., 1989). Due to these controversial findings, it has been suggested that cAMP-mediated regulation of *HSD17B1* mRNA expression is cell-type dependent (Ghersevich et al., 1994b; Lewintre et al., 1994; Tremblay et al., 1989). Rat *Hsd17b1* expression and activity are also cAMP-regulated; both expression and HSD17B activity are increased in diethylstilbestrol (DES)-treated rat granulosa cells in response to cAMP (Ghersevich et al., 1994b, 1994c). In addition, rat *Hsd17b1* mRNA expression is positively regulated by follicle-stimulating hormone (FSH), estrogens and androgens (Ghersevich et al., 1994b, 1994c). Estrogens and androgens are suggested to enhance FSH effects, whereas FSH actions occur through cAMP (Ghersevich et al., 1994b, 1994c). Negative regulation of *Hsd17b1* expression by human Chorionic Gonadotropin (hCG) has been observed in rat ovaries.

In addition to the above-mentioned factors, several other regulators of HSD17B1 have been identified. Progestin increases the human HSD17B1 protein concentration in T-47D human breast cancer cell line (Poutanen et al., 1990). Furthermore, positive regulation of HSD17B1 by retinoid acids has also been observed in choriocarcinoma, cytotrophoblast and breast cancer cells (Piao et al., 1995; Zhu et al., 2002). Positive regulation of mouse *Hsd17b1* expression by Activin A has been observed in gonadotropic cells and granulosa cells (Bak et al., 2009). Other positive regulators, such as epidermal growth factor (EGF), transforming growth factor (TGF) alpha, insulin-like growth factor (IGF) 1 and 2, tumor necrosis factor (TNF) alpha, and interleukins (Ils) 6 and 11 have been identified in humans (Duncan et al., 1994; Lewintre et al., 1994; Salama et al., 2009; Singh and Reed, 1991). The GATA motif has been observed to be a negative regulator of human *HSD17B1* expression in choriocarcinoma cells (Piao et al., 1997). In contrast to human HSD17B1 regulation, TGF alpha and EGF decrease HSD17B activity in rat granulosa cells (Ghersevich et al., 1994b; Kaminski et al., 1997).

2.2 Sex determination and its regulation

Sex determination is a biological event that determines the development of sexual characteristics. In both mice and humans, primary sex determination is chromosomal (XX in females and XY in males). During the early stages of fetal development, a bipotential gonad together with two sets of embryonic internal genitalia and undifferentiated ambisexual external genitalia, is developed (Quigley, 2006). Development of the gonads, ovaries or testes, begins with the differentiation of the bipotential gonad, which starts at week 6 of development in humans and embryonic day 10.5 in mice (Eggers and Sinclair, 2012).

2.2.1 *Genetic regulation of fetal testis development*

The main trigger for testis development from the gonadal primordium is activation of the sex-determining region Y gene (*Sry*, Fig. 1). In the individual with the XY chromosomes, activation of the *SRY* gene results in the initiation of cascades responsible for testicular development (Lovell-Badge, 1992; Sinclair et al., 1990). One of the first effects of SRY is the initiation of somatic cell migration from the mesonephros into the XY gonads, which is necessary for the formation of testicular cords (Quigley, 2006; Tilmann and Capel, 2002). Loss-of-function of or mutations in the *SRY* gene cause sex reversal from male to female, and ectopic expression of this gene in XX individuals results in female-to-male reversal (Jäger et al., 1990; Maier et al., 2003). Similar to the loss-of-function mutation in humans, deletion of

the *Sry* gene from the mouse Y chromosome results in male-to-female sex reversal with complete female external and internal genitalia in knockout male mice (Kato et al., 2013). Studies of the mouse *Sry* gene have revealed that expression of the gene is momentary in the bipotential gonads (Koopman et al., 1990) and that expression of *Sry* initiates the expression of the sex-determining region Y-Box 9 (*Sox-9*) gene in both humans and mice (Koopman et al., 1990; McClelland et al., 2012).

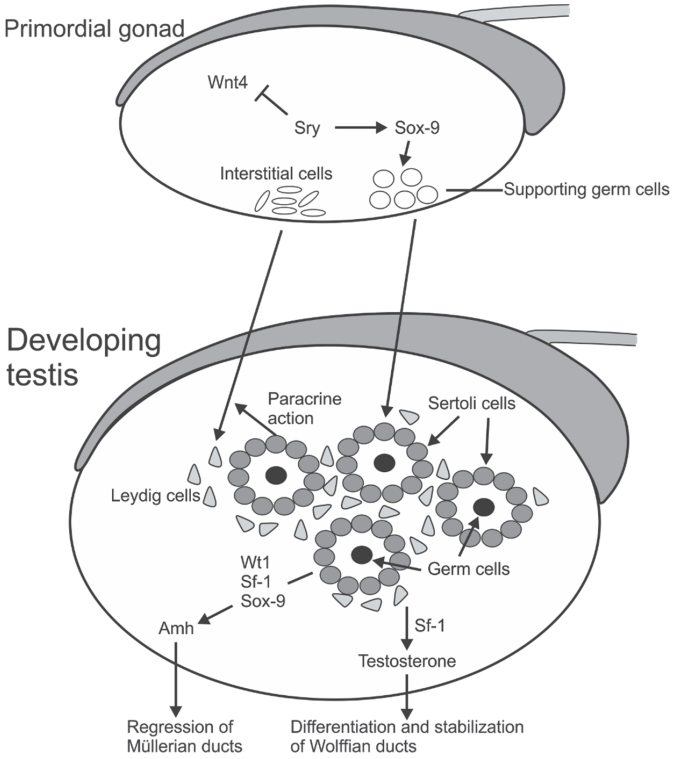

Figure 1. Model of testis determination. During the development, bipotential gonad with XY chromosomes begins to express *Sry*, which induces upregulation of *Sox-9* and inhibits *Wnt4* expression. SOX-9 induces the differentiation of Sertoli cells from supporting germ cells. Sertoli cells form the seminiferous tubules and promote the differentiation of Leydig cells from the interstitial cells. Leydig cells begin to secrete testosterone, which is controlled by SF-1, and produced testosterone stabilizes the Wolffian ducts. Anti-Müllerian hormone (Amh) secretion is induced by WT1, SF-1 and SOX-9 from Sertoli cells, resulting in the regression of Müllerian ducts. Modified from Quigley, 2006.

The *Sox-9* gene initiates the differentiation of interstitial precursor cells to Sertoli cells and is expressed in mouse Sertoli cells throughout testis development (Fig. 1, Kent et al., 1996; Morais da Silva et al., 1996). In addition, *Sox-9* regulates the organization of the seminiferous tubules (Kent et al., 1996; Morais da Silva et al., 1996). Similar to the *Sry* gene, deletion of *Sox-9* in mice causes a complete male-to-female sex reversal (Barrionuevo et al., 2005; Chaboissier et al., 2004), and 75

% of males with a heterozygous mutation in the *SOX9* gene exhibit a complete or partial sex reversal (Foster et al., 1994; Wagner et al., 1994). Sekido and Lovell-Badge have suggested that the mechanism behind male gonadal development involves three factors: steroidogenic factor 1 (SF-1), SOX-9 and SRY (Sekido and Lovell-Badge, 2008). In the genital ridges (of XX and XY individuals), SF-1 initiates low-level expression of *Sox-9*. Then, SF-1, together with SRY, binds to testis-specific enhancer of *Sox-9* core (TESCO) and upregulates the expression of *Sox-9* in the male genital ridges. The SOX-9 levels are kept high throughout the autoregulatory loop of SOX-9 itself.

Doublesex and mab-3 related transcription factor 1 (DMRT1) is an important regulator of primordial gonadal development and testicular development (De Grandi et al., 2000; Kim et al., 2003; Raymond et al., 2000). DMRT1 expression in the mouse testis is localized in Sertoli cells and germ cells, and loss of *Dmrt1* results in disrupted postnatal Sertoli cell differentiation (Kim et al., 2007; Raymond et al., 1999). Data from *Dmrt1*-deficient male mice suggest that DMRT1 is necessary for postnatal testis development but not for the fetal period because no disruptions are observed in the fetal testes (Raymond et al., 2000).

Testicular development begins with the appearance of primordial Sertoli cells. After formation of the genital ridge, proliferation of Sertoli cells occurs, and the cells agglomerate around the primitive germ cells, which later form the seminiferous tubules of the testis (Quigley, 2006). The Sertoli cells differentiate from the somatic cells of the coelomic epithelium. The fetal testis development is suggested to be Sertoli-cell -driven, as it has been demonstrated that without germ cells, development of the testis is morphologically normal (McCoshen, 1982). However, recently, it has been demonstrated that loss of germ cells in the testis results in irregular testis cord formation and a decreased number of Sertoli cells (Rios-Rojas et al., 2016). The Leydig cells and peritubular myoid cells differentiate from the mesonephric interstitial cells, and the latter participate in forming the testicular cords (Quigley, 2006). The last key phase of fetal testis development is formation of the vasculature, which transports substances from the testis to the internal and external genital, and thus enables their proper masculinization (Quigley, 2006).

2.2.2 *Hormonal regulation of male sexual development*

2.2.2.1 AMH and regression of the Müllerian ducts

The development of male internal genitalia comprises two distinct stages: regression of the Müllerian ducts and stabilization of the Wolffian ducts. The anti-Müllerian hormone (AMH) belongs to the TGFβ family, and during the fetal testicular development, high expression of AMH causes regression of the Müllerian ducts. SOX-9 is a direct regulator of AMH transcription (De Santa Barbara et al., 1998). In addition, binding of SF-1 and GATA binding protein 4 (GATA4) to the *Amh* promoter region upregulates the expression of *Amh* in Sertoli cells from the early phases of Sertoli cell differentiation until puberty (di Clemente and Belville, 2006; Lasala et al., 2011). AMH binds to two receptors, namely, anti-Müllerian hormone receptor types I and II (AMR-R1 and AMH-R2, Quigley, 2006). These receptors form a tetraheteromer of two molecules of each (Josso and Di Clemente, 2003). AMH-R2 is a transmembrane receptor with serine/threonine kinase activity, which when activated, phosphorylates the type 1 receptor (Quigley, 2006). The type 2 AMH receptor is expressed in fetal Müllerian ducts, Sertoli cells and adult ovarian granulosa cells (di Clemente et al., 1994). *AMH-R1*, identified as bone morphogenetic protein 1 receptor (BMPR1A), is expressed more widely in several tissues and has been suggested to also bind other TGFβ members (Jamin et al., 2002; Josso and Di Clemente, 2003). In the Müllerian ducts, which are formed from epithelial and mesenchymal cells, *AMR-R2* is expressed in mesenchymal cells (Baarends et al., 1994). Müllerian duct regression begins with the initiation of *AMR-R2* transcription by SF-1 and Wnt family member 7a (Wnt7a) in the mesenchymal cells surrounding the Müllerian duct (Parr and McMahon, 1998; De Santa Barbara et al., 1998). The AMH-dependent mesenchymal regression then causes apoptosis in the epithelia of the Müllerian ducts, which results in regression of the cranial part of the duct, while the caudal part of the duct continues to grow (Allard et al., 2000). Regression of the cranial part correlates with the inclining levels of AMH-R2, from cranial to caudal. The importance of both AMH and AMH-R2 has been demonstrated with knockout mouse models, where the male mice deficient in *Amh* or *Amh-r2* or double (*Amh/Amh-r2)* possess both female and male duct structures (Behringer et al., 1994; Mishina et al., 1996). In humans, a similar phenotype is known as persistent Müllerian duct syndrome type 2, where either the gene encoding *AMH* or *AMH-R2* is mutated resulting in Müllerian duct-derivatives in XY males (Belville et al., 1999).

2.2.2.2 Testosterone biosynthesis and Wolffian duct stabilization

Parallel to Müllerian ducts regression, the Wolffian ducts are stabilized, their resorption is prevented, and the external genitalia are masculinized. The main contributor to Wolffian duct stabilization is testosterone (Quigley, 2006). Testosterone is produced in fetal Leydig cells under the control of luteinizing hormone (LH). Mutation of the *LH-β* gene causes delayed puberty, low testosterone and arrested spermatogenesis (Achermann et al., 2001). Achermann *et al.* has also reported that placental hCG is enough to stimulate adequate amounts of testosterone during the important genital development period *in utero,* based on a patient with a heterozygous mutation of *LH-β* gene who had normal external genitalia. Mouse fetal Leydig cells do not require gonadotropins to develop and function, as demonstrated with mice lacking gonadotropin-releasing hormone (GnRH, O'Shaughnessy et al., 1998). Activation of LHCG-R initiates the steroidogenesis cascade by promoting the activation of the steroidogenic acute regulatory protein (StAR) preceding upregulation of several other factors such as a cytochrome P450 family 11 subfamily A member 1 (CYP11A1), hydroxysteroid-3β dehydrogenases (HSD-3β) –1 and 2, CYP17A1, HSD17B1, HSD17B3 and 5α-reductase type 2 (SRD5A2, Quigley, 2006). The steroidogenic pathway is discussed in more detail in Chapter 2.6.2.

The knockout mouse models of *Star* and *Cyp11a1* present various defects in both the gonads and reproduction. The common feature of these two models is feminized external genitalia. In the *Star*-deficient male mice, it has been suggested that the prenatal testosterone levels are altered because the male mice present with female external genitalia. Interestingly, the internal genitalia are masculinized normally, which indicates sufficient testosterone levels in the fetal testis for Wolffian duct stabilization and development (Caron et al., 1997; Hasegawa et al., 2000). The *Cyp11a1* knockout male mice have reduced testis, epididymides and vas deferentia sizes (Hsu et al., 2002). In addition, these male mice do not have prostates or seminal vesicles, and the external genitalia are feminized (Hsu et al., 2002). No mouse model has been established for the HSD-3β enzymes, but in humans, this deficiency results in decreased levels of steroids and undermasculinization of the male external genitalia (Simard et al., 2002). Mutations in the *CYP17A1* gene result in pseudohermaphroditism in males (New and Suvannakul, 1970; Rosa et al., 2010; Zachmann et al., 1972). HSD17B3 is expressed in the Leydig cells of the testis in both humans and mice (Baker et al., 1997; Geissler et al., 1994). In addition to HSD17B3, HSD17B1 has been demonstrated to be expressed in the mouse testis, though localized to the seminiferous tubules rather than interstitial cells (O'Shaughnessy et al., 2000). Mutations in the HSD17B3 enzyme disrupts testicular conversion of A-dione to T, which results in feminized external genitalia while presenting with masculinized internal genitalia (Andersson and Moghrabi,

1997; Geissler et al., 1994; Lee et al., 2007; Mendonca et al., 2000). The patients present with masculinized Wolffian duct-derivates (epididymides, vas deferentia and seminal vesicles), suggesting that there are other enzymes converting an adequate amount of testosterone close to the Wolffian ducts to ensure development. Development of the external male genitalia is dependent on dihydrotestosterone (DHT), which is produced from testosterone by 5α-reductase type 2 (SRD5A2) (Siiteri and Wilson, 1974; Wilson and Lasnitzki, 1971). *SRD5A2* is expressed in the primordia of the prostate and external genitals before differentiation, whereas there is no expression in the Wolffian ducts until they are differentiated. This suggest that testosterone is the main androgen affecting the development of Wolffian duct-derivates and that DHT has a major role in development of the external genitalia of males (Siiteri and Wilson, 1974; Thigpen et al., 1993). This is further supported by the findings that patients with mutated *SRD5A2* genes present with a uniform phenotype of feminized external genitalia and underdeveloped prostates (Mendonca et al., 2016). In contrast, male mice that are deficient in *Srd5a2* present with normal internal and external genitalia and are fertile, suggesting that testosterone is sufficient for development of the male urogenital tract in mice (Mahendroo et al., 2001).

The effects of androgens are passed through the androgen receptor (AR), which initiates the expression of target genes in androgen-dependent tissues (Brinkmann and Trapman, 1999; Quigley, 2006). Ligand-binding to AR results in a conformational change that enables AR to bind to DNA. The AR/ligand complex binds as a homodimer to androgen response elements (ARE) in DNA sequences and initiates target gene transcription (Heinlein and Chang, 2002; Prescott and Coetzee, 2006). Loss-of-function mutations in AR and the absence of AR are common causes of male pseudohermaphroditis, where patients show resistance or insensitivity to the androgen effects, known as heterogenous androgen insensitivity syndromes (AIS, Quigley, 2006). The patients demonstrate decreased masculinization with high variations, demonstrating the importance of androgens in male sexual development (Yong et al., 2003). Similar findings have been observed with a knockout mouse model of AR, where the *Ar* gene has been deleted universally from all tissues (ARKO). ARKO mice have similar phenotypes to human AIS-syndrome patients, with a female-like appearance and complete androgen insensitivity, demonstrating the importance of AR and androgen effects in male sexual development. (De Gendt et al., 2004; Notini et al., 2005).

2.2.3 *Genes regulating ovarian development*

Ovarian development in females starts from the same primordial genital bridge as male testicular development. The difference between the genders can be seen by gestational week 8, where testicular formation is completed but ovarian development has not started (Warne and Kanumakala, 2002). Until gestational week 10, ovarian development can be distinguished from testis development by the lack of testicular structures. The primordial germ cells (PGC) migrate from the yolk sac to the genital ridge, where the PGCs then become oogonia by entering into mitosis. After division, oogonia enter meiosis (Hummitzsch et al., 2013). The dividing oogonia, together with somatic cells, form ovarian cords, and later during development, these two cell types develop into primordial follicles. The oocytes and follicular epithelial granulosa cells develop from the oogonia and somatic cells, respectively (Hummitzsch et al., 2013). Theca cells, which differentiate from the mesenchymal cells of ovarian stroma, are corresponding counterparts of Leydig cells (Magoffin, 2005).

The genes regulating and controlling ovarian development are less known than the genes directing testicular development. One hypothesis suggest a two-stage regulatory process of development: expression of testis-inducing genes such as *SOX-9* is prevented, and either block the inhibitory effects towards ovarian development or activate ovarian development-promoting genes (Fig. 2, Quigley, 2006; Sarraj and Drummond, 2012).

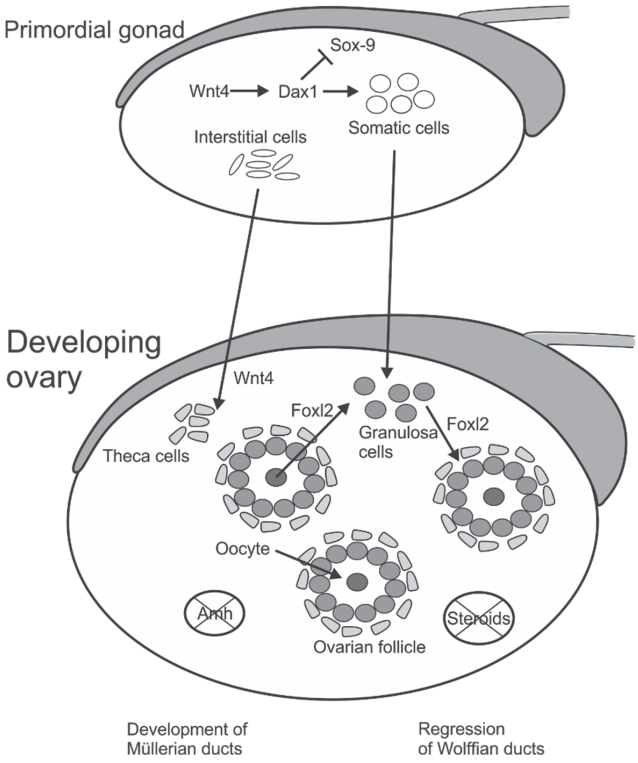

Figure 2. Model of ovary determination. The primordial gonads with XX chromosomes do not express *Sry* and *Sox-9*, which prevents the differentiation of Sertoli cells and formation of testis cord. Wnt4 induce the interstitital cells to develop into theca cells by inhibiting interstitial cells to develop into Leydig cells. Granulosa cells surround the oocytes to form ovarian follicles possibly under the influence of Foxl2. Due to the lack of Sertoli and Leydig cells, no anti-Müllerian hormone (AMH) or testosterone is produced causing the development of Müllerian ducts and regression of Wolffian ducts, respectively. Modified from Quigley, 2006.

WNT4 (wingless-type MMTV integration site family, member 4) was the first well-known regulator of ovarian development to be revealed. In mice, WNT4 is required for the beginning of Müllerian duct development in both sexes (Vainio et al., 1999). *Wnt4* expression is repressed in the testis during development but is sustained in the ovaries throughout fetal development. In females, the absence of *Sry* and its downstream target genes enables upregulation of *Wnt4* and subsequent *Dax1* (dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome, gene 1) expression, which directs the development of bipotential gonads towards ovaries (Mizusaki et al., 2003). WNT4 has been suggested to repress the vascular development of gonads, prevent steroidogenic cell migration and suppress the differentiation of Leydig cells from primordial interstitial cells (Jeays-Ward et al., 2003; Vainio et al., 1999). WNT4 together with R-

spondin1 (RSPO1), regulates cellular proliferation in the early gonads of mice, and the loss of either WNT4 or RSPO1 results in the development of ovotestes (Chassot et al., 2012, 2008; Tomizuka et al., 2008; Vainio et al., 1999). In *Wnt4* knockout mice, the knockout female mice have masculinized gonads, regressed Müllerian ducts and developed Wolffian ducts, which indicates that *Wnt4* performs its essential functions in female development by repressing testosterone synthesis and secretion via several mechanisms (Vainio et al., 1999). In addition, overexpression of human *WNT4* in male mice disrupts testicular vascularization and testosterone biosynthesis, which suggests that WNT4 regulates ovarian development by repressing male coelomic blood vessel formation and migration of steroidogenic cells to the gonads (Jordan et al., 2003). *Wnt4* knockout mouse studies have also revealed the importance of WNT4 for granulosa cell development as loss of *Wnt4* results in differentiation of pregranulosa cells to Sertoli cells instead of granulosa cells at birth (Chassot et al., 2014; Maatouk et al., 2013).

The other key factor in ovarian development is winged helix/forkhead transcription factor 2 (FOXL2), which is one of the earliest markers of ovarian development in mammals (Loffler et al., 2003). Mutations in FOXL2 in humans results in premature ovarian function failure with some cases of dysgenic development of the ovaries (Crisponi et al., 2001). The ovaries of *Foxl2* knockout female mice do not develop growing follicles and have a partial female-to-male sex-reversal (Ottolenghi et al., 2005; Uda et al., 2004). The importance of WNT4 and FOXL2 for ovarian development has been demonstrated in a double knockout mouse model of *Wnt4* and *Foxl2,* in which the female mice undergo a complete female-to-male sex reversal, including the germ cells (Ottolenghi et al., 2007).

WNT4-mediated differentiation of granulosa cells prevents the production of AMH by Sertoli cells, which in turn, prevents regression of the Müllerian ducts. Suppression of Leydig cell differentiation leads to a lack of androgens in the gonads, which leads to regression of the Wolffian ducts and promotes the development of female external genitalia (Quigley, 2006).

2.3 Sexual maturation and its endocrinology

Sexual maturation, *i.e.*, puberty of females and males, is a process of analogous changes in endocrinological and physiological events. Sexual maturation is caused by elevated production of steroids. In females, elevated steroidal production mainly includes ovarian estradiol and progesterone and adrenal androgen precursors. In males, increased steroidal production mainly includes testosterone, dihydrotestosterone and estradiol.

Neuroendocrine factors are the main regulators of gonadal sex steroid production. The neuroendocrine factors include GnRH, which is produced by the hypothalamus, and LH and FSH which are produced by the pituitary. All of these factors are released in a pulsatile manner, which involves a regulated feedback loop (Odell and Jameson, 2006). The gonadotropin levels already increase before the onset of puberty during fetal development and during postnatal development. In addition, another neuroendocrine regulator, KISS-1 metastasis-suppressor (KISS1), is essential for proper onset of puberty. Mutations resulting in loss-of-function of the KISS1 receptor (KISS1R) or targeted deletion of either *Kiss1* or *Kiss1r* causes hypogonadotropism with delayed or absent puberty in both humans and mice (Funes et al., 2003; García-Galiano et al., 2012; Lapatto et al., 2007; Topaloglu et al., 2012).

2.3.1 *Female sexual maturation*

The increase in E2 production and consequent increase in the serum concentration causes the development of secondary sexual characteristics at the target organs, such as breast tissue growth, linear bone growth and epiphyseal plate fusion (DiVall and Radovick, 2009). In addition, deposition of body fat and development of the genitalia (vagina and uterus) and pubic hair are affected. The rise in E2 levels negatively regulates hypothalamic production of GnRH.

The onset of puberty in females initiates the menstrual cycle and ovulation. In humans, the length of the menstrual cycle is between 25 and 30 days, whereas the estrous cycle in mice lasts approximately 4 to 5 days (Caligioni, 2009). The menstrual cycle comprises the follicular and luteal phases in humans. During the follicular phase, the ovarian follicles grow and mature, and one of the follicles becomes the dominant ovulatory follicle under FSH stimulation (Marshall, 2006). During the second half of the follicular phase, E2 is produced by the ovarian granulosa cells of the dominant follicle. In contrast to the negative feedback on GnRH, the increased E2 levels stimulate the preovulatory LH surge at the pituitary (Hall, 2009). Ovulation is initiated by the LH surge, which is followed by the luteal phase, where the *corpus luteum* is formed. If no fertilization has occurred, the luteal phase transits back to the follicular phase. The characteristics of the transition include a decline in CL function with decreases in the E2 and P4 levels (Fig. 3).

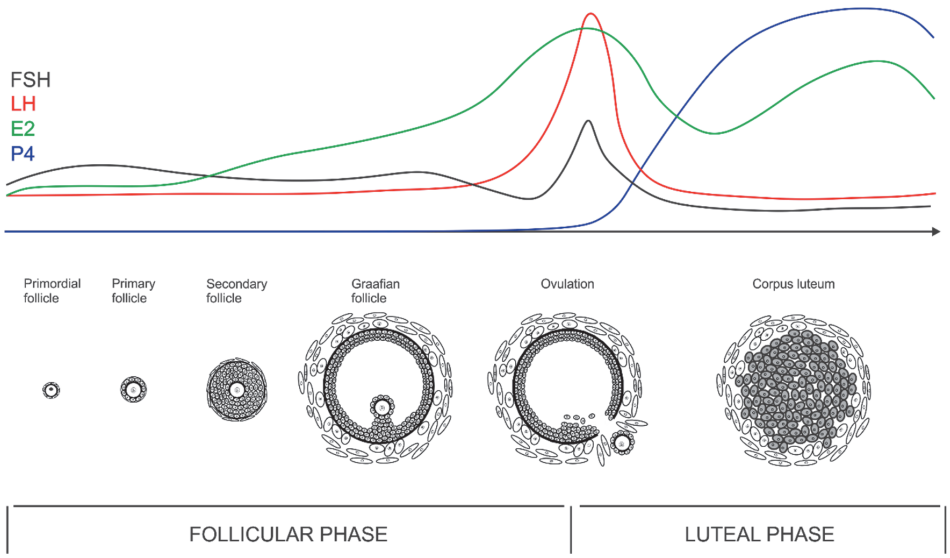

Figure 3. Menstrual cycle and associated hormonal changes. The menstrual cycle comprises two phases, follicular phase and luteal phase. E2 is produced from granulosa cells after the middle of the follicular phase. During the follicular phase, follicles develop and the preovulatory LH surge initiates ovulation. After ovulation, during the luteal phase, *corpus luteum* is formed and P4 is produced. If fertilization does not occur, *corpus luteum* regresses and cycle starts over.

In mice, the estrous cycle has four phases: proestrus, estrus, metestrus and diestrus. The proestrus and estrus stages correspond to the follicular phase of the menstrual cycle, where the follicles grow under the influence of FSH, mature and eventually ovulate after the LH surge (Caligioni, 2009). Ovulation in mice occurs during the estrus stage, and the preovulatory follicles form the *corpora lutea*, which starts producing P4. Progesterone production during metestrus and diestrus suppresses LH production and prevents ovulation (Bertolin and Murphy, 2014). If no fertilization occurs, the CLs and their function deteriorate, causing a reduction in P4 (Bertolin and Murphy, 2014). The reduced P4 concentrations alleviate the inhibitory effect of P4 on LH and enable the cycle to start again from proestrus.

2.3.2 *Male sexual maturation*

During male postnatal development, an event referred to as minipuberty occurs. Minipuberty occurs after birth due to the disappearance of placental E2. Subsequent loss of negative feedback increases the LH levels and elevates androgens to the pubertal level (Fig. 4.). Minipuberty affects long-term testicular functions and spermatogenesis by increasing the testicular volume and mass of Sertoli cells (Baştuğ, 2014). At puberty, increased levels of LH and FSH promote testosterone

production in Leydig cells, which in turn, exhibit negative feedback on the pituitary gonadotrophins and GnRH (Kerr and de Kretser, 2006). Similar to the E2 effect on female secondary sexual characteristics, T promotes genitalia and pubic hair growth, increases bone mass and muscle strength and causes a growth spurt (Odell and Jameson, 2006). In addition, sexual maturation of males initiates spermatogenesis, which will be discussed in Chapter 2.5.1.

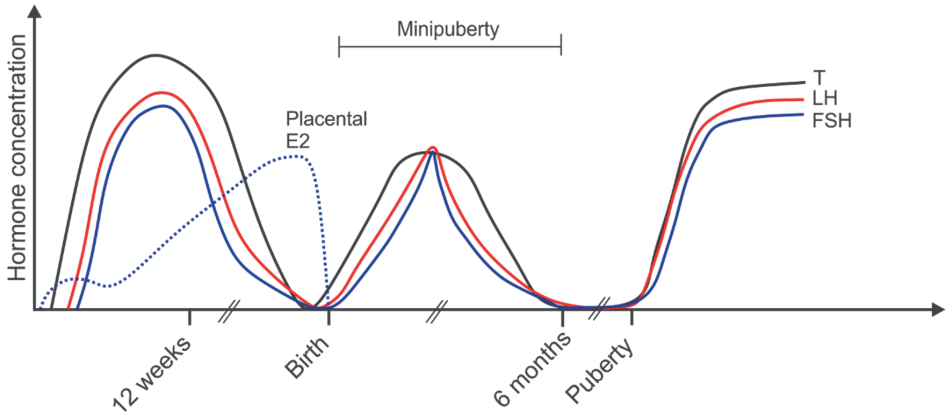

Figure 4. Pituitary gonadotrophins and testosterone during fetal, postnatal and pubertal development. The loss of placental E2 after birth causes a loss of negative feedback on the hypothalamus-pituitary-gonadal axis. Induction of the LH levels causes minipuberty, where testosterone level is elevated to the pubertal level, peaking at the three months of age in humans.

2.4 Female reproduction

2.4.1 *Folliculogenesis*

The ovaries undergo constant changes from the onset of puberty to menopause due to folliculogenesis, where primordial follicles develop into preovulatory follicles of which some are eventually ovulated. Follicular development can be divided into two separate phases: the preantral phase and the antral phase (Erickson, 2006).

The preantral phase of folliculogenesis comprises the development of primordial follicles to preantral follicles. The preantral phase is also known as the gonadotropin-independent phase. The preantral phase begins when primordial follicles are recruited from the pool of quiescent follicles. The pool of quiescent follicles is formed during the fetal period, and no stem cells are present to produce more oocytes after birth. The primordial follicle consists of an oocyte, a single squamous granulosa cell layer and a basal lamina membrane (Erickson, 2006). Recruitment of primordial

follicles from the pool requires several factors, such as growth differentiation factor 9 (GDF-9), FOXL2 and AMH. The importance of GDF-9 for follicular recruitment has been demonstrated by treating rats with recombinant GDF-9; follicular activation was increased (Kim, 2012). In addition, when introduced to human ovarian follicles *in vitro*, recombinant GDF-9 promoted follicular growth and development with an increased survival rate of the follicles (Hreinsson et al., 2002). The importance of GDF-9 has also been shown by Dong *et al.* with a GDF-9-deficient mouse model, where the follicles of the knockout female mice did not develop further than the primary follicle stage (Dong et al., 1996). As mentioned in Chapter 2.2.3, FOXL2 is required for the development of growing follicles. The *Foxl2* knockout female mice did not develop secondary follicles even though normal primordial follicle development was present (Schmidt et al., 2004). The data demonstrated that *Foxl2* was necessary for granulosa cell development as the impaired follicular development in knockout mice was observed to be due to an improper transition of squamous granulosa cells to cuboidal granulosa cells (Schmidt et al., 2004). Granulosa cells express AMH and its type 2 receptor (AMH-R2) postnatally (Baarends et al., 1995; Hirobe et al., 1992). The primordial follicles are depleted from the pool prematurely in *Amh* knockout female mice, indicating an inhibitory effect of AMH on the growth of primordial follicles (Durlinger et al., 1999).

Primordial follicles develop further into primary follicles, during which the oocyte grows in size (Erickson, 2006). The primary follicle consists of a single layer of one or more cuboidal granulosa cells and a layer of basal lamina. During the transition from the primordial to primary follicle, some critical steps occur, such as expression of FSH receptor (FSHR) in granulosa cells, formation of gap junctions between the oocyte and granulosa cells and deposition of the zona pellucida. Development of the primary follicle initiates the expression of FSHR (Oktay et al., 1997). FSHR expression is important for the follicles to be able to advance further in folliculogenesis (Palermo, 2007).

The secondary follicles, which contain the fully grown oocyte and zona pellucida, are developed from the primary follicles. The appearance of the theca cell layer in the secondary follicles is the key step in this stage of folliculogenesis. The theca cells have been suggested to originate from fibroblast-like precursors from the stroma of the ovaries (Honda et al., 2007). The granulosa cells have been observed to promote stromal cell differentiation into theca cells (Orisaka et al., 2006). In the secondary follicles, there are two to eight layers of cuboidal or columnar granulosa cells, and the theca cell layer is located next to the basal lamina (Erickson, 2006). Vascular formation is another important factor that occurs in the secondary follicles (Young and McNeilly, 2010). Angiogenesis enables exposure of the secondary follicle to hormones, such as LH and FSH, and angiogenesis is also necessary for follicular survival (Erickson, 2006; Young and McNeilly, 2010).

The next step from the secondary follicle is the formation of the antral follicle (also known as the Graafian follicle). During the last phases of secondary follicle development, follicular fluid accumulation begins and is followed by opening of the crescentic antrum (Erickson, 2000). During cavitation of the Graafian follicle, theca cells undergo differentiation to two distinct subpopulations: *theca interna* and *theca exerna*. The theca interna cell is a steroid-producing cell with an epithelioid shape, and the theca externa cell resembles smooth muscle cell (Tajima et al., 2007). The LH receptor is expressed in the theca interna cells, and in addition, granulosa cells begin expressing aromatase under FSH stimulation (Minegishi et al., 2008; Whitelaw et al., 1992; Zeleznik, 2004).

The last step before ovulation in folliculogenesis is selection of the dominant follicle at the end of the luteal phase from the group of small Graafian follicles. The selected dominant follicle is recognized by its maintenance of high mitotic activity during maturation. The group of small Graafian follicles show increased mitotic activity in the granulosa cells, but the difference between the selected and nonselected follicles is that the selected dominant follicle undergoes rapid cell division, while mitosis in the nonselected follicles slows down (Erickson, 2006). Selection of the dominant follicle requires increased plasma FSH levels, which directs the development of early antral follicles further (Zeleznik, 2004). The increase in FSH levels promotes the synthesis of E2 by increasing aromatase gene expression in granulosa cells. The rising levels of E2 have a negative feedback effect on FSH and thus reduce the FSH levels. This reduction in FSH by E2 prevents the maturation of inferior follicles. LH receptor expression in granulosa cells of the Graafian follicle enables these cells to respond to the preovulatory LH surge (Minegishi et al., 1997). The LH surge initiates expression of genes, such as progesterone receptor (*Pgr*) and CCAAT/enhancer-binding protein β (*Cebpb*), which are critical for a proper ovulation process (Lydon et al., 1995; Sterneck et al., 1997). Knockout mutations of either *Pgr* or *Cebpb* result in infertility caused by failure to ovulate completely or efficiently, respectively (Lydon et al., 1995; Sterneck et al., 1997).

The last step of folliculogenesis is ovulation of the dominant follicle. Meiosis is dormant in developing follicles, but during ovulation, the preovulatory LH surge initiates meiosis again (Erickson, 2006). The LH surge in the middle of the cycle induces the production of progesterone, which functions through the PGR to induce the prostaglandin synthesis. Prostaglandins bind to prostaglandins receptors in the epithelial cells at the surface of the follicle, causing the release of lysosomal enzymes. The lysosomal enzymes degrade ovarian tissues at a localized area known as the stigma, which causes the follicle to rupture and the oocyte to be released (Erickson, 2006; Strauss and Williams, 2013).

2.4.2 *Luteogenesis*

Luteogenesis is an event where the ovulated follicle transforms into the *corpus luteum*. The ovulated follicle contains theca interstitial cells, which transform into theca lutein cells (small luteal cells) and granulosa cells transform into granulosa lutein cells (large luteal cells, Strauss and Williams, 2013). Luteogenesis can be divided into two separate phases of the *corpus luteum* life cycle: luteinization and luteolysis.

Luteinization is regulated by LH which initiates the expression of several genes. A key event during luteinization is vascularization of the CL, where vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) gene expression is initiated by LH (Strauss and Williams, 2013). In addition to vascularization, expression of *STAR*, *CYP11A1*, *HSD-3β* and *CYP19A1* is induced in large luteal cells by LH (Erickson, 2006). These genes are involved in steroidogenesis, and their expression induces high production of E2 and P4. In cases of fertilization and subsequent implantation of the embryo in humans, the *corpus luteum* grows in size and becomes the *corpus luteum of pregnancy* (Erickson, 2006; Strauss and Williams, 2013). For the *corpus luteum* to become the *corpus luteum of pregnancy*, human chorionic gonadotrophin (hCG) needs to be produced from the trophoblasts (Stouffer et al., 1977; Strauss and Williams, 2013). In cases of improper CL formation or function, pregnancy cannot be maintained. Several mouse models, *i.e.,* the frizzled class receptor 4 (FZD4), prolactin receptor (PRLR) and nuclear enriched abundant transcript 1 (NEAT1) knockout mice, have demonstrated the importance of the *corpus luteum* for maintenance of pregnancy as infertility in these models was due to improper formation of the *corpus luteum* (Grosdemouge et al., 2003; Hsieh et al., 2005; Nakagawa et al., 2014).

If fertilization does not occur, luteolysis occurs, and the CL regresses. In rodents, two-phase regression of the CL occurs: the first phase is functional regression, and second phase is structural regression (Stocco et al., 2007). During functional regression of the CL, P4 production is terminated, which reduces circulating P4 levels and thus enables parturition to occur. Two important factors have been suggested to be involved in functional regression of the CL: prostaglandin F2alpha (PGF2A) and LH. In 1969, Pharriss and Wyngarden (Pharriss and Wyngarden, 1969) demonstrated that when PGF2A was administered to pseudopregnant rats, circulating P4 levels decreased. In addition, mice with the null mutation of the PGF2A receptor do not give birth due to steady levels of P4 (Sugimoto et al., 1997). Removal of the ovaries enables PGF2A-null female mice to undergo parturition. It has been demonstrated that PGF2A abundantly increases the expression of 20α-hydroxysteroid dehydrogenase (20α-HSD, Stocco et al., 2000). PGF2A is produced mainly in the uterus, but a minor proportion is also produced by the CL itself (Arosh et al., 2004; Hayashi et al., 2003; Stocco et al., 2007). Even though

luteinizing hormone has a known role as a luteotropic factor, there is also evidence that LH is induced at the end of pregnancy and that LH reduces P4 production by stimulating 20α-HSD activity (Morishige et al., 1973; Stocco and Deis, 1996).

During structural regression, the size and weight of the CL decreases due to apoptosis, and the *corpus luteum* transforms into the *corpus albicans*. The programmed cell death that is responsible for structural regression of the CL is regulated by two pathways: the death receptor mediated-pathway and mitochondrial pathway. The death receptor-mediated pathway functions through so-called death receptors, including the tumor necrosis factor (TNF) and Fas receptors and their ligands (Henkes et al., 2008; Kuranaga et al., 2000; Pru et al., 2003; Sakamaki et al., 1997; Sakumoto et al., 2011). The mitochondrial pathway functions through initiator caspases, such as caspase-8, which initiates a stress stimulus that changes mitochondrial permeability and eventually results in activation of an apoptotic factor, caspase-9 (Stocco et al., 2007). Studies with rat luteal cells have demonstrated a decrease in Bh3 interacting domain death agonist (Bid), which mediates mitochondrial damage through caspase-8 and increases cleavage of procaspase-9 under starvation conditions, indicating mitochondrial pathway activation (Goyeneche et al., 2006). Both of the pathways leading to programmed cell death activate the final effectors of programmed cell death or executioner caspases, *i.e.*, caspase-3, caspase-6 and caspase-7 (Stocco et al., 2007). Caspase-3 is located in the CL of mice, and a null mutation in caspase-3 results in impaired cell death and delayed atrophy of the CL (Carambula et al., 2002). However, loss of caspase-3 does not prevent involution of the CL, suggesting that caspase-3 is not the only factor regulating cell death in the CL. Studies have indicated that PGF2A and prolactin are involved in structural regression of the CL. Luteal apoptosis induced by PGF2A in pseudopregnant rats associated with increased BAX (BCL2 associated X, an apoptosis regulator) and BCL-2 (BCL-2, an apoptosis regulator) and with elevations in the protein and activity levels of caspace-3 and caspase-9 (Yadav et al., 2005). Additionally, prolactin induces structural regression through apoptotic cell death (Endo et al., 1993; Matsuyama et al., 1996).

2.5 Male reproduction

Male reproduction comprises the production of male germ cells. The process known as spermatogenesis is responsible for male germ cell production, which continues throughout the reproductive lifetime of males (Griswold, 2016). Spermatogenesis occurs in the seminiferous tubules, which consist of the seminiferous epithelium. All cell populations in the seminiferous epithelium are highly organized and appear along the tubule either in sequence or in specific stages (Fig. 5). The seminiferous tubule cycles consists of 6 stages (I – VI) in humans and 12

stages in mice (I – XII) (Hermo et al., 2010a; Smith and Walker, 2015). The seminiferous tubules cycle is continuous, and the duration of each cycle is 16 days in humans and 8.6 days in mice (Hermo et al., 2010a; Oakberg, 1956). The stages of the seminiferous tubule cycle follow each other in a wavelike fashion in humans and mice (Smith and Walker, 2015). In humans, the cycle spirals through the seminiferous tubule, while in rats the cycle is arranged segmentally. The seminiferous epithelium consists of multiple layers of germ cells at different stages, Sertoli cells and peritubular myoid cells (Kerr and de Kretser, 2006).

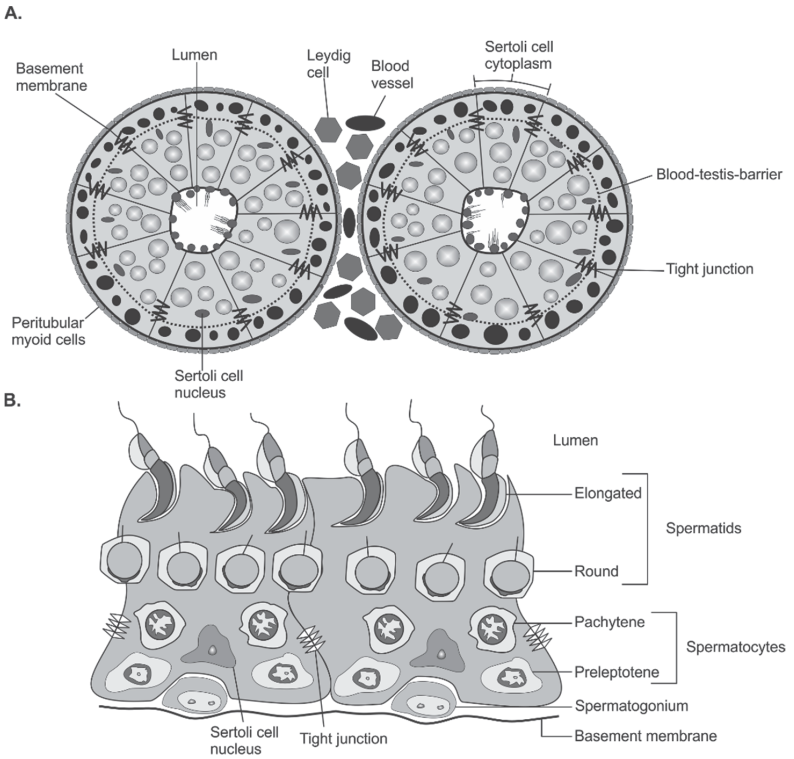

Figure 5. Structure of seminiferous tubules and spermatogenesis. A., A cross-sectional visualization of two seminiferous tubules. Different stages of germ cells are embedded in Sertoli cell cytoplasm and the mature spermatozoa is shown in the lumen. Seminiferous tubules are surrounded by peritubular myoid cells, and Leydig cells and blood vessels are located in the interstitial space between the tubules. Dashed line presents the blood-testis-barrier and saw-edged line presents a tight junction. B., Visualization of Sertoli cells and germ cells. Sertoli cells surround the germ cells with cytoplasm, which extends from the basement membrane to the lumen. Tight junctions form a blood-testis barrier between Sertoli cells, indicated by the saw-edged figure. During the development, germ cells migrate from the basement membrane towards the lumen. Modified from Smith and Walker, 2015.

2.5.1 *Spermatogenesis*

2.5.1.1 Proliferative and meiotic phases

In contrast to female oogenesis, male spermatogenesis does not begin until the onset of puberty (Feng et al., 2014). It has been calculated that in mice, spermatogenesis from the spermatogenic stem cell pool to spermiation takes approximately 35 days, while in humans, it takes approximately 74 days (Amann, 2008; Griswold, 2016). Spermatogenesis can be divided roughly into three distinct phases, namely, the proliferative phase, meiotic phase and differentiation phase (known as spermiogenesis).

Spermatogenesis encompasses two distinct models describing the early development of germ cells in mice and humans: the A_s model for mice and the A_{dark}/A_{pale} for humans. A_s germ cells are rare stem cells in the rodent testes that are distributed regularly along the basal lamina membrane of the seminiferous tubules (Tegelenbosch and de Rooij, 1993; Valli et al., 2015). These stem cells produce A_{paired} spermatogonia which can either produce two new A_s spermatogonia through complete cytokinesis (known as self-renewal) or stay connected to the intercytoplasmic bridge and produce $A_{aligned}$ spermatogonia (up to 16 or sometimes even 32 $A_{aligned}$ spermatogonia) after the sequential divisions. The A_s, A_{paired} and $A_{aligned}$ are termed as $A_{underdifferentiated}$. Development continues with differentiation of chained 4, 8 or 16 $A_{aligned}$ spermatogonia into A1 spermatogonia which occurs in stage VII of the seminiferous epithelium cycle. This is then further continued by consecutive mitotic division to produce A2, A3 and A4 spermatogonia, B spermatogonia and eventually, preleptotene spermatocytes, which then enter meiosis and migrate from the basal lamina towards the lumena of the seminiferous tubules (Valli et al., 2015).

The human model consists of two distinct undifferentiated A spermatogonia, namely, A_{dark} and A_{pale}, based on their haematoxylin staining and nuclear architecture (Valli et al., 2015). It has been suggested by Clermont *et al.* that these two subpopulations represent the quiescent (A_{dark}) and active (A_{pale}) stem cells (Clermont, 1969). The A_{pale} cells maintain the spermatogonial stem cell pool with self-renewal divisions and also differentiate into B spermatogonia while the A_{dark} spermatogonia remain dormant and become active only if the pool of A_{pale} is depleted (Amann, 2008; Clermont, 1969; Dym et al., 2009). The distribution between A_{dark} and A_{pale} differs as the A_{dark} is evenly spread along the seminiferous tubules similarly to the mouse A_s and A_{paired} spermatogonia while the A_{pale} spermatogonia are concentrated at mid-cycle, similarly to the mice $A_{aligned}$ spermatogonia (Valli et al., 2015). It has been shown in nonhuman primates that the A_{pale} spermatogonia

precede the differentiating B spermatogonia, which appear in stage X (Fouquet and Dadoune, 1986; Valli et al., 2015). The difference between nonhuman primate and human B spermatogonia is that primates have four generations of B spermatogonia (B1 – B4) while humans have only one generation of B spermatogonia (Valli et al., 2015). Type B spermatogonia then give rise to the preleptotene spermatocytes, which are involved in the differentiation phase.

2.5.1.2 Differentiation phase

The differentiation phase or spermiogenesis comprises four separate phases: the Golgi, cap, acrosome and maturation phases (Clermont et al., 1993; Kerr and de Kretser, 2006). During the Golgi and cap phases, the acrosome is formed at the cranial side of the nucleus causing flattening of the head, and the acrosome eventually covers the anterior part of the sperm head (Kerr and de Kretser, 2006). In addition, at the opposite side of the nucleus, the axoneme is formed by two centrioles. At this time, the cell-cell junctions between the spermatids and Sertoli cells are also formed (O'Donnell, 2014). During the third phase, also known as the acrosome phase, the nuclear chromatin condenses, and modification of the species-specific shape of the sperm head occurs. The microtubular structure, known as the manchette, determines the sperm head shape (O'Donnell, 2014). Development of the middle and principal pieces is associated with elongation of the caudal cytoplasm, which then forms the flagellum. During the last phase (maturation), the spermatid cytoplasm forms residual bodies around the sperm head and tail, and prior to the release of spermatozoa from the Sertoli cells, these residual bodies are phagocytosed by the Sertoli cells (Kerr and de Kretser, 2006).

2.5.1.3 Hormones and spermatogenesis

Spermatogenesis and its hormonal regulation by FSH, T or E2 has been studied widely. Analyses of knockout mouse models FSHRKO and FSHβKO have demonstrated that loss of FSH during development reduces the number spermatogonia, spermatocytes and spermatids, which indicates that FSH increases the number of spermatogonia and their entrance into the first meiotic division (Abel et al., 2008; Wreford et al., 2001). It has been suggested that FSH also plays a role in the completion of spermiogenesis; however, the studies by Abel *et al.* with FSHRKO further demonstrate that FSH does not play a role in spermiogenesis as the ratio of round spermatids to mature sperm is comparable to that of the control (Abel et al., 2008).

Androgens are key factors in the development and maintenance of spermatogenesis, as has been demonstrated using knockout mouse models, such as ARKO and Sertoli cell-specific AR knockout (SCARKO) mice and luteinizing hormone receptor knockout (LuRKO) mice or with mouse models with markedly lowered androgen levels, such as gonadotrophin-deficient mice (*hpg* mouse model, Cattanach et al., 1977; De Gendt et al., 2004; Haywood et al., 2003; Yeh et al., 2002; Zhang et al., 2001). In all of the models, spermatogenesis is disrupted, and in the model with lowered androgen levels, spermatogenesis can be restored with testosterone replacement (Haywood et al., 2003; Pakarainen et al., 2005). In addition, it has been demonstrated by Zhang *et al.* that the low levels of T without LH stimulation are sufficient for maintenance of spermatogenesis (Zhang et al., 2003). LuRKO male mice present with qualitatively full spermatogenesis at the age of 12 months regardless of the lack of LH action and with markedly low testosterone (only 2 % of the control level). Because the observed changes in the AR knockout models can be due to the effect of *Ar* deletion that occur during development and during the first wave of spermatogenesis, Willems *et al.* have studied these changes by using an inducible AR knockout mouse model (iARKO, Willems et al., 2011). In this model, deletion of *Ar* is induced by tamoxifen, which itself has potential to disrupt spermatogenesis (D'Souza, 2003). However, tamoxifen treatment of the control animal does not have significant effects on spermatogenesis, while impaired spermatogenesis is observed in tamoxifen-treated iARKO testes (Willems et al., 2011). The role of androgens in the later phases of spermatogenesis, such as spermiogenesis and spermiation, has been studied with androgen-withdrawal models (Donnell et al., 1994; Kerr et al., 1993; O'Donnell et al., 1996; Saito et al., 2000). It was shown with these models that androgens are necessary for adhesion between Sertoli cells and spermatids and that androgens inhibit phagocytosis of elongated spermatids and prevent the premature release of round spermatids. All of these models indicate that androgens are necessary for spermatogenesis.

Estrogens are also needed for proper spermatogenesis. One of the key enzymes that converts androgens into estrogens is aromatase which, is expressed in Leydig cells and germ cells (Carreau et al., 2009; Levallet et al., 1998; Nitta et al., 1993). Aromatase knockout (ArKO) male mice undergo progressive spermatogenic failure and become completely infertile at the age of one year (Robertson et al., 1999). Spermatogenesis is halted during the early steps of spermatogenesis with degenerating round spermatids and no elongating spermatids (Robertson et al., 1999). In the ArKO testis, abnormal acrosomal development is also observed, which indicates a role for E2 in acrosomal development. In addition, analyses with an aromatase overexpressing mouse model ($AROM^+$) have also demonstrated the importance of estrogens in spermatogenesis as the $AROM^+$ male mice present with arrested spermatogenesis at the pachytene spermatocyte stage together with ele-

vated E2 and reduced T concentrations in the serum (Li et al., 2001). It was hypothezised by Li *et al.* that the cause of the arrested spermatogenesis was partly due to the suppression of FSH secretion by excessive E2. However, as the AROM$^+$ male mice were all cryptorchid, the cause of the impaired spermatogenesis could also be due to cryptorchidism. ERαKO male mice exhibit infertility that is caused by a structural defect rather than by a direct impact on spermatogenesis as the lack fluid reabsorption in the efferent ductules causes backpressure to the testis and thus leads to germ cell death (Hess et al., 1997). This has been further studied by Sinkevicius *et al.* who demonstrated, surprisingly, that the fluid reabsorption is regulated by ligand-independent ERα signaling and that estrogen-dependent ERα signaling is essential for viability of germ cells (Sinkevicius et al., 2009a). It was hypothesized that impaired germ cell viability was the result of altered Sertoli cell function. The first two estrogen receptor β knockout mouse models developed by Krege *et al.* and Dupont *et al.* demonstrated normal fertility in the male KO mice (Dupont et al., 2000; Krege et al., 1998). These knockout models were produced with insertion of a *Neo* cassette into exon 3 with slightly different approaches. In both models, several splice variants of estrogen receptor β were detected. This led Antal *et al.* to produce a mouse model where the entire exon 3 was deleted to remove the possibility that other transcript variants caused the observed phenotype (Antal et al., 2008). These male KO mice were reported to be infertile, but no abnormalities in the testis histology were evident. However, when the male mice were bred, they did not produce copulatory plugs, which indicated that the infertility problem in this case could be due to sexual behavior rather than spermatogenesis (Antal et al., 2008). Taken together, the data from these models show that estrogens through ERα have an important role in the early development of spermatogenesis and for overall spermatogenesis.

2.5.2 *Sertoli cell function*

Sertoli cells are supporting cells for germ cell development. The sperm producing ability of men is dependent on the number of Sertoli cells, as shown in studies by Johnson *et al.* and Orth *et al*. in 1984 and 1988, respectively (Johnson et al., 1984; Orth et al., 1988). Sertoli cells are located inside of the seminiferous tubules beside the peritubular myoid cells and basal lamina membrane (Smith and Walker, 2015). From the basal lamina membrane, Sertoli cells extend towards the lumena of the seminiferous tubules and envelope the germ cells. The columnar shape of the Sertoli cells is supported by an actively changing cytoskeleton (Kerr and de Kretser, 2006). The cytoskeleton also regulates the intracellular localization of organelles and maintains adhesion between germ cells and Sertoli cells (Fig. 5, Kerr and de Kretser, 2006).

2.5.2.1 Formation of the blood-testis barrier

Sertoli cells support germ cell development by nourishing and supporting the germ cells. In addition to the germ cell support, Sertoli cells form the blood-testis barrier (BTB), which provides a unique environment for the germ cells to develop. The BTB is formed by special junctions, known as basal ectoplasmic specialization (ES), between neighboring Sertoli cells and the basement membrane (Kerr and de Kretser, 2006). The blood-testis barrier consists of tightly packed bundles of actin-filaments, which are positioned vertically to the plasma membrane, and actin-binding proteins, such as ESPIN (Bartles et al., 1996; Cheng and Mruk, 2012). The bundles are between the cisterna of the endoplasmic reticulum and the plasma membrane of adjacent the Sertoli cell. The epithelium of the Sertoli cells is divided into two different compartments known as the basal and adluminal regions (Kerr and de Kretser, 2006). These regions separate germ cells (young and more mature) into two distinct anatomical and functional sections, where the spermatogonia and spermatocytes up to the leptotene stage reside in the basal section and those of the latter stages inhabit the adluminal region. The tight junctions between the neighboring Sertoli cells and germ cells prevent intercellular transport between adjacent Sertoli cells and form the adluminal compartment where maturation (meiosis) and spermiogenesis is completed under the regulation of Sertoli cells (Kerr and de Kretser, 2006).

2.5.2.2 Cell-cell tight junctions in germ cell movement and development

Sertoli cells regulate germ cell movement and development throughout spermatogenesis with cell-cell tight junctions, which are composed of actin, intermediate filaments and microtubules (Mruk and Cheng, 2004a). The specialized tight junctions in the testis between the Sertoli and germ cells are known as apical ectoplasmic specialization (aES). These ectoplasmic specializations have several different functions during spermatogenesis, such as adhesion, spermatid orientating and head shaping (Mruk and Cheng, 2004b).

The adhesive function of apical ES exists between Sertoli cells and elongating spermatids, and this function is lost during spermiation (Mruk and Cheng, 2004b). Supportive data for the adhesive function has been obtained from studies where mechanical separation of spermatids from the epithelium does not break the attachment of the apical ES to spermatids (Guttman et al., 2000; Vogl, 1996). The adhesive function has also been studied by using a fungal toxin, cytochalasin D (Russell et al., 1988). Cytochalasin D inhibits polymerization of actin, and *in vivo* administration of the compound extricates the ES from spermatids in rats. It has

also been demonstrated by several groups that adhesion molecules, such as N-cadherin, are located in the apical ES, supporting the adhesive function of apical ectoplasmic specialization (Johnson and Boekelheide, 2002; Lee et al., 2004; Wine and Chapin, 1999).

The apical ES has been shown to emerge with step 8 spermatids (O'Donnell et al., 2000). During step 8, the round spermatids begin to elongate and become polarized (Berruti and Paiardi, 2014). One junctional adhesion molecule (JAM), JAM-C, has been linked to round spermatid differentiation, more specifically to round spermatid polarization (Gliki et al., 2004). JAM-C is expressed in several spermatogenic cells, such as premeiotic spermatocytes, haploid spermatids and differentiated elongated spermatids. JAM-C was observed to be limited to the cell-cell junctions of elongated spermatid heads. Loss of JAM-C in male mice results in infertility, which is caused by defective polarization of the round spermatids (Gliki et al., 2004). Furthermore, abnormal positioning of spermatids in the seminiferous epithelium has been observed in Nectin-2 (adhering protein) knockout male mice (Ozaki-Kuroda et al., 2002).

The microtubules of the apical ES have been demonstrated to be essential for germ cell positioning and shaping through blockage of microtubule assembly with colchicine, which binds to the tubulin heterodimer (Vogl et al., 1983). The manchette, as mentioned in Chapter 2.5.1.2., is a microtubular-structure associated with spermatid head shaping (O'Donnell and O'Bryan, 2014). The manchette is a bundle of microtubules formed in skirt-like fashion that protrude from the perinuclear ring (below the acrosome) into the cytoplasm of the spermatid (Hermo et al., 2010b). The manchette appears as the spermatids begin to elongate and disappears as nuclear elongation and condensation is completed (Clermont et al., 1993; Hermo et al., 2010b). The manchette contains several different proteins, such as the α-tubulin and β-tubulin heterodimers and γ-tubulin. In addition, there are also several motor proteins, such as kinesins and dyneins, that are involved in protein transfer. Mice that express truncated nonfunctional forms of HOOK1 (hook microtubule tethering protein 1) present with abnormalities in their sperm heads (Mendoza-Lujambio et al., 2002). The HOOK1 protein is necessary to form the connection between the manchette and cellular structures. Another linker protein, cytoplasmic linker protein of 170 kDa (CLIP-170), is expressed in undifferentiated spermatogonia and the spermatid manchette (Akhmanova et al., 2005). Knockout CLIP-170 male mice are subfertile with abnormal sperm head shapes as loss of CLIP-170 affects the formation and maintenance of the spermatid manchette. Several other knockout mouse models associated with microtubular manchette formation or function, such as kinesin family member 3A (KIF3AKO), sperm flagellar 2 (SPEF2KO) and sperm associated antigen 4 (SPAG4/SUN4KO), present with defective sperm head

shaping (Lehti et al., 2013, 2017; Pasch et al., 2015). Another microtubular structure that is regulated by the manchette is the formation of tail. The manchette transfers the needed proteins for tail formation along the microtubules and actin (O'Donnell and O'Bryan, 2014). The importance of motor proteins for proper sperm tail development has also been demonstrated using knockout mouse models, such as KIF3AKO (Lehti et al., 2013).

2.5.2.3 Phagocytosis

The residual cytoplasms of the spermatids and degenerating germ cells are phagocytozed by Sertoli cells during spermatogenesis (Kerr and de Kretser, 2006). The residual bodies have been demonstrated to merge with endosomes and lysosomes, which then move to the base of the Sertoli cells (Kerr and de Kretser, 1974). Furthermore, the germ cells that display improper characteristics, such as improper meiosis, are phagocytozed by Sertoli cells (Arandjelovic and Ravichandran, 2015). Phagocytosis is necessary for proper spermatogenesis, as has been demonstrated by Elliott *et al.* with engulfment and cell motility 1 (ELMO1) knockout mice (Elliott et al., 2010). The ELMO1 knockout male mice have disrupted seminiferous epithelia, multinucleated giant cells, accumulated apoptotic germ cells and lowered sperm production.

2.6 Gonadal steroid synthesis

2.6.1 *Gonadotropins regulate steroid hormone synthesis*

Ovarian steroid biosynthesis is controlled by FSH and LH. Treatment with recombinant FSH in patients with isolated gonadotropin deficiency results in follicular growth up to the preovulatory follicle but does not produce an increase in estrogen or androgen biosynthesis (Schoot et al., 1992). Similar results have been obtained from mouse models with either deletion of the FSH β-subunit or FSHR receptor. Analyses of FSH receptor knockout mice demonstrate atrophic uteri and closed vaginas, which indicates reduced levels of estrogens (Abel et al., 2000). However, FSH β-subunit knockout mice have no changes in their E2 levels, but the P4 levels are decreased by 50 % (Kumar et al., 1997). The LH receptor and LHβ-subunit knockout have reduced levels of E2, and the LH receptor knockout mice have reduced levels of P4 (Lei et al., 2001; Ma et al., 2004; Zhang et al., 2001). During the last stages of follicular maturation, when follicular growth has been initiated by FSH, either FSH or LH is able to maintain E2 production (Sullivan et al., 1998).

In addition, LH regulates progesterone production through StAR (Devoto et al., 2001).

Leydig cells are testosterone-producing cells of the testis that are localized to the intertubular space of the testis. In the intertubular space, Leydig cells are in close association with interstitial blood capillaries to enable distribution of androgens into the bloodstream (Haider, 2004). LH stimulates testosterone production in the Leydig cells through luteinizing hormone/chorionic gonadotropin receptor (LHCGR). LH induces cAMP production, which then catalyzes synthesis of protein kinases A and C (PKA and PKC, respectively, Haider, 2007; Smith and Walker, 2015). PKA initiates steroidogenesis in Leydig cells through GATA4, cAMP response element binding (CREB) protein and cAMP response element modulator (CREM), which then initiate the expression of their downstream target genes, such as *StAR* and peripheral-type benzodiazepine receptor (*PBR*, Manna et al., 2002; Tremblay and Viger, 2003).

2.6.2 *Gonadal hormone biosynthesis*

Steroid biosynthesis begins with the acquisition of cholesterol. In ovarian steroidogenic cells, cholesterol is either synthesized *de novo* or acquired via uptake of lipoprotein-carried cholesterol (Ho and Strauss, 2006). Testicular cholesterol transfer is mediated through PBR and StAR (Hauet et al., 2002; Krueger and Papadopoulos, 1990; Lin et al., 1995; Miller, 2007). The StAR protein is involved in the transfer of cholesterol into the mitochondria by transferring the cholesterol from the outer mitochondrial membrane to the inner membrane (Lin et al., 1995; Miller, 2007; Strauss et al., 1999). Loss of StAR causes congenital lipoid adrenal hyperplasia (lipoid CAH) in humans, and similar findings have been obtained from a *Star* knockout mouse model, where the knockout mice exhibit histological changes that are consistent with the pathogenesis of lipoid CAH (Caron et al., 1997). After cholesterol is transferred into the inner mitochondrial membrane, it is converted to pregnenolone by CYP11A1 (Hall, 1985). The conversion of cholesterol to pregnenolone is limited by several factors, such as the amount of cholesterol delivered into mitochondria and its transition to inner membrane (Ho and Strauss, 2006). Additionally, the quantity of the CYP11A1 enzyme and its activity affects to the conversion rate. Pregnenolone is converted further by two different enzymes: HSD-3β (3β-hydroxysteroid/Δ^4-Δ^5 isomerase) and CYP17A1 (P450c17, 17α-hydroxylase/17,20 desmolase). In the ovaries, CYP17A1 is localized to the follicular theca cells and theca lutein cells of the *corpus luteum*, while in the testis, CYP17A1 is localized to Leydig cells (Sasano *et al.*, 1989; Tamura *et al.*, 1992; Uhlen *et al.*, 2015, Human Protein Atlas, available from www.proteinatlas.org).

CYP17A1 functions as a catalyzer in two conversions: hydroxylation of pregnenolone to 17α-hydroxypregnenolone to dehydroepiandrosterone (DHEA) and progesterone to 17α-hydroxyprogesterone to androstenedione (Payne and Hales, 2004). HSD-3β is localized to the ovarian theca cells and *corpus luteum* and the the Leydig cells in the testis, where the enzyme converts pregnenolone to progesterone, 17α-hydroxypregnenolone to 17α-hydroxyprogesterone and DHEA to androstenedione (Baker et al., 1999; Dupont et al., 1990; Ho and Strauss, 2006; Teerds and Dorrington, 1993).

2.6.2.1 Estrogen biosynthesis in the granulosa cells

Ovarian steroid hormone biosynthesis continues further via aromatase. Aromatase is expressed in granulosa cells and luteal cells and is responsible for estrogen biosynthesis in the ovaries (Stocco, 2008). Androstenedione, which is produced in theca cells by HSD-3β, is transferred to granulosa cells, where aromatase uses it as a substrate for estrogen synthesis (Ho and Strauss, 2006). Aromatase expression in granulosa cells is not evenly distributed; the mural granulosa cells express higher levels of aromatase than the proliferating cells near the antrum (Turner et al., 2002). In addition to aromatase, HSD17B1 catalyzes the final step of estrogen biosynthesis in granulosa cells. The function of HSD17B1 in steroid hormone synthesis was discussed earlier in Chapter 2.1.4.

2.6.2.2 Testosterone biosynthesis in Leydig cells

The terminal step for testicular steroid hormone biosynthesis is the conversion of androstenedione to potent testosterone by HSD17B3 (Fig. 6, Payne and Hales, 2004). Loss of catalytic activity due to a point mutation in exon 9 of the *HSD17B3* gene results in pseudohermaphroditism (Andresson et al., 1996; Geissler et al., 1994). Furthermore, mutations in exon 3 of the *HSD17B3* gene cause a decreased cofactor binding ability, which also results in pseudohermaphroditism (Andresson et al., 1996; Geissler et al., 1994). In addition to HSD17B3 in the testicular testosterone conversion, HSD17B5 (also known as AKR1C3) is known to function in the A-dione to T conversion (Dufort et al., 1999). Expression of *HSD17B5* occurs mainly in the liver and prostate but has also been shown in the Leydig cells of the adult human testis, which indicates a possible role for testosterone production in the testis (Azzarello et al., 2008; Dufort et al., 1999; Penning et al., 2001).

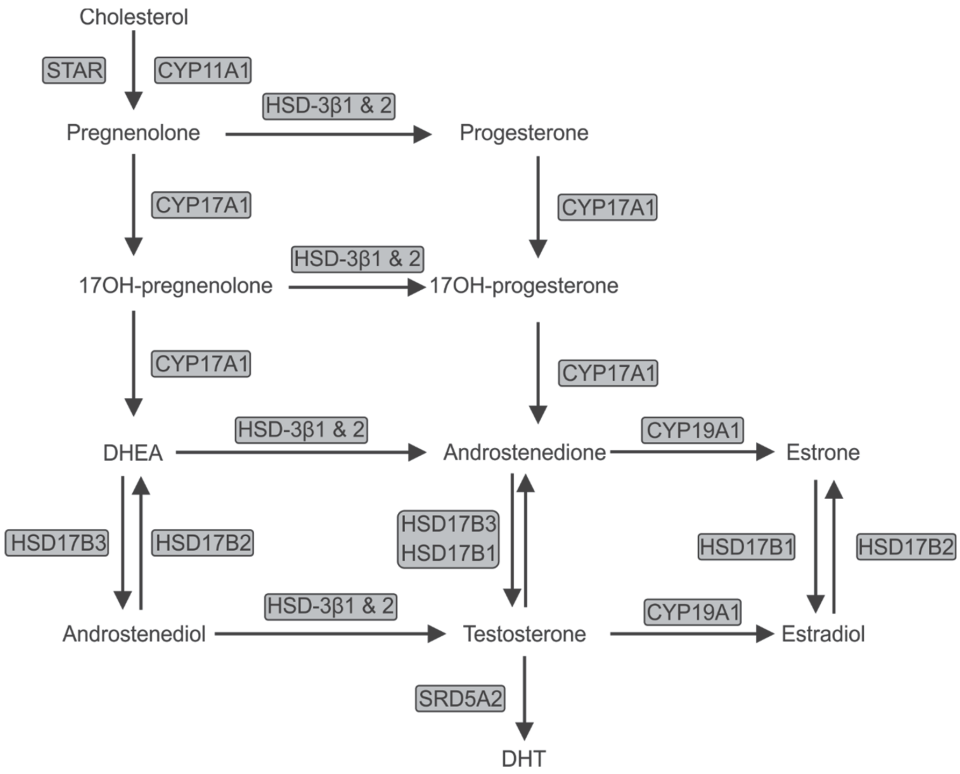

Figure 6. Steroidogenic pathway. Steroidogenic pathway with associated proteins and enzymes marked with gray boxes.

3 AIMS OF THE STUDY

This study aimed to analyze hormonal regulation of steroid synthesis and reproduction by the HSD17B1 enzyme. To address the study questions, we generated a *Hsd17b1*-deficient knockout mouse model (HSD17B1KO). After proper validation and characterization of the model, we focused our study on defining the role of the enzyme in the ovaries and testis and female and male and reproduction. This study is part of a larger investigation aimed at further validating HSD17B1 as a drug target to inhibit estrogen actions.

Specific aims:

1. To validate the HSD17B1 global knockout mouse model (HSD17B1KO).
2. To evaluate the role of HSD17B1 in the development of reproductive tissues, fertility and steroid biosynthesis in female mice.
3. To evaluate the role of HSD17B1 in the development of reproductive tissues, fertility and steroid biosynthesis in male mice.

4 MATERIALS AND METHODS

4.1 Methods

Table 2. Used methods in studies

Method	Used in
Mouse housing conditions	I, II
Establishment of HSD17B1 knockout mouse model	I
Naglu knockout mouse model	II
Serum and tissue processing	I, II
HE and PAS staining	I, II
Immunohistochemistry	I, II
Immunofluorescence	II
TUNEL staining & quantification	I, II
Beta-galactosidase staining	I
RNAscope *in situ* staining	II
Ovarian follicle counting	I
Electron microscopy analysis	II
Gonadotropin measurements	I, II
Measuring intratissue steroid concentrations	I, II
Measuring serum concentrations of hormones	I, II
RNA extraction and cDNA synthesis	I, II
Quantitative real-time PCR	I, II
Anogenital distance measurement	II
Onset of puberty test	I, II
Fertility test	I, II
Pseudopregnancy test	I
Morphological analyses of spermatozoa	II
Statistical analyses	I, II

4.2 RT-qPCR primers for the genes of interest

Table 3. The RT-qPCR primers for the analyses of the genes of interest.

Gene	Foward	Reverse	Used in
Actb	CGTGGGCCGCCCTAGGCACCA	TTGGCCTTAGGGTTCAGGGGG	I, II
L19	GGACAGAGTCTTGATGATCTC	CTGAAGGTCAAAGGGAATGTG	II
Ppia	CATCCTAAAGCATACAGGTCCTG	TCCATGGCTTCCACAATGTT	I, II
Hsd17b1	TTGTTTGGGCCGCTAGAAG	CACCCACAGCGTTCAATTCA	I, II
Hsd17b2	GAGCGTCTTTCAGTGCTCC	CCTT-GGACTTTCTAAGTAGAGGCA	I
Hsd17b3	CACGGGGATAAAGACCAGGT	GATCGCAGGAAAGAGCTTGG	II
Hsd17b5	CAGACAGTGCGTCTAAGTGATG	CGGATGGCTAGTCC-TACTTCCT	II
Hsd17b6	TTTGGAGGATTCTACAGTTGCTC	TCACCCCGAAATCTTGAACCT	II
Hsd17b7	TGGCAGAAGACGATGACCTC	GGCAGGATTCCAGCATTCAG	I, II
Hsd17b12	AGCAAAACGTGGAATGAAGATT	TTTCTTGATGGTGTT-GTCCAAG	I, II
Star	CAGGGAGAGGTGGCTATGCA	CCGTGTCTTTTCCAATCCTCTG	I, II
Cyp11a1	AGATCCCTTCCCCTGGCGACAATG	CGCATGAGAAGAGTATCGAC-GCATC	I, II
Cyp17a1	CAAGCCAAGATGAATGCAGA	AGGATTGTGCACCAGGAAAG	I, II
Cyp19a1	GCAATCCTGAAGGAGATCCA	GCCGTCAATTACGTCATCCT	I, II
20α-Hsd	GCTATAGATGTTGGGTTCTGCC	TCTGGACGATGGGAAGTTGA	I
Hsd3b1	CAGGAGCAGGAGGGTTTGTG	GTGGCCATTCAGGACGAT	I, II
Esr1	TTGACAAGAACCGGAGGAAG	ATAGATCATGGGCGGTTCAG	I
Esr2	GACAAGAACCGGCGTAAAAG	GGACGGCTCACTAGCACATT	I
Fshr	TGTCATTGCTCTAACAGGGTCT	TGGTGAGCACAAATCTCAG-TTC	I, II
Lhcgr	GCCCTGAGCCCTGCGACTGC	AAAGCGTTCCCTGG-TATGGTGGTT	I
Pgr	CTCCGGGACCGAACAGAGT	ACAACAACCCTTTGGTAGCAG	I
Prlr	CCACAAATGTCGTTCCCCTG	GAATTGGGGCCACTGGTTTT	I
Ar	GTCTCCGGAAATGTTATGAA	AAGCTGCCTCTCTCCAAG	II
Gdf-9	GCTCTATAAGACGTATGCTACC	CAGAGTGTATAGCAAGAC-CGAT	I
Ccdn2	GAGTGGGAACTGGTAGTGTTG	CGCACAGAGCGATGAAGGT	I
Cebpb	TGCGGGGTTGTTGATGTTTT	TGCTCGAAACGGAAAAGGTT	I
Amh	CCACACCTCTCTCCACTGGTA	GGCACAAAGGTTCAGGGGG	II
Sox-9	AAGAAAGACCACCCCGATTACA	CAGCGCCTTGAAGATAGCATT	II
Sf-1	AGGTGTCGGGCTACCACTAC	CCACCCCGCATTCGATCAG	II
Gata1	TGGGGACCTCAGAACCCTTG	GGCTGCATTTGGGGAAGTG	II
Gata4	CCCTACCCAGCCTACATGG	ACATATCGA-GATTGGGGTGTCT	II
Rhox5	ACTCGGAAGAACAGCATGATG	CCCTGGTGCCACTATCCTT	II
Dmrt1	GACCCCGCCTACTACAGCA	GTCTGAGCAGGCACGTAAGG	II
Thra	CTGACCTCCGCATGATCGG	GGTGGGGCACTCGACTTTC	II
Ptgfr	CTGGACTCATCGCAAACACAA	AGGAAGCCTTT-GACTTCTGTCTA	I

4.3 Antibodies used in immunohistochemical analyses of proteins of interest

Table 4. Primary antibodies used for immunohistochemistry against the proteins of interest.

Antigen	Species	Manufacturer	Dilution	Used in
Cyp17a1	Rabbit	Proteintech Group	1:2000	I
Hsd3b2	Rabbit	gift from J. I. Mason	1:3000	I
Sf-1	Rabbit	Transgenic	1:100	I

4.4 Antibodies used in immunofluorescence analyses of proteins of interest

Table 5. Primary antibodies used for immunofluorescence analyses against proteins of interest.

Antigen	Species	Manufacturer	Dilution	Cat. #	Used in
α-tubulin	Mouse	Thermo Scientific	1:1000	MS-581.P	II
Rhodamine-conjugated peanut agglutinin (PNA)		Vector Laboratories	1:4000	RL-1072	II
Hyperacetylated Histone H4	Rabbit	Merck Millipore	1:500	06-946	II
Bromodomain Testis Associated (BRDT)	Rabbit	Abcam	1:200	ab5157	II
Espin	Mouse	BD Transduction Laboratories	1:400	611656	II

Table 5. Secondary antibodies used for immunofluorescence analyses.

Antibody	Manufacturer	Dilution	Used in
Alexa fluor 488, 594 (anti-mouse, anti-rabbit)	InVitrogen	1:750	II

4.5 Antibodies used in the analysis of testicular populations with flow cytometry

Table 6. Primary antibody used in flow cytometry analysis of testicular populations.

Antigen	Species	Cat. #	Manufacturer	Dilution	Used in
γH2AX-Ser139	Mouse	05-636	EMD Millipore	1:500	II

Table 7. Secondary antibody used in flow cytometry analysis.

Antigen	Manufacturer	Dilution	Used in
Alexa 488 (anti-mouse)	Thermo Fisher Scientific	1:500	II

Table 8. Nuclei stain used in flow cytometry analysis.

Dye	Manufacturer	Concentration	Used in
FxCycle Far Red Stain	Thermo Fisher Scientific	200 nM	II

4.6 Probes used for localization of *Hsd17b1* using RNAscope *in situ* hybridization

Table 9. Probes used in RNAscope *in situ* hybridization assay.

Target gene	Cat. #	Target region	Manufacturer	Used in
Hsd17b1	476331	2 – 924	Advanced Cell Diagnostics	II
Ubiquitin C (positive control)	310771	34 – 860	Advanced Cell Diagnostics	II
Basillus subtilis (dihydrodipicolinate reducatese, negative control)	310043	414 – 862	Advanced Cell Diagnostics	II

4.7 Reagents used in HSD17B activity measurement

Table 10. Reagents used in HSD17B activity measurement with HPLC.

Reagent	Specific activity	Manufacturer	Concentration	Used in
Estrone, [2, 4, 6, 7-^{3}H(N)]	74.0 Ci/mmol	PerkinElmer	6 nM (500 000 cpm)	I, II
Androst-4-ene-3, 17-dione, [1β-^{3}H(N)]	98.2 Ci/mmol	PerkinElmer	6 nM (500 000 cpm)	I, II
β-Nicotinamide adenine dinucleotide – NADH		Sigma-Aldrich	1.4 mmol/l	I, II
β-Nicotinamide adenine dinucleotide 2'-phosphate – NADPH		Sigma-Aldrich	1.4 mmol/l	I, II

5 RESULTS

5.1 Generation of the HSD17B1 knockout mouse model and localization of HSD17B1 (I & II)

To be able to study the role of HSD17B1 in mouse reproduction, a knockout mouse model was generated. All coding exons of the *Hsd17b1* gene were deleted by inserting a targeting construct consisting of the beta-galactosidase gene, *lacZ,* and *Neo* gene into the translation start site of the *Hsd17b1* locus (I: Fig. 1A). The RT-qPCR analysis of the ovaries demonstrated lack of expression of the *Hsd17b1* gene (I: Fig. 1C). Additionally, our group has demonstrated that deletion of HSD17B1 by replacing the genomic region with a LacZ reporter causes downregulation of the α-N-acetylglucosaminidase (*Naglu*) gene, which is upstream of the *Hsd17b1* gene (Jokela et al., 2017).

The expression profiles of various tissues demonstrated that *Hsd17b1* was expressed mainly in the adult ovaries (I: Fig. 1B), and that the testis contained the third highest expression level of *Hsd17b1*. However, expression was observed to be markedly higher in the newborn testis than in the adult testis (II: Fig. 1A). Beta-galactosidase staining was performed to confirm successful deletion of the *Hsd17b1* gene and to observe the localization of HSD17B1 in the ovaries. Beta-galactosidase staining showed localized expression of *lacZ* in the ovarian follicles, indicating that the insertion cassette successfully replaced the *Hsd17b1* gene (I; Fig. 1D). Localization of HSD17B1 in the ovaries was confirmed with an RNAscope *in situ* hybridization assay, where ovarian expression was demonstrated in the granulosa cells of the Graafian follicles (data not shown). Testicular expression was localized to Sertoli cells in the newborn testis (II: Fig. 1B), and Sertoli cell-specific expression was also observed in adult mice but with a faint signal only (data not shown).

5.2 Loss of HSD17B1 impairs fertility in females and males (I & II)

5.2.1 *Female HSD17B1KO mice suffer from severe subfertility (I)*

Due to high expression of *Hsd17b1* in the ovaries, we determined whether deletion of *Hsd17b1* affected reproduction in HSD17B1KO female mice. The wild-type (WT) and HSD17B1KO female mice were bred with known fertile NMRI male mice for 2 months. The data demonstrated severely impaired fertility as the

HSD17B1KO female mice produced only 6 litters in comparison to 23 litters produced by the WT littermates. The litter size was significantly smaller, and the total number of offspring was greatly reduced in the HSD17B1KO females: 32 pups and 232 pups were observed from the HSD17B1KO and WT female mice, respectively (I: Table 1). Furthermore, the HSD17B1KO mice required six times more matings to produce one litter, indicated by the plug/litter ratios (WT: 1.4 plugs/litter; HSD17B1KO 9.3 plugs/litter, I: Table 1). The observed increase in the plug/ratio prompted us to examine the ability of the knockout mice to maintain pregnancy. To this end, the WT and HSD17B1KO female mice were bred with sterile males for 1 month, and the matings and lengths of the pseudopregnancies were analyzed. The data revealed that the female HSD17B1KO mice were unable to maintain pseudopregnancy. As expected, the WT female mice were observed to maintain pseudopregnancy 10 to 12 days before mating again; however, the female HSD17B1KO mice were observed to mate regularly at 3 – 4 day intervals (I: Fig. 3).

Histological analysis of the ovaries revealed the existence of all follicle stages of folliculogenesis and the *corpus lutea* in the HSD17B1KO female mice (I: Fig. 6A-D). The number of follicles during the different stages of folliculogenesis showed no significant difference between WT and HSD17B1KO at the proestrus stage. However, at the estrus stage the number of preovulatory follicles was higher in HSD17B1KO. The most striking difference was observed in the number of *corpora lutea* in the pseudopregnant HSD17B1KO mice, where the number of CLs was markedly diminished. Closer examination of the CL in the HSD17B1KO ovaries revealed less distinct basal lamina of the *corpus lutea* compared to the WT ovaries (I: Fig. 6A´& 6B´).

5.2.2 *Male HSD17B1KO are infertile and present with azoospermia (II)*

Similar to the expression levels of *Hsd17b1* in the ovaries, high expression of *Hsd17b1* in the Sertoli cells of the newborn testis prompted us to analyze the reproductive performance of the HSD17B1KO male mice. For this purpose, WT and HSD17B1KO male mice, were bred with two known fertile NMRI females each. Similar mating indicated by the plugs was observed between the groups, but none of the seven HSD17B1KO male mice produced litters compared to 12 litters produced by the seven WT male mice (II: Table 1).

The infertility of the HSD17B1KO male mice suggested testicular defects, which were confirmed with the histological analyses. The early steps of spermatogenesis and differentiation of spermatogonia, spermatocytes and round spermatids were observed to be normal (II: Fig. 2A). This was supported by the flow cytometry

analysis, which demonstrated parallel cell populations in the testis (1C, 2C and 4C) between the WT and HSD17B1KO testis (II: Fig. 2B). The most striking finding was that the number of the step-16 elongating spermatids was greatly reduced in the knockout seminiferous epithelium (II: Fig. 2 A, stage VII – VIII). However, the number of elongating spermatids was observed to be already decreased at stages IX – X, suggesting that the problem originated during the early phases (steps 9 – 10) of elongating spermatid differentiation (II: Fig. 2A). Furthermore, the organization of the differentiating germ cells in the seminiferous epithelium was disordered, indicated by a regular mixture of elongating spermatids and spermatocytes at stages IX – X (II: Fig. 2A). The histological analysis of the epididymides of the male mice was in line with the defects in spermatogenesis and in the azoospermia of the HSD17B1KO male mice as a radically reduced number of mature spermatozoa was observed in the cauda epididymides (II: Fig. 2C). Closer examination using cell spreads from specific stages of the seminiferous epithelial cycle (stage-specific cell spreads) revealed abnormalities in the structures of the elongating spermatids of the HSD17B1KO testis (II: Fig. 3A and 3B). We also used the α-tubulin antibody to visualize the appearance and disappearance of the spermatid-specific manchette, which briefly formed around the heads of the elongating spermatids and took part in the head shaping. With these analyses, no disturbances were observed in manchette formation and disappearance in the elongating spermatids of the HSD17B1KO mice (II: Fig. 3A and 3B). As the head shaping of the elongating spermatids is dependent on chromatin condensation, we continued to analyze a few selected processes that occur before the histone-protamine transition. Hyperacetylation of histone H4 in the early elongating spermatids and the association between bromodomain testis-associated (BRDT) protein and the acetylated chromatin demonstrated no notable alterations. In addition, nuclear staining with DAPI revealed that compacting of the chromatin in the spermatids of HSD17B1KO occurred (II: Fig. 3A).

The seminiferous tubules of HSD17B1KO demonstrated an increased apoptotic signal in early stage germ cells and elongating spermatids (II: Fig. 2D and 2E). The defect in spermatid differentiation was further supported by morphological examination of the cauda epididymal spermatozoa. The HSD17B1KO mice presented only a few normal sperm heads, whereas most of the spermatozoa had severe aberrations, such as missing or malformed heads and abnormal tail bends. Additionally, some of the HSD17B1KO spermatozoa were observed to be missing mitochondrial sheats (Fig. 3C).

Due to downregulation of the *Naglu* gene, we wanted to ensure that the reproductive phenotypes were specific to deletion of *Hsd17b1*. We analyzed fertility in female and male Naglu knockout mice (NAGLUKO). Using the NAGLUKO female

mice, we demonstrated that, comparably to the WT mice, these mice reached puberty, and their estrous cycles were similar to the WT littermates (Jokela et al., 2017). In addition, the NAGLUKO female mice were fertile, while the NAGLUKO ovaries presented with lysosomal accumulation in the stroma. The male NAGLUKO fertility and histological analyses of the testis revealed normal fertility and comparable testicular histology to that of WT (II: Supplementary Fig. 1). These results indicate that both the female and male reproductive defects in the HSD17B1KO mouse model were specific for the loss of *Hsd17b1*.

5.3 Female and male HSD17B1KO mice develop and reach puberty normally (I & II)

The HSD17B1KO mice were born according to the Mendelian ratio at the age of 20 days; 26 % of the born offspring were WT, 46 % were HEZ and 28% were HOZ. These ratios clearly indicate that HSD17B1 is not crucial for fetal or postnatal development. The analysis of the onset of puberty in HSD17B1KO by vaginal opening demonstrated, surprisingly, that no significant difference between the WT (d 30 ± 0.8) and HSD17B1KO (d 33 ± 3.4) female mice was evident. After puberty, the uterine responses to estrogens were analyzed macroscopically and with the wet weights, which were comparable between the WT and HSD17B1KO female mice (I: Fig. 2A and 2B). In contrast, the relative ovarian weights were observed to be higher in the knockout mice at the estrus stage and at pseudopregnancy (I: Fig. 2C). Examination of the estrous cycle with vaginal smears revealed no change between the WT and HSD17B1KO mice (I: Fig. 2E). The length of estrous cycle was similar between the analyzed groups (WT: 5 d ± 1.3 and HSD17B1KO: 6 d ± 2.2), and the distributions of time spent in the different phases of the estrous cycle were similar between the WT and HSD17B1KO female mice (I: Fig. 2D). Altogether, the data indicated that loss of HSD17B1 is not vital for the fetal, postnatal or pubertal development in female mice.

High expression of *Hsd17b1* during development and peak expression of *Hsd17b1* in the newborn testis suggested a role for the enzyme in the fetal and neonatal testis development. In addition, disturbances during the fetal development can cause infertility in adults. The potential changes in androgen actions were analyzed in the newborn male mice by analyzing the anogenital distance (AGD). However, the HSD17B1KO male mice had similar AGDs to those of the WT littermates, indicating that loss of *Hsd17b1* does not cause disturbances in androgen actions during the fetal period (II: Fig. 4A). We then continued with an analysis of the onset of puberty by measuring the balanopreputial separation, and demonstrated that the timing of the onset of puberty in the HSD17B1KO (28.5 d ± 0.5) male mice was

similar to that of the WT (29.5 d ± 0.5) mice (II: Fig. 4B). We also analyzed the weights of the androgen-dependent tissues. Similar to the data obtained from fetal development and puberty, no significant changes were observed in any of the extragonadal androgen-dependent tissues (II: Fig. 4C). The data suggested that the cause of infertility did not originate from disturbances in androgen-regulated actions during the fetal, postnatal and pubertal periods in the HSD17B1KO male mice.

5.4 Steroid environment in the HSD17B1KO ovaries and testis (I & II)

5.4.1 *HSD17B1 is essential for ovarian and testicular HSD17B activity (I & II)*

Due to the known function of HSD17B1 in the E1-to-E2 and A-dione-to-T conversion, we examined HSD17B activity in adult ovaries using a tritium-labelled substrate. Loss of HSD17B1 resulted in a significant reduction in ovarian HSD17B activity demonstrating that HSD17B1 was the major HSD17B enzyme involved in estradiol biosynthesis in the ovaries (I: Fig. 4A).

The exact role of HSD17B1 in the testis is not known. Thus, we studied the role of HSD17B1 in steroid biosynthesis in the testis. HSD17B activity was examined in the newborn and adult testes. Similar to the ovaries, the E1-to-E2 conversion was reduced in the newborn testis of HSD17B1KO, which together with the ovarian data, indicated that HSD17B1 contributed significantly to the E1-to-E2 conversion in both tissue types (II: Fig. 5A). The adult testis showed no difference between the WT and HSD17B1KO groups (Fig. 7). Surprisingly, the conversion of E1 to E2 in the newborn testis was very low considering the high level of mRNA expression of *Hsd17b1*. The A-dione-to-T conversion was also analyzed in the testis at both time points. The newborn groups did not show differences, but the adult HSD17B1KO testis had markedly elevated conversion of A-dione to T *in vitro*, being in line with the increased *Hsd17b3* mRNA expression level in the adult HSD17B1KO testis (Fig. 7, II: Fig. 5B - 5D).

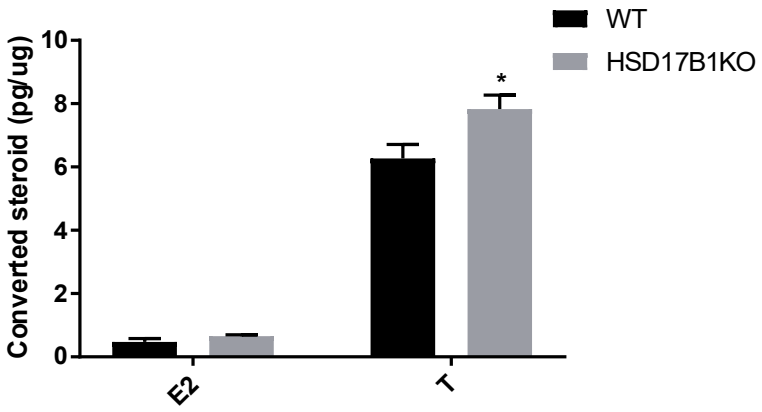

Figure 7. The intratesticular HSD17B activity in adult HSD17B1KO male mice. A-dione-to-T conversion in HSD17B1KO male testis is increased while no change was observed in E1 to E2 conversion. * Statistical significance at $P < 0.05$. Values represented as mean ± SEM, n = 4.

5.4.2 *Serum gonadotropin and intratissue steroid measurements of the HSD17B1KO mice (I & II)*

Although the analyses of the systemic steroid concentrations showed no obvious alterations in the HSD17B1KO mice, we wanted to examine the steroid milieu more carefully by determining the serum gonadotropin concentrations and intratissue steroid concentrations.

The 3-month-old HSD17B1KO female mice showed similar serum FSH concentrations to those of the WT female mice (Table 11). While the serum LH concentration was elevated 4-fold in the HSD17B1KO mice during the estrus phase, no difference between the knockout and WT was observed in the proestrus phase (Table 11).

Table 11. Serum gonadotropin levels in HSD17B1KO females.

		FSH	LH
Proestrus	WT	7.9 ± 2.62 ng/ml	4.2 ± 3.82 ng/ml
	HSD17B1KO	9.0 ± 2.60 ng/ml	5.3 ± 4.68 ng/ml
Estrus	WT	8.9 ± 2.30 ng/ml	0.1 ± 0.03 ng/ml
	HSD17B1KO	8.0 ± 2.86 ng/ml	0.4 ± 0.13 ng/ml *

The ovarian steroid concentrations during the different phases of the estrous cycle and in pseudopregnant mice were analyzed. The individual steroid metabolite concentrations were equivalent between the WT and HSD17B1KO groups. Conversely, the E1-to-E2 and A-dione-to-T ratios were significantly increased, which supported the observed decline in HSD17B activity. The most striking difference was the A-dione-to-T ratio in the pseudopregnant group, where the A-dione-to-T ratio was observed to be 40-fold higher in the HSD17B1KO ovaries than in the WT ovaries (I: Fig. 4C). Consistent with the observed the reduction in the number of *corpora lutea* in the HSD17B1KO ovaries, the intraovarian P4 concentrations were clearly reduced in the estrus phase and in the pseudopregnant HSD17B1KO female mice (I: Fig. 4D).

The male serum gonadotropins and intratesticular steroids showed equal concentrations in the HSD17B1KO and WT male mice. No differences were observed in either the E1-to-E2 or A-dione-to-T ratios.

5.4.3 *Expression of several steroidogenic enzymes is altered due to the loss of HSD17B1*

5.4.3.1 Corpus luteum-related steroidogenic enzymes are altered in HSD17B1KO female mice

Since the steroid ratios and P4 concentration were changed in the HSD17B1KO ovaries, we conducted an RT-qPCR analysis of the mRNA expression levels of several steroidogenic enzymes. First, we focused our evaluation on the other enzymes of the HSD17B family, namely, types 2, 7 and 12 (*Hsd17b2, Hsd17b7, Hsd17b12*), which have been shown to possess an ability to convert E1 to E2 that is similar to that of HSD17B1. However, none of these other HSD17B types showed compensative upregulation in the HSD17B1KO ovaries during the proestrus or estrus stages (I: Fig. 5). In contrast, in the pseudopregnant HSD17B1KO ovaries, expression of *Hsd17b7* was 30-fold downregulated (I: Fig, 5C). The pro-

estrus phase of the estrous cycle also demonstrated various changes in gene expression; *Star, Cyp11a1* and *Ccnd2* were upregulated. However, changes were not observed in the estrus phase of the HSD17B1KO ovaries. *Cyp11a1* and *Lhcgr* were both downregulated in the pseudopregnant HSD17B1KO ovaries. The most prominent finding was the several-fold upregulation of *Cyp17a1*, which was observed in both estrous cycle phases and in pseudopregnant knockout ovaries (I: Fig. 5). Upregulation of *Cyp17a1* expression was verified with immunohistochemistry, where an increased signal intensity was observed in the theca cells of the HSD17B1KO ovaries (I: Fig. 7C).

5.4.3.2 Compensatory expression of other HSD17B enzymes due to loss of HSD17B1 in the testis

The analysis of the expression levels of the testicular steroidogenesis-related enzymes was also focused on the other HSD17B enzymes, such as *Hsd17b3* and *Hsd17b5*, which are both known to be involved in testosterone biosynthesis. *Hsd17b3* expression was markedly upregulated in the HSD17B1KO testes of both the newborn and adult mice (II: Fig. 5C and 5D). There was also a tendency towards upregulation of *Hsd17b5* expression in both the newborn and adult knockout testes (II: Fig. 5C and 5D). Both upregulations support the finding that the systemic androgen concentration was comparable to that of WT. In addition, the proteins involved in androgen synthesis prior HSD17B actions, such as *Star, Cyp11a1* and *HSD-3β1,* also demonstrated a trend towards upregulation in the newborn HSD17B1KO testis (II: Fig. 5C). However, only *Cyp11a1* was significantly upregulated in the adult HSD17B1KO testis (II: Fig. 5D).

5.5 Premature luteolysis of the *corpus luteum* results in severe subfertility in HSD17B1KO females

The further analysis of the *corpora lutea* in 3.5-day *post coitum* (dpc) pseudopregnant female ovaries with TUNEL staining revealed apoptosis in the HSD17B1KO *corpora lutea* while the WT *corpora lutea* did not show any positive TUNEL staining (Fig. 8). This suggested that the *corpora lutea* of the HSD17B1KO females underwent premature regression, resulting in an inability to maintain pregnancy. CL regression and luteolysis, is associated with prostaglandin F2α and its receptor (PTGFR) and 20α-HSD. During luteolysis, prostaglandin F2α binds to its receptor in the CL and induces the expression of *20α-HSD* and apoptosis in the CL (Stocco et al., 2000; Wang et al., 2003). 20α-HSD inactivates P4 by converting it to 20-α-hydroxyprogesterone. RT-qPCR analysis of *Ptgfr* and *20α-HSD* in the ovaries of

the 3.5-day dpc pseudopregnant HSD17B1KO female mice demonstrated an almost 2-fold decrease in expression of *Ptgfr* and a similar trend towards upregulation of *20α-HSD* (Fig. 9).

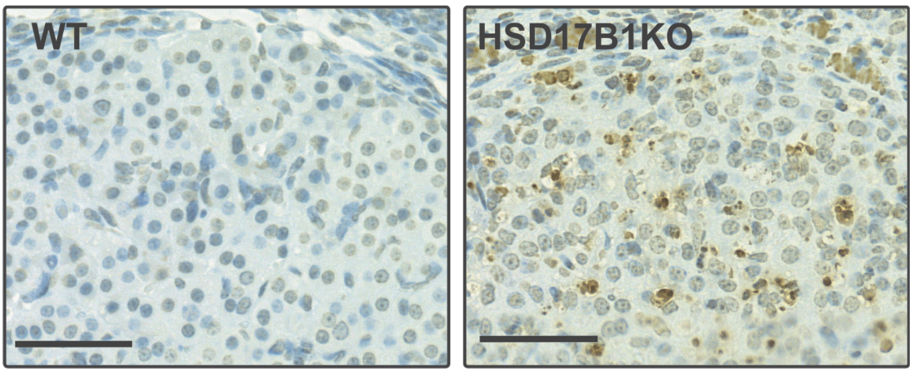

Figure 8. Increased apoptosis in *corpus luteum* in ovaries of 3.5 dpc pseudopregnant HSD17B1KO female mice. TUNEL staining of ovaries of pseudopregnant female mice indicated increased apoptosis in the HSD17B1KO female mice while no apoptosis was observed in CL of WT littermates. Scale bar is 50 μm.

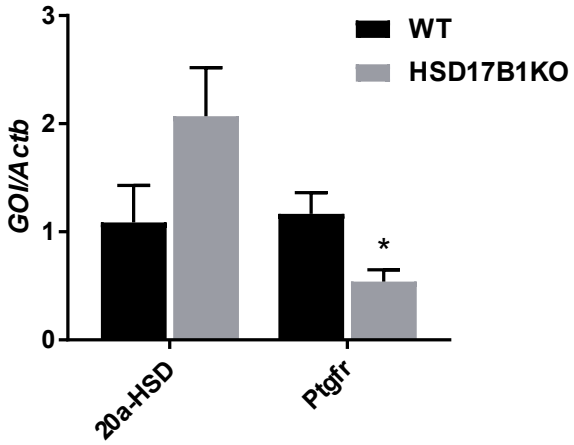

Figure 9. The expression of luteolytic factors in ovaries of 3.5 dpc pseudopregnant female mice. The expression of *Ptgfr* was reduced 2-fold in HSD17B1KO pseudopregnant ovaries while the *20α-HSD* gene expression demonstrated trend toward upregulation. * Statistical significance at $P < 0.05$. Values represented as mean ± SEM, $n \geq 8$.

5.6 Defective Sertoli cell maturation results in impaired function of adult Sertoli cells in HSD17B1KO mice, causing azoospermia

The mixed layers of spermatocytes and spermatids in the HSD17B1KO testis indicated that the tight junctions between the cells, which are needed for Sertoli cell function in positioning the germ cells, might be disrupted. We examined this by immunostaining using antibodies against ESPIN and PNA to visualize the aES and acrosomes, respectively. The apical ectoplasmic specializations are tight junctions that connect Sertoli cells to elongating spermatids. These junctions regulate the positioning and location of the elongating spermatids in the seminiferous epithelium and prevent the untimely release of spermatids. In the WT testis, the acrosomes of spermatids in the epithelium were polarized appropriately towards the basal lamina, and the tail extended in the direction of lumen. In contrast, the elongating spermatids in the HSD17B1KO epithelium were frequently mispolarized, with acrosomes pointing in various directions, indicating that control of spermatid positioning in the epithelium was insufficient (II: Fig. 6A & 6B). In addition, premature release of spermatids into the lumena of the HSD17B1KO seminiferous tubules was observed (II: Fig. 6A). Further analysis demonstrated that ESPIN-positive junctions formed between the elongating spermatids and Sertoli cells in the HSD17B1KO testis. However, the junctions were observed to be irregular, and ESPIN was frequently observed outside of the acrosome (II: Fig. 6C). The presented data supported the findings of the problems in maintaining cell-cell tight junctions and of inadequate Sertoli cell function.

The high expression levels of *Hsd17b1* in the Sertoli cells of the developing and newborn testes, as well as the normal systemic steroid environments, motivated us to concentrate on examining Sertoli cell function and the possible role of HSD17B1 in it. Several markers associated with immature and mature Sertoli cells, such as *Amh, Sox9, Sf-1, Gata4, Gata1, Fshr, Dmrt1* and *Ar,* were examined. Surprisingly, *Sox9, Sf-1* and *Gata4* were upregulated in the newborn HSD17B1KO testis, while *Amh, Fshr* and *Dmrt1* were displaying a trend towards upregulation (*Amh,* $p = 0.056$; *Fshr*, $p = 0.095$; *Dmrt1*, $p = 0.056$; II: Fig. 7A). Similar upregulation was observed in the adult HSD17B1KO testis for *Amh* and *Sox9* (II: Fig. 7B). *Amh* expression is associated with immature Sertoli cells and the upregulated expression level in the HSD17B1KO adult testis demonstrates improper maturation of the Sertoli cells and disruption of Sertoli cell function in the context of HSD17B1 deficiency.

6 DISCUSSION

6.1 Localization of HSD17B1 in the reproductive organs (I, II)

Mouse *Hsd17b1* has been demonstrated to be expressed mainly in the ovaries, while in other female reproductive tissues, such as uterus and vagina, no signal has been detected (Pelletier et al., 2004). Ovarian expression is localized to the granulosa cells of growing follicles of all stages, but no signal is evident during the earlier-stages follicles, such as the primordial or primary follicles, or in the *corpus luteum* (Akinola et al., 1997; Pelletier et al., 2004). These previous observations were supported by our findings, where mouse *Hsd17b1* was observed to be mainly expressed in adult ovaries. *Hsd17b1* localization was confined to the follicles and, more precisely, to the granulosa cells per beta-galactosidase staining and RNAscope *in situ* hybridization analyses, respectively. Although mouse *Hsd17b1* expression was mainly localized to the ovaries, the human enzyme has been demonstrated to also be expressed in placenta (Uhlen et al. 2015, Human Protein Atlas available from www.proteinatlas.org).

The tissue distribution of *Hsd17b1* revealed that its expression in the adult testis was minimal. Surprisingly, expression in the fetal testis was high, peaking in the newborn testis with an expression level that was 101-fold higher than in the adults testis. Testicular expression has also been reported previously by O'Shaugnessy *et al.* and Pelletier *et al.* (O'Shaughnessy et al., 2000; Pelletier et al., 2004). Both studies detected HSD17B1 inside the seminiferous tubules, while the cellular localization was suggested to occur in germ cells by Pelletier *et al.* and in Sertoli cells by O'Shaughnessy *et al.* In line with the studies by O'Shaughnessy *et al*., our investigations with RNAscope *in situ* hybridization showed mouse *Hsd17b1* mRNA in the Sertoli cells.

6.2 Importance of HSD17B1 in reproductive performance (I, II)

Reproductive performance in females and males requires balanced hormonal homeostasis where both estrogens and androgens are needed during reproduction. The infertility problems in humans are emerging with a clear association between a defective hormonal environment and impaired reproduction in both women and men. Both female and male HSD17B1KO mice showed impaired reproduction, with females being severely subfertile and having problems maintaining pregnancy. The female HSD17B1KO mice required significantly more matings to produce offspring, and the ability to maintain a pseudopregnancy was also decreased.

Furthermore, a detailed histological analysis revealed a reduced number of *corpora lutea* in the pseudopregnant HSD17B1KO ovaries. Male HSD17B1KO mice were completely infertile, with a major defect in spermatogenesis comprising observed abnormalities in the head shapes of the elongating spermatids, a mixed organization of the germ cell layers and a reduced number of mature spermatozoa. Similar findings have been observed for genes coding proteins involved in regulation of estrogen actions in other knockout mice, such as female ArKO mice, where disrupted folliculogenesis and ovulation failure resulted in infertility (Britt et al., 2002). Additionally, the estrogen receptor α knockout mouse models by Lubahn *et al.* and Dupont *et al.* demonstrated complete infertility with no *corpora lutea* present (Dupont et al., 2000; Lubahn et al., 1993). The ENERKI (estrogen-nonresponsive estrogen receptor α knockin) mouse model has a mutation in the ligand-binding domain of ERα, thus preventing ligand-dependent pathway activation.The ENERKI female mice further confirmed the importance of estrogens in female reproduction as the female ENERKI mice were infertile with haemorrhagic follicular cysts, and no *corpora lutea* were observed (Sinkevicius et al., 2009b). The importance of androgens for female reproduction has been demonstrated with the granulosa cell-specific androgen receptor knockout (GCARKO) mice, which are subfertile due to impaired preantral- and antral-stage follicular development (Walters et al., 2012).

In men, the main steroid regulating reproduction is testosterone. However, estrogens play crucial roles in spermatogenesis as has been demonstrated in a knockout mouse model of aromatase which showed declining fertility upon ageing, resulting in infertility. However, in men with aromatase deficiency, no clear association between loss of aromatase activity and infertility has been diagnosed. In addition to the ArKO mouse model, the importance of estrogens and androgens in spermatogenesis has been noted with several other models, such as ARKO, SCARKO, and ENERKI which all exhibit disrupted spermatogenesis and either subfertility (ENERKI) or complete infertility (ARKO, SCARKO, De Gendt et al., 2004; Sinkevicius et al., 2009; Yeh et al., 2002).

The knockout mouse models of steroidogenic factors with impaired reproduction demonstrate the importance of estrogens and androgens in both female and male reproduction. Our data on female and male reproduction evidently demonstrates the significance of HSD17B1 on reproduction in both sexes. It can be hypothesized that HSD17B1 regulates the steroid hormone environment at the gonadal level, and is thus needed for proper reproductive performance.

6.3 Role of HSD17B1 in regulation of the steroid hormone environment at the cellular level in gonads (I & II)

A balanced hormonal environment is already necessary during the fetal period and disturbances during fetal development often cause impaired fertility in adulthood. In males, the Wolffian ducts are stabilized by testosterone, and the developed external genitalia are under the influence of DHT. If biosynthesis of these steroids is disturbed or if loss-of-function or absence of AR occurs, males present with pseudohermaphroditis and feminized external genitalia (Kerr and de Kretser, 2006). Excessive exposure to estrogens during fetal development in male mice, such as in aromatase overexpressing male mice, causes cryptorchidism, arrested spermatogenesis and infertility (Li et al., 2003). Although HSD17B1 is known to function in both estrogen and androgen biosynthesis *in vitro* and although the expression of *Hsd17b1* was observed to be high in fetal and newborn testes, the current study demonstrated that *in vivo* HSD17B1 was not essential for fetal programming and development as no changes were observed in the AGDs of the HSD17B1KO male mice. Similar to the WT mice, the HSD17B1KO male mice also reached puberty, and no changes were observed in the adult androgen-dependent tissue weights or intratesticular steroid concentrations. Together, these findings indicate that loss of HSD17B1 does not alter global estrogen or androgen metabolism in male mice. Surprisingly, the RT-qPCR analysis of steroidogenic genes in the newborn and adult testes demonstrated upregulation of *Hsd17b3*, which is known to convert A-dione to T. This compensatory expression in the HSD17B1KO testis may underlie the unaltered systemic steroid environment.

Female gonadal development during the fetal period is similarly dependent on a balanced hormonal environment. Excessive exposure to androgens during the fetal period causes occasional or completely absent ovarian cycles in adults (Abbott et al., 1998; Sullivan and Moenter, 2004). A lack of androgens during the fetal period has been studied with StAR knockout female mice, in which deletion of StAR results in failure of folliculogenesis and a lack of *corpora lutea* (Hasegawa et al., 2000). It has been postulated that estrogens do not have specific role in fetal programming. This has been demonstrated using different knockout mouse models such as ERαKO and ERβKO which do have clear ovarian phenotypes but do not have major defects in fetal development (Dupont et al., 2000). Surprisingly, the αβERKO double knockout ovaries have testicular structures and express Sertoli cell markers *Sox-9* and sulfated glycoprotein-2, as well as Leydig cell marker *Hsd17b3* (Couse and Korach, 2001). However, the αβERKO female mice do have female reproductive tracts. This suggest that estrogens are more necessary for maintenance of somatic cell phenotypes in adults than for fetal development. The effects of excessive estrogen exposure in females are similar to those of excessive androgen exposure during fetal development. Fetal exposure to estrogens in

women causes an intermittent or absent cycle with reduced fertility (Bibbo et al., 1977; Haney et al., 1979). Overexpressing HSD17B1 female mice (HSD17B1TG) have structures that resemble Wolffian ducts, which indicates excessive androgen exposure as the Wolffian ducts are stabilized under the influence of testosterone (Saloniemi et al., 2009). The HSD17B1KO female mice have normal female reproductive tracts and no changes are evident at the onset of puberty, indicating that HSD17B1 does not play a major role during fetal development or during puberty. However, in the HSD17B activity analysis, a significant reduction in the E1-to-E2 conversion was observed in the HSD17B1KO ovaries *in vitro*. In addition, the E1-to-E2 and A-dione-to-T ratios were increased, which indicated important functions for these precise steroid conversions. No changes were observed in the absolute intraovarian estrogen or androgen concentrations but, unexpectedly, markedly decreased P4 levels were observed in the knockout ovaries during the estrus phase of the estrous cycle and in the pseudopregnant HSD17B1KO female mice. The main site of P4 production in ovaries is the *corpus luteum,* and the number of CLs was markedly reduced in the HSD17B1KO female mice. This suggests that the observed reduction in P4 production is actually due to a reduced number of CLs. This finding is in line with the observed subfertility and inability to maintain pseudopregnancy as the CL-produced P4 is the key factor that governs these physiological events.

6.4 HSD17B1 is important for the *corpus luteum* and Sertoli cell function (I & II)

6.4.1 *HSD17B1 is necessary for proper maintenance of CL (I)*

Pregnancy and pseudopregnancy have been thought to be maintained in mice by CL-produced P4; in humans, CL contributes to P4 production in early pregnancy, and the placenta takes over P4 production after gestational week 8 (Malassiné et al., 2003). However, recently Naruse *et al.* demonstrated that the placenta produces P4 during mid-gestation in mice, which is accompanied by a simultaneous temporary reduction in ovarian P4 production (Naruse et al., 2014). They also demonstrated the importance of P4 reduction prior to parturition with sushi-ichi retrotransposon homologue 7/leucine zipper downregulated in cancer 1 (*Sirh7/Ldoc1*) knockout mice, where placental P4 production was excessive and resulted in delayed parturition.

When gestation approaches parturition, the P4 levels decrease through functional and structural luteolysis of the CL, which involves several factors, such as PTGFR

and 20α-HSD. Prostaglandin F2α (PGF2α) is produced mostly in the uterus, and its receptors are localized in the mouse CL (Sugatani et al., 1996; Sugimoto et al., 1992; Unezaki et al., 1996). The knockout mouse model of prostaglandin F receptor ($FP^{-/-}$) does not deliver offspring, and the P4 levels of these mice do not drop before the expected parturition (Sugimoto et al., 1997). These KO females have normal estrous cycles, ovulation and fertilization. Ovariectomy on the 19th day of pregnancy enables successful parturition. It has also been shown that $FP^{-/.}$ female mice are not able to respond to exogenous oxytocin, but the response is restored with ovariectomy. These findings indicated the importance of prostaglandin F2α in the initiation of luteolysis. PGF2α regulates the P4 reduction by inducing 20α-HSD expression in the *corpora lutea*, which has been demonstrated by administrating PGF2α to rats on 19th day of pregnancy (Stocco et al., 2000). Similarly, when the 20α-HSD gene and protein levels are analyzed in WT and $FP^{-/-}$ female mice, no elevation is observed in the KO mice, while the WT mice exhibit elevation in 20α-HSD on days 19th and 20th prior to parturition (Stocco et al., 2000). Additionally, administration of PGF2α to pseudopregnant mice causes increased apoptotic signaling, indicating that PGF2α induces apoptosis in the CL (Wang et al., 2003). 20α-HSD inactivates P4 by converting it to 20α-dihydroprogesterone (20α-OHP). Deletion of this enzyme results in a prolonged estrous cycle, pseudopregnancy and pregnancy (Ishida et al., 2007). The KO female mice produce offspring; however, the number of offspring is significantly reduced. The finding of reduced intraovarian P4 concentrations in pseudopregnant HSD17B1KO female mice supports the hypothesis of premature luteolysis of the CL. As the number of CLs in HSD17B1KO is reduced, the lower P4 concentration may be due to an inability to produce the CL after ovulation. However, as pseudopregnant HSD17B1KO ovaries show an average of two CLs, it is likely that the problem occurs in luteolysis rather than in luteogenesis. HSD17B1KO mice also demonstrate a reduction in *Ptgfr* expression in pseudopregnant ovaries, suggesting excessive production of PGF2α, which is also supported by upregulation of *20α-HSD* expression in the ovaries of pseudopregnant HSD17B1KO female mice. In addition to these findings, the CLs of the pseudopregnant HSD17B1KO ovaries exhibited increased apoptotic signals, which may be due to excessive PGF2α signaling. Our data, together with the findings of increased apoptosis in the CL after PGF2α administration, indicates that the *corpus luteum* in HSD17B1KO mouse undergoes premature luteolysis which underlies the reduced intraovarian P4 concentrations and the inability to maintain pseudopregnancy or pregnancy.

6.4.2 *HSD17B1 is essential for the function of Sertoli cells (II)*

The function of Sertoli cells is to provide nourishment and support for germ cells during their development. The localization of *Hsd17b1* to mouse Sertoli cells encouraged us to analyze the Sertoli cells and their function in the HSD17B1KO testis further. The supportive functions of Sertoli cells includes regulation of movement and development of germ cells with cell-cell tight junctions, which are known as ectoplasmic specializations. The apical ectoplasmic specializations are microtubular structures, formed between Sertoli and germ cells, that regulate germ cell adhesion, polarization and head shaping (Mruk and Cheng, 2004b). These tight junctions are particularly essential for spermatid development. The HSD17B1KO testis phenotype demonstrates disordered layers of germ cells and premature release of spermatids, which indicates a disturbance in aES function. The organization and orientation of the germ cells, especially the spermatids, are regulated by functional aES. Interfering with the aES, *i.e.*, by knocking down *Ezrin* with RNA interference (RNAi) or by knocking out *Spata31*, causes improper organization and polarization of spermatids and infertility with premature release of spermatids, respectively (Gungor-Ordueri et al., 2014; Wu et al., 2015). Similarly, a dysfunctional aES was observed in HSD17B1KO mice, where the elongating spermatids were mixed with spermatocytes near the basal lamina instead of near the luminal sides of the seminiferous tubules. Furthermore, polarization of the elongating spermatids was disrupted. Although the protein components of the cell-cell junctions were expressed, these junctions often appeared irregularly organized, evidenced by ectopic localization of ESPIN outside of the acrosomal region. In addition to adhesion and organization, aES also regulate the head shaping of elongating spermatids (Mruk and Cheng, 2004b). The head shapes of the elongating spermatids of the HSD17B1KO mice were visibly disrupted, which supports the hypothesis of disrupted aES and dysfunctional Sertoli cells.

Adult Sertoli cell function is markedly dependent on proper proliferation and maturation. Due to the high expression level of *Hsd17b1* in the fetal testis, with peaking expression in the newborn testis, and due to *Hsd17b1* localization to Sertoli cells, an analyze of Sertoli cells maturation was performed in the HSD17B1KO testis. Proliferation and maturation of Sertoli cells occurs prior to puberty, and several factors, such as AMH, SOX-9, SF-1, GATA1 and GATA4, are involved. The HSD17B1KO newborn testis expressed significantly elevated levels of *Sox-9, Sf-1* and *Gata4*, and the *Amh, Fshr* and *Dmrt1* expression levels showed clear upregulation trends. Interestingly, the adult HSD17B1KO testis expressed upregulated levels of *Amh* and *Sox-9*. AMH is a known immature Sertoli cell marker that is regulated by FSH signaling together with SOX-9, SF-1 and GATA4 (Arango et al., 1999; Giuili et al., 1997; Lasala et al., 2011; De Santa Barbara et al., 1998; Viger et al., 1998). Sustained expression of *Amh* in the adult HSD17B1KO testis

can be associated with improper maturation of the Sertoli cells. A similar link between improper maturation and defective spermatogenesis has been demonstrated with A-kinase anchoring protein 9 knockout male mice (AKAP9KO, Schimenti et al., 2013). Adult AKAP9KO male testes express markers of immature Sertoli cells, *Amh* and thyroid hormone receptor alpha (*Thra*), and the gap and tight junctions of the testes are disrupted. These results suggest that the apparently defective spermatogenesis and aES function are due to improper maturation of Sertoli cells.

In addition to maturation of Sertoli cells, it has been demonstrated that both androgens and estrogens regulate tight junctions in the testis. The probable hypothesis for defective spermatogenesis in HSD17B1KO male mice is that the local hormonal balance of Sertoli cells is affected due to loss of HSD17B1 which causes improper aES function. The role of testosterone in spermatogenesis and tight junctions has been demonstrated with several different knockout mouse models of AR and with an androgen withdrawal model (Chang et al., 2004; De Gendt et al., 2004; Holdcraft and Braun, 2004; O'Donnell et al., 1996). The Sertoli cell-specific AR knockout (S-AR$^{-/y}$) male mice by Chang *et al.* have elevated *Amh* levels in the adult testis, which is similar to HSD17B1KO male mice. In addition, the S-AR$^{-/y}$ testis shows increased apoptosis, which is also present in the HSD17B1KO testis. Sertoli cell-specific removal of AR function, performed by Holdcraft & Braun, further supports the importance of androgens for Sertoli cells function and spermatogenesis as the *Ar$^{flox(ex1\text{-}neo)Y}$;Amh-cre* male mice exhibit loss of elongating spermatids. Luteinizing hormone receptor knockout (LuRKO) male mice have decreased testosterone levels, and spermatogenesis is arrested at the round spermatid stage, supporting the importance of testosterone in spermatogenesis (Zhang et al., 2001). Futhermore, the infertility of the LuRKO male mice can be partially rescued by testosterone replacement (Pakarainen et al., 2005). The estrogenic effect has been demonstrated with β-estradiol 3-benzoate (E2B) which causes morphological disruption of elongating spermatids and disorganization of aES in step-8 and step-9 spermatids (Toyama et al., 2001). In addition, aromatase knockout male mice develop infertility over time, and ENERKI male mice do not have round or elongating spermatids (Robertson et al., 1999; Sinkevicius et al., 2009a). ENERKI mice also exhibit increased apoptosis. The *Amh* upregulation in the HSD17B1KO adult testis may be due to reduced ligand availability for AR in Sertoli cells, which is supported by the S-AR$^{-/y}$ mouse model. Our results from the HSD17B1KO testis, together with the observed local steroidogenic effects in the ovaries of HSD17B1KO mice, suggest improper aES function in the HSD17B1KO testis is also due to an unbalanced hormonal environment in Sertoli cells.

6.5 HSD17B1 and regulation of cellular hormone actions (I & II)

One of the major findings in the HSD17B1KO ovaries is the strongly upregulated expression of *Cyp17a1* which is known to convert pregnenolone to 17α-hydroxy-pregnenolone to DHEA and to convert progesterone to 17α-hydroxyprogesterone to androstenedione (Payne and Hales, 2004). The ovarian *Cyp17a1* localizes to the theca cells and theca lutein cells of the CL (Sasano et al., 1989; Tamura et al., 1992). Ovarian steroidogenesis between the theca and granulosa cells is known as the two cell – two gonadotropin model where steroid synthesis is regulated by LH and FSH and where steroid metabolites are synthesized and transferred between these two cell types. It has been demonstrated with ERαKO that loss of ERα induces the expression of *Cyp17a1* (Couse et al., 2003). This was further examined *in vitro* with ERαKO follicles where Taniguchi *et al.* observed that late-stage follicles without ERα showed a high upregulation of *Cyp17a1* and produced a higher amount of A-dione than the WT follicles (Taniguchi et al., 2007). The WT follicles exhibited the same upregulation of *Cyp17a1* when treated with an aromatase inhibitor or ER-antagonist (ICI182,780). Even though no changes in the absolute A-dione concentration in the ovaries were observed, the increase in the A-dione-to-T ratio in the HSD17B1KO ovaries and the upregulation of *Cyp17a1* indicates ERα-mediated regulation. These findings, together with our observations, have led us to hypothesize that communication between the theca and granulosa cells and balanced ovarian steroidogenesis requires HSD17B1. Loss of HSD17B1 results in loss of the ERα ligand, which then reduces the ability of ERα to suppress *Cyp17a1* expression, resulting in upregulation of *Cyp17a1* (Fig. 10A).

In adult males, HSD17B3 is a major enzyme that converts A-dione to T in Leydig cells, and the observation by O'Shaughnessy *et al.* and Shima *et al.* demonstrated that fetal Leydig cells produce only A-dione which is then converted to T in fetal Sertoli cells (O'Shaughnessy et al., 2000; Shima et al., 2013). It has been demonstrated that the fetal Leydig cells (E18.5) do not express *Hsd17b3*, but expression has been observed in fetal Sertoli cells (E18.5, O'Shaughnessy *et al.*, 2000; Shima *et al.*, 2013). Fetal Leydig cells produce A-dione through CYP17A1 and A-dione is transferred to fetal Sertoli cells for further conversion to T. The fetal and newborn mouse testis shows high expression of *Hsd17b1*, and loss of HSD17B1 results in upregulation of *Hsd17b3*, which suggest that HSD17B1 has a role in the A-dione-to-T conversion in the fetal testis. Based on the results of the present study and the literature, we hypothesize that fetal testicular testosterone production is regulated by HSD17B3 and HSD17B1 together and that upregulation of *Hsd17b3* compensates for loss of HSD17B1 in fetal Sertoli cells to maintain the proper T level in Sertoli cells (Fig. 10B).

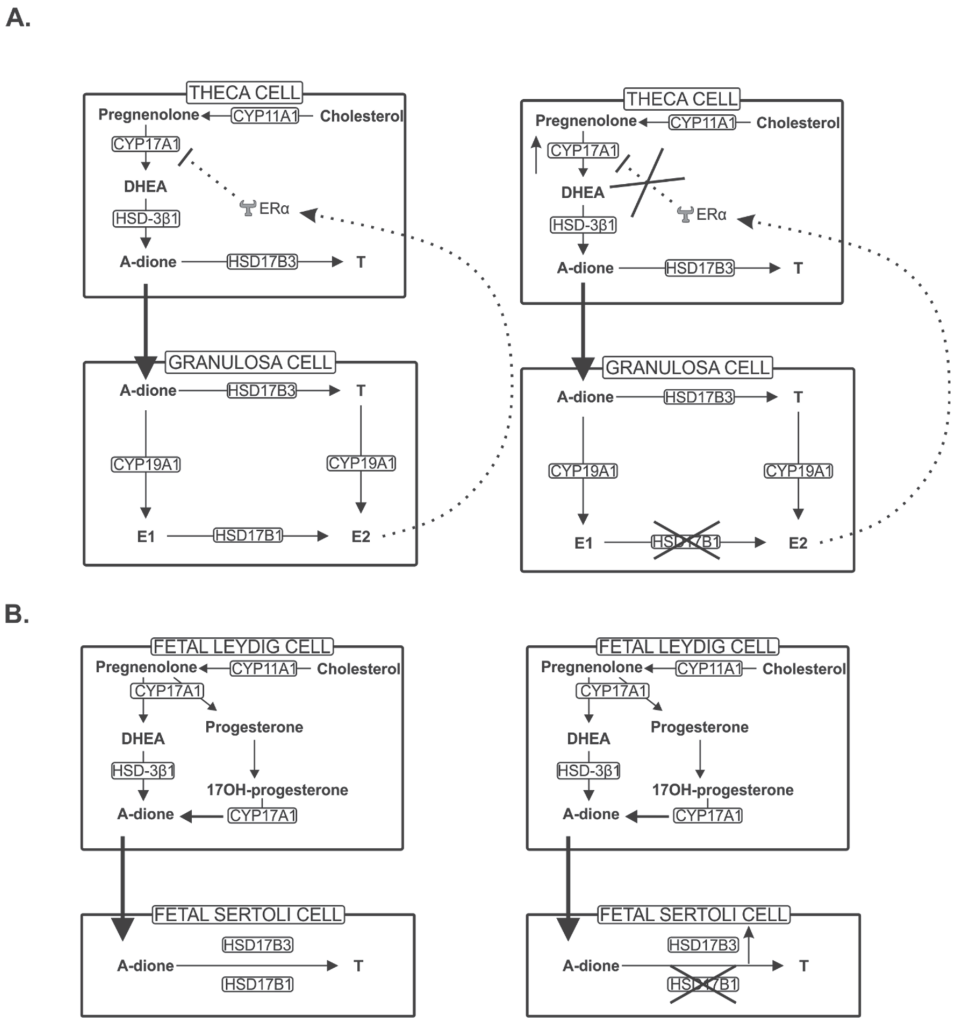

Figure 10. Proposed regulation of hormonal action by HSD17B1 in the ovaries (A.) and in the fetal testis (B.). A., The two cell - two gonadotropin model demonstrating the ovarian steroidogenesis and the inhibitory effect of ERα on *Cyp17a1* expression. In the HSD17B1KO ovaries, we hypothesize that the loss of HSD17B1 results in the reduction of ERα ligand, which in turn decreases the inhibitory effect on *Cyp17a1* expression and thus causes upregulation of *Cyp17a1* in ovarian theca cells. B., Regulation of testosterone production in fetal testis by HSD17B1 and HSD17B3 together. The loss of HSD17B1 causes upregulation of HSD17B3 in fetal Sertoli cells to maintain the necessary T level.

6.6 Novelty of the thesis

The present study demonstrates for the first time the role of hydroxysteroid (17β) dehydrogenase 1 in the development of testis and in Sertoli cell maturation. The deletion of *Hsd17b1* gene in males causes an improper maturation of Sertoli cells,

which affected the spermatogenesis and reproductive function of the adult male mice. It has been previously shown that the maturation of Sertoli cells during development affects the function of Sertoli cells in the adulthood, and thus, affects the spermatogenesis and reproduction (Juul et al., 2014; Schimenti et al., 2013). However, no data indicating any role of HSD17B1 in the function of Sertoli cells or in male reproduction has been previously published. Furthermore, we revealed that HSD17B1 has a cooperative role with HSD17B3 in the testosterone production together in the fetal Sertoli cells, as the deletion of *Hsd17b1* increased markedly the expression of *Hsd17b3*. HSD17B3 has been shown to produces testosterone in the Sertoli cells during fetal age while during adulthood HSD17B3 produce the testosterone in the Leydig cells (Shima et al., 2013). Findings of the present study provide novel knowledge of the HSD17B1 in male reproduction and factors affecting the testicular dysgenesis and azoospermia in men.

It has been shown by Nokelainen *et al.* in 1996 that mouse HSD17B1 catalyzes the reactions of E1-to-E2 and A-dione-to-T with similar catalytic efficiency (Nokelainen et al., 1996). Our data confirmed the role of HSD17B1 as a major enzyme in ovarian E2 biosynthesis. In addition to the role in the E2 steroid production, we have demonstrated the importance of HSD17B1 in the ovarian hormone balance. The granulosa and theca cells are known to cooperate together in steroid hormone synthesis. Previously discussed in Chapter 6.5, the *Cyp17a1* expression is regulated by estrogens through ERα. Aromatase is known to be one of the enzymes regulating the ligand availability to ERα, however, our data revealed that the HSD17B1 is also regulating the ligand availability for the ERα. This regulation through ERα controls the *Cyp17a1* expression, and thus, regulates the hormonal balance in ovaries. In the HSD17B1KO, the expression of *Cyp17a1* is upregulated due to the loss of ligand availability to ERα. One of the novel and surprising findings of HSD17B1 was its requirement for the maintenance of CL. Previous studies and our data revealed that the HSD17B1 is expressed in the granulosa cells but not in the CL. However, the loss of HSD17B1 results in premature luteolysis of CL, and thus inability to maintain the CL and pregnancy.

Taken together, these data from our female and male HSD17B1KO studies indicate that HSD17B1 plays significant roles in the regulation of local steroid actions in granulosa cells and Sertoli cells. In both cell types, HSD17B1 regulates steroid actions by functioning in estrogen biosynthesis (granulosa cells) or androgen biosynthesis (Sertoli cells). The present study provides novel knowledge on reproduction and on the intracrine and paracrine regulation of steroid actions at the target-cell level.

7 CONCLUSIONS

The reproduction and function of the ovaries and testis are dependent on the proper balance of steroid hormones. However, regulation of hormonal actions in reproduction and the gonads is not completely understood. In this study, we investigated the role of HSD17B1 in the steroid hormone biosynthesis of the gonads and its role in reproduction by using a global knockout mouse model of *Hsd17b1*. The main conclusions of the study are the following:

1. HSD17B1 is required for female reproduction and maintenance of the *corpus luteum*. Loss of HSD17B1 results in severe subfertility and an inability to maintain pseudopregnancy due to premature luteolysis of the *corpora lutea*.

2. HSD17B1 is essential for Sertoli cell maturation, spermatogenesis and male reproduction. A deficiency of *Hsd17b1* in Sertoli cells causes improper maturation of Sertoli cells and disrupts the cell-cell tight junctions, known as apical ectoplasmic specializations, between the Sertoli cells and elongating spermatids causing infertility and azoospermia.

3. HSD17B1 controls hormonal action via paracrine and intracrine regulation in the ovaries and testis, respectively. Granulosa cells require HSD17B1 for sufficient E2 production, which, in turn, is required in theca cells for proper ERα-mediated inhibition of *Cyp17a1* expression. Testosterone synthesis in fetal Sertoli cells is regulated together by HSD17B1 and HSD17B3, as indicated by compensatory upregulation of *Hsd17b3* due to loss of HSD17B1.

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