TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 843

MEDICA - ODONTOLOGICA

NOVEL INSIGHTS INTO THE ROLE OF HYDROXYSTEROID (17β) DEHYDROGENASE 2 (HSD17B2) AS REVEALED BY TRANSGENIC MICE EXPRESSING THE HUMAN ENZYME

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Turku 2009

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ISBN 978-951-29-3843-8 (PRINT)
ISBN 978-951-29-3844-5 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2009

ABSTRACT

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Novel insights into the role of human hydroxysteroid (17β) dehydrogenase 2 (HSD17B2) as revealed by the transgenic mice expressing the human enzyme

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Annales Universitatis Turkuensis, Medica-Odontologica
2009, Turku, Finland

In vitro studies have indicated that HSD17B2 possesses predominantly oxidative HSD17B activity to inactivate both estrogens and androgens and activate 20α-hydroxyprogesterone to progesterone (P), which is considered important for the inactivation of 17β-hydroxy steroids, e.g. estradiol (E_2) and testosterone (T) in various peripheral tissues, including sex steroid target tissues. To understand the function of HSD17B2 *in vivo*, we generated transgenic (TG) mice ubiquitously expressing human HSD17B2. This model gave an excellent possibility to study the consequences of HSD17B2 expression *in vivo* in detail.

In the present study, both female and male HSD17B2TG mice showed growth retardation and delayed eye opening at the post-natal age, and resulted in delayed puberty.

HSD17B2TG males showed infertility after the age of 3 months, and at the age of 6 months the seminiferous tubules showed a Sertoli cell only phenotype. Disrupted spermatogenesis was evident in the presence of normal serum and intratesticular T, P and normal circulating luteinizing hormone (LH) concentrations. Proper androgen action in the target tissues was confirmed by the normal histological appearance of the prostate and the epididymis. Furthermore, quantitative RT-PCR analysis indicated only a slight decrease in androgen-dependent gene expression in the prostate. The disrupted spermatogenesis was not associated with increased germ cell apoptosis as analyzed by caspase-3 activation.

Ubiquitous human HSD17B2 expression inhibits skeletal development in male mice at prepubertal age. The disturbances in skeletal development partially resulted from growth retardation, showing a decreased bone formation rate associated with shorter bone length and a lower density of both the trabecular and cortical bones. These changes were, at least partially, explained by lower serum Igf1, osteocalcin and T concentrations, and were normalized at adult age.

The HSD17B2TG female mice presented with ovary dysfunction and mammary gland hyperplasia associated with increased expression of multiple pregnancy associated genes. The macroscopic phenotype observed in the mammary glands was dependent on ovarian function, while a normal histological appearance was observed in transgenic mammary glands transplanted into a wild-type host. However, the significant suppression of several known estrogen target genes in the HSD17B2TG mammary transplants in WT females was observed, suggesting that HSD17B2 modulates estrogen action *in vivo*.

Bi-TG mice expressing human HSD17B1 and HSD17B2 showed growth retardation similar to the HSD17B2TG mice. The bi-TG females presented the masculinized phenotype reported for HSD17B1TG females and mammary gland hyperplasia observed in HSD17B2TG females. Furthermore, the bi-TG males showed severely disrupted spermatogenesis, similar to the HSD17B2TG males.

In conclusion, the data suggest that HSD17B1 and HSD17B2 primarily act on different signaling pathways *in vivo*. HSD17B1 is mainly involved in the sex steroid metabolism and HSD17B2 acts primarily on pathways other than on sex steroids but is also capable of modulating sex steroid action. Interestingly, the testicular phenotype and some of the other phenotypic changes observed, together with the rescue of the testis phenotype by a synthetic retinoic acid receptor agonist (TTNPB) suggest a role for HSD17B2 in the action of retinoids, in addition to its oxidative HSD17B activity on sex steroids.

Key words: Hydroxysteroid (17β) dehydrogenase 2, transgenic mice, growth retardation, testis, ovary, mammary gland, skeletal development, androgen, estrogen

TIIVISTELMÄ

Zhongyi Shen

Hydroxysteroidi (17β) dehydrogenaasi 2 (HSD17B2) -entsyymin toiminnan selvittäminen ihmisen entsyymiä ilmentävien siirtogeenihiirten avulla

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Annales Universitatis Turkuensis, Medica-Odontologica, 2009, Turku, Suomi

In vitro -työt ovat osoittaneet, että HSD17B2-entsyymi katalysoi estradiolin ja testosteronin muuntumista ääreiskudoksissa heikompi tehoisiksi hormoneiksi estroniksi ja androstendioniksi, ja entsyymi myös aktivoi 20α-hydroksiprogesteronin progesteroniksi (P). HSD17B2-entsyymin toiminnan ymmärtämiseksi paremmin olemme tuottaneet siirtogeeniset hiiret, jotka tuottavat ihmisen entsyymiä kaikissa kudoksissa. Mallin avulla kykenemme tutkimaan HSD17B2-entsyymin ilmentymisestä mahdollisesti johtuvia fysiologisia muutoksia hiiren elimistössä. Tulokset osoittivat, että sekä uros että naarashiirten kasvu sekä silmien aukeaminen oli viivästynyt. Lisäksi sekä uros että naarashiirten puberteetti ilmaantui kontrollihiiriä myöhäisemmällä iällä. HSD17B2TG-urokeet olivat hedelmöityskyvyttömiä, ja niiden sukusolut eivät kypsyneet normaalisti. Tämä johti hiirten hedelmättömyyteen kolmen kuukauden iässä, ja kuuden kk iässä lähes kaikki sukusolut olivat tuhoutuneet huolimatta eläinten normaaleista testosteroni- ja gonadotropiinihormonipitoisuuksista. Spermatogeneesin häiriintymiseen liittyi voimakas apoptoosin lisääntyminen sukusoluissa. Osoitimme myös, että hiirten androgeenivasteisten kudosten (esim. eturauhanen ja lisäkives) histologia oli normaali, mutta lähetti-RNA tasolla tehdyt mittaukset osoittivat eräiden androgeenivasteisten geenien ilmentymisen olleen heikentynyt. Havaitsimme myös, että ihmisen HSD17B2-geenin ilmentyminen esti luuston normaalia kehittymistä nuorella iällä. Osoitimme, että sekä trabekulaari- että kortikaaliluun tiheys oli siirtogeenihiirillä alentunut. Nämä muutokset voitiin yhdistää alentuneeseen Igf1-kasvutekijän, osteokalsiinin, ja testosteronikonsentraation laskuun. Nämä kaikki muutokset normalisoituivat aikuisiässä.

Naarashiirissä HSD17B2:n ilmentyminen johti munasarjojen toiminnan häiriöön sekä rintarauhasen epiteelin hyperplastiseen kasvuun, ja siihen liittyi myös useiden tyypillisesti raskauden aikana ilmentyvien geenien aktiivisuuden lisääntyminen. Rintarauhasen rakenne kuitenkin normalisoitui kokeessa, jossa siirsimme HSD17B2-siirtogeenihiiren rintarauhasen epiteeliä kasvamaan normaaliin hiireen. Rakenteen normalisoitumisesta huolimatta lisääntynyt paikallinen HSD17B2-aktiivisuus vaikutti estrogeenivasteisten geenien ilmentymiseen. Tulokset siten osoittivat, että kohdekudoksessa HSD17B2-entsyymiaktiivisuus voi muuntaa estrogeenivasteisten geenien ilmentymistä in vivo olosuhteissa, mutta useat tulokset myös antoivat epäsuoria viitteitä siitä, että HSD17B2-entsyymi osallistuu retinoidien aineenvaihduntaan.

Tutkimme myös siirtogeenihiiriä, jotka ilmensivät sekä ihmisen HSD17B1- että HSD17B2-geeniä yhtaikaa. Entsyymien on oletettu toimivan toistensa vastavaikuttajina siten, että HSD17B1-entsyymi aktivoi heikkoja 17-ketosteroideja voimakkaammin vaikuttaviksi 17-beta-hydroksisteroideiksi, kun taas HSD17B2-entsyymi katalysoi käänteistä reaktiota. Tuloksemme kuitenkin osoittivat, että kyseiset entsyymit eivät ilmentyessään voineet korjata toistensa siirtogeenihiirissä aiheuttamia fysiologisia häiriötiloja. Johtopäätöksenä voitiin todeta, että kyseiset entsyymit toimivat pääosin eri signaaliteissä. HSD17B1 toimii erityisesti sukupuolisteroidien aineenvaihdunnassa, kun taas HSD17B2 toimii pääasiallisesti muissa signaaliteissä, mutta kykenee muokkaamaan myös sukupuolisteroidien vasteita.

Avainsanat: androgeeni, estrogeeni, hydroksisteroidi (17β) dehydrogenaasi 2, kasvu, kives, luusto, munasarjat, rintarauhanen, siirtogeeninen hiiri

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ABBREVIATIONS

Official symbol*	Gene name*	Official name
ADT		3α-hydroxy-5α-androstan-17-one (Androsterone)
ADHs		*Adhs* Alcohol dehydrogenases
3α-diol		5α-androstane-3α, 17β-diol (3α androstanediol)
A-dione		5α-androstane-3, 17-dione (androstanedione)
AFs		Activation functions of transcription
AKR		Aldo-keto reductase
AKR1C (Akr1c)	*AKR1C* (*Akr1c1)*	Aldo-keto reductase family 1, member C (3α-HSD)
ALB (Alb)	*ALB* (*Alb)*	Albumin
ALDH1A (Aldh1a)	*ALDH1A* (*Aldh1a)*	Aldehyde dehydrogenase 1 family, member A **(**RALDH)
AR (Ar)	*AR* (*Ar)*	Androgen receptor
at(RA)		All-*trans* (retinoic acid)
CCND1 (Ccnd1)	*CCND1* **(***Ccnd1)*	Cyclin D1 (CycD1)
(c)DNA		(complementary) Deoxyribonucleic acid
CDKN1B (Cdkn1b)	*CDKN1B* **(***Cdkn1b)*	Cyclin-dependent kinase inhibitor 1B (p27)
Cortisol		11β, 17, 21-trihydroxy-4-pregnene-3, 20-dione
Cortisone		17, 21-dihydroxy-4-pregnene-3, 11, 20-trione
CRABP (Crabp)	*CRABP* (*Crabp)*	Cellular retinoic acid binding proteins
CYP11A1 (Cyp11a1)	*CYP11A1* (*Cyp11a1)*	Cytochrome P450, family 11, subfamily A, polypeptide 1 (P450scc)
CYP17A1 (Cyp17a1)	*CYP17A1* (*Cyp17a1)*	Cytochrome P450, family 17, subfamily A, polypeptide 1 (P450c17)
CYP19A1 Cyp19a1)	*CYP19A1* (*Cyp19a1)*	Cytochrome P450, family 19, subfamily A, polypeptide 1 (P-450arom)
CYP21A2 (Cyp21a2)	*CYP21A2* (*Cyp21a2)*	Cytochrome P450, family 21, subfamily A, polypeptide 2 (P450c21B)
CYP11B1 (Cyp11b1)	*CYP11B1* (*Cyp11b1)*	Cytochrome P450, family 11, subfamily B, polypeptide 1 (P450c11)
CYP11B2 (Cyp11b2)	*CYP11B2 (Cyp11b2)*	Cytochrome P450, family 11, subfamily B, polypeptide 2 (P450c18)
CYP26 (Cyp26)	*CYP26 (Cyp26)*	Cytochrome P450, family 26
DHT		5α-dihydrotestosterone
DHEA		Dehydroepiandrosterone
DHEAS		Dehydroepiandrosterone sulfate
$\triangle^4$-dione		4-androstene-3, 17-dione
$\triangle^5$-diol		$\triangle^5$-androstene-3β, 17β-diol (androstenediol)
3β-diol		5α-androstane-3β, 17β-diol (3β-androstanediol)
DSD		Disorders of sex development
E_1		Estrone
E_2		17β-estradiol
E_3		Estriol
ESR1 (Esr1)	*ESR1* (*Esr1)*	Estrogen receptor 1 (ERα)

ESR2 (Esr2)	*ESR2* (*Esr2)*	Estrogen receptor 2 (ERβ)
SULT1E1 (Sult1e1)	*SULT1E1 (Sult1e1)*	Sulfotransferase family 1E, estrogen-preferring, member 1 (EST, Estrogen sulfotransferase)
FGF2 (Fgf2)	*FGF2* (*Fgf2)*	Fibroblast growth factor 2 **(**FGFB)
FSH (Fsh)	*FSH* (*Fsh*)	Follicle-stimulating hormone
GABA		γ-aminobutyric acid
hpg		Hypogonadal
HSD3B (Hsd3b)	*HSD3B* (*Hsd3b)*	Hydroxy-δ-5-steroid dehydrogenase, 3β- and steroid δ-isomerases (3β-HSD)
HSD17B (Hsd17b)	*HSD17B (Hsd17b)*	Hydroxysteroid (17β) dehydrogenases **(**17β-HSDs)
HSP (Hsp)	*HSP* (*Hsp)*	Heat shock proteins
INSL3 (Insl3)	*INSL3* (*Insl3)*	Insulin-like 3 (RLF**)**
kb		Kilobase pair(s)
kDa		Kilodalton
KO		Knockout (mouse)
LDL (Ldl)	*LDL* (*Ldl)*	Low density lipoprotein
LH (Lh)	*LH* (*Lh*)	Luteinizing hormone
LHCGR (Lhcgr)	*LHCGR* (*Lhcgr)*	Luteinizing hormone /choriogonadotropin receptor (LHR)
mRNA		Messenger RNA
NAD		Nicotinamide-adenine dinucleotide
NADP		Nicotinamide-adenine dinucleotide phosphate
NRs		Nuclear receptors
NR3C1 (Nr3c1)	*NR3C1 (Nr3c1)*	Nuclear receptor subfamily 3, group C, member 1 (Glucocorticoid receptor)
NR5A1 (Nr5a1)	*NR5A1* (*Nr5a1)*	Nuclear receptor subfamily 5, group A, member 1 (SF-1)
NR6A1 (Nr6a1)	*NR6A1 (Nr6a1)*	Nuclear receptor subfamily 6, group A, member 1 (Germ cell nuclear factor)
P		Progesterone
PBS		Phosphate buffered saline
PGR (PR)	*PGR* (*Pgr)*	Progesterone receptor (PR)
PGR-A (Pgr-A)		Progesterone receptor-A (PR-A)
PGR-B (Pgr-B)		Progesterone receptor-B (PR-B)
PIAS (Pias)	*PIAS* (*Pias)*	Protein inhibitor of activated STAT
POMC (pomc)	*POMC (Pomc)*	Proopiomelanocortin (ACTH)
Pregnenolone		3β-hydroxy-5-pregnen-20-one
Pregnenolone-S		3β-hydroxy-5-pregnen-20-one sulfate
PRL (Prl)	*PRL* (*Prl)*	Prolactin
PRLR (Prlr)	*PRLR* **(***Prlr)*	Prolactin receptor
PSA (Psa)	*PSA* (*Psa)*	Prostate-specific antigen
RAR (Rar)	*RAR* (*Rar)*	Retinoic acid receptor
RBP (Rbp)	*RBP* (*Rbp)*	Cellular retinol binding proteins
RBP4 (Rbp4)	*RBP4* (*Rbp4)*	Plasma retinol binding protein
RIA		Radioimmunoassay
RNA		Ribonucleic acid
RT-PCR		Reverse transcription polymerase chain reaction
RXR (Rxr)	*RXR* (*Rxr)*	Retinoid X receptor
SDR		Short chain dehydrogenase/reductase
SERPINA6 (Serpina6)	*SERPINA6 (Serpina6)*	Serpin peptidase inhibitor, clade A

		(Transcortin)
SHBG (Shbg)	*SHBG* (*Shbg)*	Sex hormone-binding globulin
SHRs		Steroid hormone receptors
SRD5A (Srd5a)	*SRD5A* (*Srd5a)*	Steroid-5α-reductase, α-polypeptides (5α-reductases)
STAR (Star)	*STAR* (*Star)*	Steroidogenic acute regulator (STARD1)
STAT5 (Stat5)	*STAT5* (*Stat5)*	Signal transducer and activator of transcription 5
STS (Sts)	*STS* (*Sts)*	Steroid sulfatase (microsomal), isozyme (Steroid sulfatase)
SULT2A1 (Sult2a1)	*SULT2A1 (Sult2a1)*	Sulfotransferase family, cytosolic, 2A, DHEA-preferring, member 1 (DST, DHEA-sulfotransferase)
SUMO (Sumo)	*SUMO (Sumo)*	SMT3 suppressor of mif two 3 homolog (Small ubiquitin-like modifier)
T		Testosterone
Tfm		Testicular feminized mouse
TG		Transgene/transgenic
TGFB (Tgfb)	*TGFB* (*Tgfb*)	Transforming growth factor, β (TGF-β)
THR (Thr)	*THR* (*Thr*)	Thyroid hormone receptor
TTNPB		4-[(E)-2-(5, 6, 7, 8-tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid
VAD		Vitamin A deficiency
WT		Wild-type

***** Official symbol and gene name are indicated for human, and in the brackets they are for rodent

LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the original articles, which will be referred to in the text by the Roman numerals given below.

I. **ZhongYi Shen, Pia Rantakari, Tarja Lamminen, Jorma Toppari, Matti Poutanen** 2007 Transgenic male mice expressing human hydroxysteroid dehydrogenase 2 indicate a role for the enzyme independent of its action on the sex steroids. Endocrinology 148:3827-3836.

II. **ZhonYi Shen, ZhiQi Peng, Yi Sun, H. Kalervo Väänänen, Matti Poutanen** 2008 Overexpression of Human Hydroxysteroid (17β) Dehydrogenase 2 Induces Disturbance in Skeletal Development in Young Male Mice. J Bone Miner Res 23:1217-1226.

III. **ZhonYi Shen, Taija Saloniemi, Aino Rönnblad, Pirjo Pakarinen and Matti Poutanen** Transgenic mice expressing Hydroxysteroid (17beta) dehydrogenase 2 enzyme reveal that the enzyme principally acts on both sex steroid independent pathway. Submitted to Endocrinology on July, 2008

1. INTRODUCTION

Human sex steroids, estrogens and androgens, are essential in reproduction. In addition, a large proportion of androgens and estrogens in men and women are synthesized locally in peripheral target tissues (*Labrie F 1991*). Transformation of the adrenal precursor steroids DHEA-S and DHEA into androgens and/or estrogens in peripheral target tissues depends upon the level of expression of the various steroidogenic and metabolizing enzymes in each of these tissues. Among the sex steroids, the highly active steroids are the 17β-hydroxy forms (e.g. E_2, T and DHT), whereas the biologically less active steroids are 17-keto forms (E_1, $\triangle^4$-dione and A-dione, respectively). HSD17Bs catalyze the conversion between 17-keto and 17β-hydroxy steroids and regulate the formation of T and E_2 (*Luu-The V 2001; Mindnich R et al 2004*). HSD17B activity is not only present in classical steroidogenic tissues such as the ovaries, placenta and testes, but more importantly, HSD17B activities are identified in various non-gonad tissues, thus indicating a role of HSD17Bs in the formation of T and E_2 both in the gonad and peripheral target tissues (*Labrie F et al 2003; Luu-The V 2001*). In recent years, more evidence has been obtained indicating the importance of this type of local activation and inactivation of sex steroids, as one of the key determinants for hormone action in the target tissues. Furthermore, HSD17Bs has been shown to be involved in the pathogenesis of human disorders such as various hormone-sensitive cancers (*Gunnarsson C et al 2001; Harkonen P et al 2003*), 46, XY Disorders of Sex Development (DSD, *Geissler WM et al 1994*), bifunctional enzyme deficiency (*de Launoit Y and Adamski J 1999*), polycystic kidney disease (*Maxwell MM et al 1995*) and Alzheimer's disease (*He XY et al 2002*). Recently, the different tissue and sub-cellular distribution, catalytic preference, substrate specificity and biochemical properties have been characterized in twelve HSD17B enzymes (*Moeller G and Adamski J 2006*). There is only a limited sequence identity with each other in most of the HSD17Bs, but many of them have overlapping substrate spectra. Among the twelve HSD17B enzymes, the HSD17B5 is a member of the aldo-keto reductase (AKR) family (*Dufort I et al 1999; Lin HK et al 1997*), while the rest of the HSD17B enzymes belong to the short chain dehydrogenase/reductase (SDR) family (*Penning TM 1997*).

The *HSD17B2* gene is located on chromosome 16 at loci q24.1-q24.2, and is at least 40 kb in length including seven exons and six introns (*Labrie Y et al 1995*). DNA sequence analysis indicated that its cDNA is 1.5 kb in size and encodes 387 amino acids long-protein. HSD17B2 predominantly catalyzes E_2 to E_1, T to $\triangle^4$-dione, DHT to A-dione and 20α-dihydroprogesterone to P (*Wu L et al 1993; Puranen TJ et al 1999*). HSD17B2 has been identified in a wide variety of tissues (*Casey ML et al 1994*). Typically, the HSD17B2 enzyme is highly expressed in the surface epithelia of gastrointestinal organs (*Mustonen MV et al 1998*). Thus, this enzyme may restrict access of the active sex steroids to the blood circulation, and protect the target tissues of hormone action from excessive sex hormone influence. Specially, in the placenta HSD17B2 may limit the access of fetal androgens to maternal tissue and the access of maternal estrogen into the fetus, and thus act as a barrier between the fetus and mother (*Moghrabi N et al 1997*). HSD17B2 in the endometrium plays critical role for normal endometrial growth and differentiation in the presence of P (*Yang S et al 2001*). The release of paracrine factors, such as retinoids, is induced by P, and further paracrine factors stimulate HSD17B2 expression in the endometrium (*Li XH et al 2002; Ito K et al 2001*).

Steroid hormone-related carcinogenesis is due to hormone induced cell proliferation, resulting in an increased opportunity for the accumulation of random genetic errors.

The development of cancers of the colon, breast and prostate have been linked to the loss of oxidative HSD17B2 activity, and accordingly to a rise in the reductive HDSD17B activity that would increase the potency of estrogens and androgens. Thus, the HSD17B2 enzyme has been suggested to play an important role in the pathogenesis of various hormone-dependent cancers (*Härkönen P et al 2003; English MA et al 2000; Oduwole OO et al 2004*). In prostate, breast and colon cancers, the reductive HSD17B activity was dominant over oxidative HSD17B activity and the expression of HSD17B2 was decreased dramatically as compared with normal tissues or benign hyperplasias (*Gunnarsson C et al 2001; Oduwole OO et al 2002*), and this decreased expression of HSD17B2 *in situ* in carcinoma was considered to increase estrogen-dependent proliferation.

Although many studies showed the properties of HSD17B2 *in vitro*, the physiological function of HSD17B2 *in vivo* is superficially investigated. It will be important to further analyze the physiological role of HSD17B2 *in vivo*, and the role of HSD17B2 in the regulation of local sex steroid availiability, thus, transgenic mice ubiquitously expressing human *HSD17B2* under the CMV-enhanced chicken β-actin promoter provide a novel tool to study the physiological function of HSD17B2 *in vivo*.

2. REVIEW OF THE LITERATURE

Steroid hormones, such as estrogens (E), progesterone (P) and androgens are synthesized and secreted by endocrine and extra-endocrine cells. They act locally within the cells or travel via the blood stream to their target cells, enter these cells by simple or facilitated diffusion, and then bind to specific receptors. Steroid hormones have important regulatory roles in a wide variety of biological processes including reproduction, differentiation, development, cell proliferation, apoptosis, imflammation, metabolism, homeostasis, and brain function (*Edwards DP, 2005; Tsai MJ and O'Malley BW 1994*).

2.1 Physiological function of sex steroids

2.1.1 Physiological function of estrogens

It is well established that estrogens play an important role not only in the development and function of the reproductive system but also in non-classical target tissues, such as the brain, skeletal, cardiovascular system, immune system, muscle, skin, liver, intestine and adipose tissues (*Manolagas SC and Kousteni S 2001; Couse JF and Korach KS 1999b*). The high intraovarian estrogen concentration is achieved during follicular maturation and corpus luteum function in the ovaries. The physiological role of estrogens within the primate ovaries and mechanisms are matters of debate, the events of which result in a mature oocyte capable of developing into a viable embryo after fertilization which may require estrogen action on granulosa cells, oocyte, or both after fertilization (*Palter SF 2001 and refs therein*). However, at the midcycle, increased estradiol creates a positive feedback on the hypothalamus and leads to an LH surge from the pituitary, leading to ovulation of the dominant follicle and the formation of the corpus luteum (*Filicori M and Cognigni GE 2001*). Estrogens have a well-defined role in the endometrial development of the uterus forming the receptive window for implantation at the appropriate phase of the menstrual cycle (*Martel D et al 1987; Fazleabas AT et al 1997*). Estrogens have a central integrative role in modulating the communication between the placenta and the fetus that results in primate fetal-placental development (*Pepe GJ et al 2001; Ni X 2002*), Likewise, the functions of estrogens are well known in the mammary glands, where they induce ductal growth and branching as well as formation of terminal end buds, (*Hennighausen L and Robinson GW 2001; 2005*). The increased interest in the role of estrogens in the male is largely due to the widespread distribution of estrogen receptors (ESRs) and studies carried out in men or transgenic animals deficient in genes coding for these receptors or for *CYP19A1*. The results obtained are changing our perception of the roles of estrogens in the male (*Sharpe RM 1998a and refs therein; Nilsson S et al 2001*).

In the human, the biosynthesis of estrogens occurs in a number of tissue sites. The principal sites are the granulosa cells of the ovaries in premenopausal women, stromal cells of adipose tissue in postmenopausal women, and the syncytiotrophoblast of the placenta in pregnant women. Additionally, estrogens are produced in a number of sites in the brain, breast and testes (*Ackerman GE and Carr BR 2002*). The ovaries mainly synthesizes E_2, adipose tissue mainly E_1 and the placenta mainly synthesizes E_3. In the blood circulation, estrogens are specifically bound to sex-hormone binding globulin (SHBG, *Petra P 1991*) and non-specifically to albumin. It is generally accepted that the unbound form is the biologically important fraction because of its freedom to diffuse into tissues. The protein bound fraction is thought to act as a reservoir for estrogens.

2.1.2 Physiological function of androgens

Androgens are important in every phase of male life. During the embryonal stage androgen determines the differentiation of sexual organs, and during puberty regulates further development and maintenance towards the adult male phenotype including the regulation of male sexual behavior. The epididymides, vas defrens, seminal vesicles, penis, testes and prostate are dependent on continuous androgen action, and androgen establishes the male phenotype in several extragenital tissues, notably muscle, brain, kidneys, liver, immune system, skin, hematopoietic system, salivary gland, mammary glands and bone (*Mooradian AD et al 1987 and refs therein*). Fetal Leydig cell-derived testosterone (T) induces transformation of the Wolffian duct to the epididymides, vas deferens and seminal vesicles. Development of external genitalia is also androgen-dependent. However, in contrast to internal genitalia, external genitalia are induced by DHT originating from T by SRD5A. DHT has been shown to be mainly responsible for masculinization of the prostate, urethra, penis and scrotum in men (*Andersson S et al 1991*), and DHT is a pivotal regulator of development, morphogenesis and growth of the prostate (*Steers WD 2001*). Spermatogenesis has been thought to depend on local T concentration (*Zhang FP et al 2003*). However, the germ cells are completely devoid of Ar, suggesting that androgens have an indirect effect on germ cells through Sertoli cells (*Zhou Q et al 2002; De Gendt K et al 2004*). In the epididymides, seminal vesicles, vas deferens and penis the lack of androgens can result in regression of the secretory epithelia, eventually leading to aspermia. Most of these alterations can be reversed by adequate androgen replacement. In human and primate ovaries, AR is mainly expressed in the granulosa cells of the follicles and, to a lesser extent, also in theca cells and stroma, suggesting that thecal androgens have a number of effects on primate ovaries. Androgens have complex actions on granulosa cells and potentially induce atresia during follicular development (*Weil S et al 1999; Hillier SG and Tetsuka M 1997*).

Androgens are mainly produced in the testes and in human to a lesser extent in the adrenal gland. Androgens also are produced in minor amounts in women by the ovaries. Additionally, DHEA and $\triangle^4$-dione predominantly originating from the adrenal cortex constitutes a large reservoir of precursors for extragonadal conversion to bioactive sex steroids in extragonadal tissues both in men and in women. T circulates in blood mostly bound to SHBG and other low-affinity, high-capacity binding proteins (albumin, α_1 acid glycoprotein, transcortin), and only a small percentage remain unbound. According to the free hormone hypothesis, only 1-2% of the free fraction is the most biologically active. The loosely protein-bound T constitutes a larger bioavailable fraction of circulating T (*Van den Beld AW 2000*).

2.1.3 Physiological function of progestins

Progestins are required for female fertility and play numerous roles in female reproduction. The target tissues of progestin action include the ovaries, uterus, mammary glands and brain. Consistent with this, biological changes in response to progestin are observed in these sites. P has diverse roles in the uterus and ovaries at every stage of reproductive function (*Clarke CL and Sutherland RL 1990*). P is also required for normal release of mature oocytes. In the maintenance of pregnancy, P also promotes uterine growth and reduces myometrial contractility, and the normal breast undergoes extensive growth, branching and lobuloalveolar development (*Graham JD and Clarke CL 1997*).

The major sites of synthesis of P are the granulosa-lutein cells of the corpus luteum, the syncytiotrophoblast of the placenta and, to a lesser extent, the zona fasciculata and zona reticularis of the adrenal cortex. In blood, P is largely (95%-98%) bound to plasma proteins.

2.2 Molecular mechanisms of sex steroid and retinoid action

The effects of sex steroids and retinoids are mediated through interaction with specific intracellular receptors that are members of the nuclear receptor (NR) superfamily of transcription factors (*Tsai MJ and O'malley BW 1994*). NR family is one the most abundant classes of transcriptional regulators (*Robinson-Rechavi M et al 2001*). NRs can be phylogenetically divided into six groups (*Laudet V 1997*). All steroid hormone receptors (SHRs) belong to group III and retinoid receptors belong to groups I and II (Table 1). The members of the nuclear receptor family are closely related in their primary amino acid sequence and in the organization of functional domains, suggesting that many aspects of their mechanism of action are conserved.

Table 1. Phylogenetic Classification of Some of the Mammalian Nuclear Receptors on the Basis of the Sequence Similarity of DNA-binding Domain, Hinge Region and Ligand-binding Domain

Class	Representative member(s)	Ligand	Response element	Binding
Class I	THR RAR	Thyroids *all-trans* -RA, *9-cis* -RA	Palindrome, DR-4 inverted palindrome	Heterodimer, Heterodimer
Class II	RXR	9-*cis* -RA	Palindrome, DR-1	Heterodimer
Class III	AR ESR1 and ESR2 NR3C1 PGR	Androgens Estrogens Glucocorticoids Progestins	Palindrome Palindrome Palindrome Palindrome	Homodimer Homodimer Homodimer Homodimer
Class IV	NGF-induced clone B	Unknown	Palindrome, DR-5	Monomer, Homodimer, Heterodimer
Class V	NR5A1	Oxysterols	Hemisite	Monomer
Class VI	NR6A1	Unknown	DR-0	Homodimer
Class 0	Small heterodimeric partner	Unknown		Heterodimer

THR: Thyroid hormone receptor, RAR: Retinoic acid receptor, RXR: Retinoid X receptor, AR: Androgen receptor, ESR: Estrogen receptor, NR3C1: Glucocorticoid receptor, PGR: Progesterone receptor, NR5A1: Steroidogenic factor 1, NR6A1: Germ cell nuclear factor

Androgen receptor (*AR*) is encoded by a single gene, and there is no evidence for alternately spliced forms (*Hirata S et al 2003*). In contrast, there are two genes for ESRs, *ESR1* (ERα) and *ESR2* (ERβ) (*Kuiper GG et al 1996; Couse JF and Korach KS 1999b*), and receptor isoforms (*PGR-A* and *PGR-B*) for P arise from a single gene. The PGR-B protein contains an additional amino acid sequence at its N-terminus that is lacking in PGR-A (*Kastner P et al 1990*). Diversity in the control of gene expression by retinoid signals originates from the existence of two families of retinoid receptors: the three RAR isotypes (A, B and G), RXR isotypes (A, B and G), and their numerous isoforms resulting from alternate promoter usage and splicing (*Chambon P 1996*). RARs bind *all trans* and 9-*cis* RA, and RXRs bind 9-*cis* RA only (*Chambon P 1996*).

SHRs consist of a C-terminal ligand-binding domain (LBD, E/F region), a centrally located and highly conserved DNA-binding domain (DBD, C region) and an N-terminal domain (A/B region) (Fig. 1). Within these domains are at least two transcription activation subdomains or functions (AFs): AF_1 and AF_3 (if it exists) reside in the N-terminal domain (Bain *DL et al 2007*) and ligand-dependent AF_2 present within the LBD (*Nolte RT et al 1998*). The LBD and DBD of SHRs are

conserved (*Wurtz JM et al 1996*), whereas the N-terminal domain is highly variable with respect to both length and primary sequence but is important for the full transcriptional activity of receptors (*Kumar R and Thompson EB 2003*). In the absence of ligands, SHRs exist as inactive oligomeric complexes with a number of other proteins including chaperon proteins, namely the heat shock proteins Hsp90 and Hsp70 and Ppidl (cyclophilin-40) and Ptges (p23) (*Pratt WB and Toft DO 1997*). The role of Hsp90 and other chaperons may be to maintain the receptors folded in an appropriate conformation to respond rapidly to hormonal signals. Upon binding to the steroid, receptors in target cells become activated through a process that involves dissociation from protein chaperons, conformational changes, dimerization and binding to *cis*-acting hormone response elements (HREs) of target genes (Fig. 2) (*Beato M and Klug JK 2000; Edwards DP 1999; Mckenna NJ and O'malley B 2002*). To regulate transcription, SHRs are subjected to multiple post-translational modifications, such as phosphorylation. The majority of the phosphorylated residues in steroid hormone receptors locate at the N-terminal domain (NTD) (*Rochette-Egly C 2003*). The physiological role of phosphorylation is not well defined but has been suggested to be involved in mediating cross-talk with other signal transduction pathways and modulating transcriptional activity of SHRs and facilitating recruitment of coactivators (*Chen D et al 2000; Lin HK et al 2001*). SHRs are also the target of sumoylation in which a small ubiquitin-like modifier (SUMO) protein is attached to a specific lysine residue of the receptor. Sumoylation of AR is ligand-dependent, and disruption of sumoylated lysines increases the transcriptional activity of the receptor (*Poukka H et al 2000*). Lysine residues of proteins may be modified by ubiquitination and acetylation in addition to sumoylation. Polyubiquitination of AR may be important for the efficient transcription of androgen-regulated genes through proteasomal degradation, such as the prostate-specific antigen (*PSA*) gene (*Kang Z et al 2002*), while monoubiquitination of AR increases its transcriptional activity (*Burgdorf S et al 2004*). The transcriptional activity of AR may also be increased by acetylation of a specific lysine residue by coactivators EP300 (p300) and TAF5L (p300/CBP-associated factor, PCAF) (*Fu M et al 2000*).

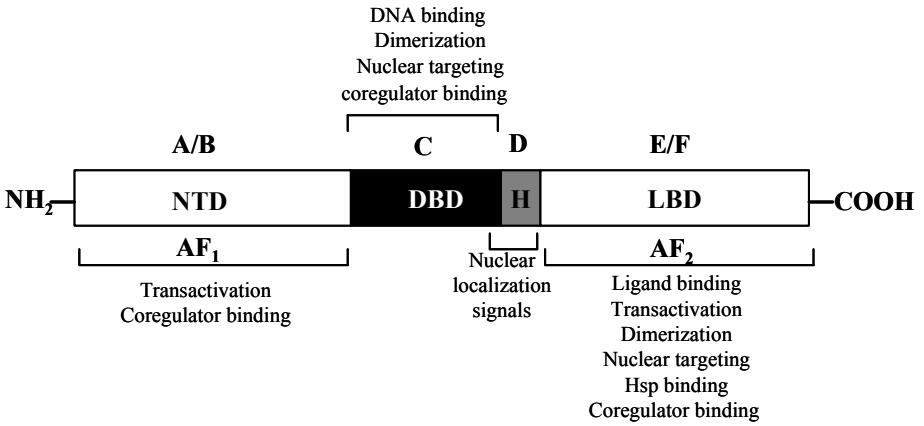


Figure 1. Domain structure of SHRs.
NTD=N-terminal domain, DBD=DNA-binding domain, H=hinge, LBD=ligand-binding domain, Hsp=heat-shock protein.

Coactivators are proteins that increase the rate of signal-specific but not basal transcription. Coactivators themselves are devoid of DNA-binding activity and are recruited through protein-protein interaction with AF_1 or AF_2 interaction surfaces of receptors and function either as enzymatic protein complexes capable of remodeling

chromatin or as protein bridging factors to facilitate assembly of the RNA polymerase II initiation complex. The function of coactivators can be divided into three classes (*McKenna NJ and O'Malley B 2002; Glass CK and Rosenfeld MG 2000; Steinmetz AC et al 2001*). Many coactivators have been found and characterized, for example, CBP/p300 proteins (*Chan HM et al 2001*), TRAP/DRIP complexes (*Acevedo ML and Kraus WL 2003*), p160 family (*Xu J and Li Q 2003*) and CARM1/PRMT1 proteins (*Strahl BD et al 2001*).

Corepressors were originally identified as proteins associated with unliganded receptors that can bind to DNA in the absence of a ligand and mediate transcriptional repression. Two of the corepressors that have been well studied, N-CoR and SMRT, interact with the C-terminal region at the SHRs (*Li H et al 1997*), recruit histone deacetylase, and convert the chromatin to an inactive form (*Fischle W et al 2002*). Additionally, RIP140 was also identified as a corepressor (*Lee CH and Wei LN 1999*), and competes with coactivators for the same binding site (*Treuter E et al 1998*) (Fig. 2).

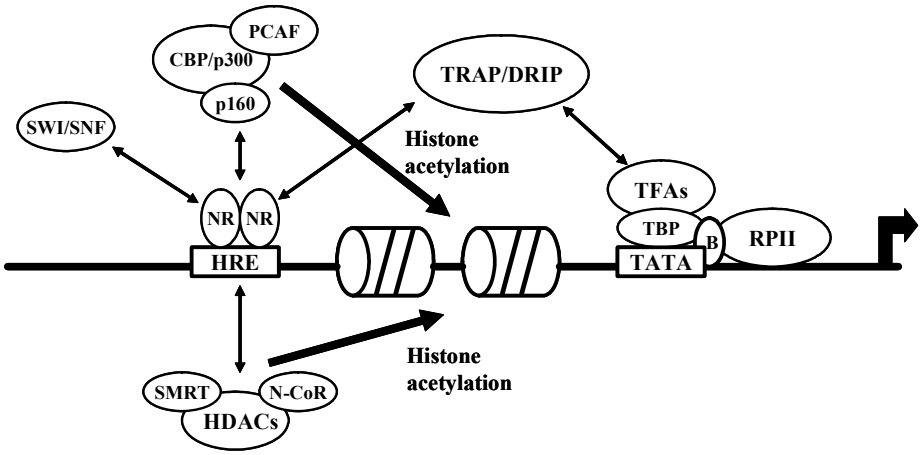


Figure 2. A model for sex hormone receptor (SHR)-dependent transcriptional activation.

HRE=hormone response element, TATA=TATA box, TBP=TATA box-binding protein, TAFs=TBP-associated factors, B=transcription factor B, RPII=RNA polymerase II. NR-dependent transcription is modulated by complexs containing chromatin remodeling (SWI/SNF), histone acetylation (p160/CBP/p300/PCAF), histone deacetylation (SMRT/N-CoR/HDACs), and coactivators (TRAP/DRIP).

The PIAS protein family consists of four members identified to date: PIAS1, PIAS3, PIASx (two splicing variants PIASxαARIP3 and PIASxβ) and PIASy (*Shuai K 2000*). The PIAS proteins modulated the transcription mediated by several nuclear receptors including the AR (*Kotaja N et al 2000*). The PIAS1 and PIASxα can act as SUMO-1 ligases in the sumoylation of AR repressing AR-dependent transcription (*Nishida T and Yasuda H 2002*). In turn, mutations in the receptor sites for the SUMO-1 enhanced the transcriptional activity of the AR (*Poukka H et al 2000*). It is possible that the sumoylation of the AR by the PIAS proteins may alter the association of the receptor with other coregulators. The effects of PIAS1 and PIASxα on AR-dependent transcription are dependent on their SUMO-binding motif and the ligase domain. Additionally, PIAS proteins have been reported to inhibit or enhance the

transcriptional activity of AR by independent sumoylation modification (*Nishida T and Yasuda H 2002; Takahashi K et al 2001*).

2.3 Overview of sex steroid metabolism

2.3.1 Pathway of sex steroid biosynthesis in classical steroidogenic tissues

Both estrogens and androgens use a common pathway of sex steroid hormone biosynthesis. The three main steroid hormones are namely C-18 estrogens, C-19 androgens and C-21 progesterones. The biochemical pathways are similar in all three main classical endocrine organs, namely the adrenals, ovaries and testes (Fig. 3). In the synthesis of P, estrogens and androgens, cholesterol is used as a precursor. Additionally, androgens are the obligate precursors of estrogens. Cholesterol can be synthesized *de novo* from acetate by steroidogenic cells, but most of their supply of cholesterol comes from plasma LDLs (*Miller WL 2002a*). Conversion of cholesterol to pregnenolone in mitochondria is the first, rate-limiting and hormonally regulated step in the synthesis of all steroid hormones (*Miller WL 1995*). The rate of formation of pregnenolone is determined by multiple factors including delivery of cholesterol to the mitochondria and the amount and catalytic activity of the cholesterol side-chain cleavage enzyme (CYP11A1). Steroid synthesis is regulated both acutely and chronically. For example, in adrenals, ACTH chronically regulates the amount of the transcription of genes encoding various steroidogenic enzymes and cofactor proteins (*Hum DW and Miller WL 1993*) but acute regulation, such as in the response of sex steroids to LH, cortisol to ACTH and aldosterone to angiotensin II, is at the level of cholesterol access to steroidogenic enzymes (*Stocco DM and Clark BJ 1996*). Steroidogenic acute regulator (STAR), which is needed for rapid and maximal steroidogenic response, appears to play a pivotal role in the translocation of cholesterol in the outer mitochondrial membranes to inner mitochondrial membranes (*Lin D et al 1995; Hasegawa T et al 2000*). Additionally, there are also low levels of STAR-independent steroidogenesis (*Lin D et al 1995*).

Enzymes involved in steroid biosynthesis and metabolism can be classified into two broad groups: the cytochrome P450s and oxidoreductases. Cytochrome P450s are heme-containing proteins with a characteristic light absorption maximum at 450 nm in the presence of carbon monoxide. The second group of steroid-metabolizing enzymes can be further divided into two families based on their structures and functions, namely the short chain dehydrogenase/reductase (SDR) and aldo-keto reductase (AKR) families (*Miller WL 2002a; 2002b*). Cholesterol is converted to pregnenolone by the side-chain cleavage enzyme (CYP11A1). Pregnenolone is then catalyzed to P by HSD3B (*Simard J et al 1996*). CYP17A1 first converts P and pregnenolone to hydroxy forms, and then catalyzes the formation of DHEA and $\triangle^4$-dione by its lyase activity. Thereafter, $\triangle^4$-dione can be converted to T or E_1 (*Miller WL et al 1997*). However, human CYP17A1 only utilizes $\triangle^5$-substrates for 17, 20-bond cleavage (Fig. 3). The two final steps in E_2 biosynthesis are catalyzed by CYP19A1 ($\triangle^4$-dione to E_1 or T to E_2, *Simpson ER et al 1997*) or by HSD17B1 (E_1 to E_2, *Poutanen M et al 1995*). The synthesis of T from $\triangle^4$-dione especially in the testes is catalyzed by testis-specific HSD17B, HSD17B3 *(Geissler WM et al 1994*) and by HSD17B5 in the ovaries (*Dufort I et al 1999*) separately. Conversion of T to its more potent derivate DHT is catalyzed by SRD5A (*Andersson S et al 1989*). In the human adrenal cortex, microsomal CYP21A2 enzyme performs the 21-hydroxylation of the $\triangle^4$-steroids, P and 17α-hydroxyprogesterone, an essential step in the biosynthesis of both mineralocorticoids and glucocorticoids (*Miller WL 2002b*). Two closely related enzymes, CYP11B1 and CYP11B2, catalyze the final synthesis of both mineralocorticoids and glucocorticoids. CYP11B1 possess 11β-hydroxylase activity

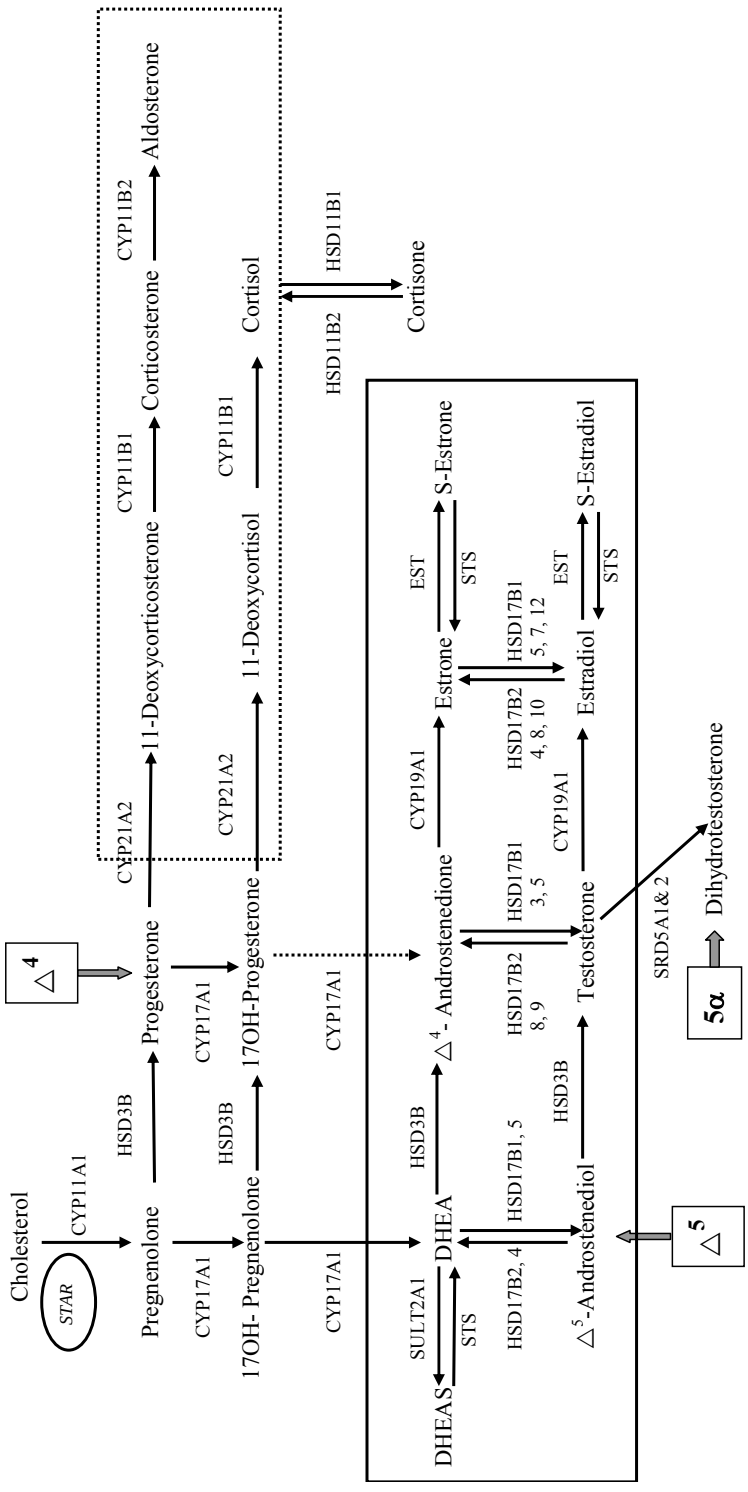


Figure 3. Major human classical and peripheral steroidogenic pathways.
Key enzymes are shown near the *arrows* indicating chemical reactions. The steroids in the first column are $\triangle^5$-steroids, which constitute the preferred pathway to C_{19} steroids in human beings. Steroids in the second column and farther right are $\triangle^4$-steroids, except the C_{18} estrogens and 5α-reduced steroids. The chemical reactions in the box of dotted line are observed in the human adrenal and the chemical reactions in the box of solid line are shown in the peripheral target tissues.

and CYP11B2 performs the two oxygenations at C18 required for aldosterone biosynthesis (*Lin L and Achermann JC 2004*) (Table 2).

2.3.2 Pathway of sex steroid synthesis in peripheral tissues

It is remarkable that humans, in addition to possessing a highly sophisticated endocrine system, possess significant sex steroid formation also in the peripheral tissues. These locally produced bioactive androgens and estrogens exert their action in the cell without release into the extracellular space (*Labrie F 1991*). The biosynthesis of sex steroids in the peripheral target tissues is dependent on the substrate supply and specific hormone activating and inactivating enzymes (Table 2) (*Suzuki T et al 2005*). The adrenals of humans and some other primates secrete large amounts of $\triangle^4$-dione, DHEA and DHEAS, this being the most abundant C_{19}-steroid circulating in human plasma (*Labrie F et al 1995; Rainey WE et al 2002*). These circulating adrenal precursors provide a substrate reservoir for the biosynthetic enzymes, which modulate local peripheral levels of sex steroids. The biochemical pathways in the formation of E_2 from DHEA and $\triangle^4$-dione are the same as found in the classical steroidogenic tissues (Fig. 3).

Instead of activation, most of the steroid-modifying enzymes in peripheral cells inactivate the steroid ligand in order either to protect the cell from unwanted steroid influence or to provide an aftercare function after completion of hormone action (*Chatterjee B et al 1994*). Two groups of enzymes are currently implicated in carrying out much of this inactivation function, one acting through oxido-reduction (e.g. HSD17B2; *Wu L et al 1993*) and the other through steroid sulfurylation (e.g. EST and DST, *Strott CA 1996; Weinshilboum RM et al 1997*). Additionally, STS can hydrolyze several sulphated steroids and regulate local sex steroid production (*Reed MJ et al 2005*).

2.3.3 Sex steroid metabolism

Major catabolic products of estrogens are the catechol estrogens, primarily 2-hydroxyestrogens but also to some extent 4-hydroxyestrogens. These generally appear in the urine as glucuronosides. A certain degree of methylation also occurs to give the 2-methoxy derivatives. The liver is the major site of 2-hydroxylation and also of glucuronidation. In addition to 2-hydroxylation, C_{18} steroids taken up by the liver can be hydroxylated to form 16α-hydroxylated derivatives. The ratio of 2α-hydroxylated to 16α-hydroxylated derivatives varies widely in a number of different physiological states. Much of the estrogen cleared by the liver ends up as diconjugates with sulfate and glucuronate. These can be secreted into the bile and undergo enterohepatic circulation. The kidneys serve essentially no role in the metabolism of estrogens, rather, it serves simply as a means of clearing the estrogenic metabolites from the blood and excreting them into urine (*Ackerman GE and Carr BR 2002 and refs. therein*). T can be metabolized by two different pathways, metabolism via the first pathway occurs in many tissues including the liver, and produces 17-ketosteroids as steroid sulfates and glucuronides, which are excreted in the urine and bile, while metabolism via a second pathway occurs primarily in target tissues and produces the potent metabolite DHT. These multiple metabolic pathways determine how fast T is cleared from the blood. P in human has several metabolic fates in addition to its conversion to androgens (*MacDonald PC et al 1991 and refs therein*). 5α- and 5β-reduction pathways are probably responsible for the most significant fraction of P metabolism. 5α- and 5β-reduction of P, both accounting for 85% of P, mainly takes place in the liver and 5α-reduction of P also takes place in extrahepatic sites. After 3α- and 20α-reduction, 5α-reduced metabolites are principally conjugated as sulfates, secreted into bile, and ultimately excreted in the feces, while 5β-reduced metabolites

Table 2. Key Human Steroidogenic Enzymes

Protein	Gene	Gene Size (Kb)	Chromosomal Locus	Location	Principal Substrates	Major Activities	Deficiency Syndromes
CYP11A1 (P450scc)	*CYP11A1*	>20	15q23-q24	ZG/ZF/ZR, gonads (L, T), placenta, brain	Cholesterol, hydroxysterols	22R-hydroxylase 20R-hydroxylase 20,22-lyase	Mild lipoid CAH in heterozygotes
CYP17A1 (P450c17)	*CYP17A1*	6.6	10q24.3	ZF/ZR gonads (L, T), brain	Preg, 17OH-preg prog, (17OH-prog) 5α-reduced C_{21} steroids	17α-hydroxylase 17, 20-lyase [16α-hydroxylase] [Δ^{16}-synthase]	17-hydroxylase deficiency isolated 17, 20-lyase deficiency
CYP21A2 (P450c21B)	*CYP21A2*	3.4	6p21.1	ZG/ZF/ZR	Prog, 17OH-prog	21-hydroxylase	21-hydroxylase deficiency
CYP11B1 (P450c11)	*CYP11B1*	9.5	8q21-q22	ZF/ZR, brain	11-deoxycortisol 11-DOC	11β-hydroxylase [18-hydroxylase]	11-hydroxylase deficiency
CYP11B2 (P-450c18)	*CYP11B2*	9.5	8q21-q22	ZG, brain, heart	Corticosterone 11-DOC, 18OH-corticosterone	11β-hydroxylase 18-hydroxylase 18-oxidase	CMO I deficiency CMO II deficiency
CYP19A1 (P-450arom)	*CYP19A1*	>52	15q21.1	Gonads L, G), placenta, brain, bone, fat	Δ^4-dione, T	19-hydroxylase 19-oxidase, aromatization	Aromatase deficiency
HSD3B1 (3β-HSD1)	*HSD3B1*	7.8	1p13.1	Placenta, liver, brain	Preg, 17OH-preg DHEA, Δ^5-diol	3β-dehydrogenase $\Delta^{5/4}$-isomerase	Not described (presumed lethal)
HSD3B2 (3β-HSD2)	*HSD3B2*	7.8	1p13.1	ZG/ZF>ZR, gonads (L, T)	Preg, 17OH-preg DHEA, Δ^5-diol	3β-dehydrogenase $\Delta^{5/4}$-isomerase	3β-HSD deficiency
HSD17B1 (17β-HSD1)	*HSD17B1*	3.3	17q21.2	Gonad (G), placenta, breast	E_1, [DHEA]	17β-ketosteroid reductase	Not described
HSD17B2 (17β-HSD2)	*HSD17B2*	>40	16q23.2	Endometrium, broadly	T, E_2, DHT	17β-hydroxysteroid dehydrogenase	Not described
HSD17B3 (17β-HSD3)	*HSD17B3*	>60	9q22	Gonad (L)	Δ^4-dione, A-dione, ADT, [DHEA]	17β-ketosteroid reductase	17-ketosteroid reductase deficiency
AKR1 C1-4 (3α-HSDs)	*AKR1C1-4*	13-25 each	10p14-p15	Liver, broadly	DHT, A-dione, 5α-reduced C_{21} steroids [17β-HSD: DHEA, Δ^4-dione, ADT]	3α-ketosteroid reductase 17β-ketosteroid reductase	Not described
SRD5A1 (5α-Reductase1)	*SRD5A1*	>35	5p15	Liver, brain, skin	T, Δ^4/C_{21}-steroids	5α-reductase	Not described
SRD5A2 (5α-Reductase2)	*SRD5A2*	>35	2p23	Prostate, genital skin	T, Δ^4/C_{21}-steroids	5α-reductase	5α-reductase deficiency
HSD11B1 (11β-HSD1)	*HSD11B1*	9	1q32.2	Liver, brain, placenta, fat, broadly	Cortisone, 11-Dehydro-Corticosterone [products]	11β-ketosteroid reductase	Cortisone reductase deficiency (plus H6PDH mutations)
HSD11B2 (11β-HSD2)	*HSD11B2*	6.2	16q22	Kidney, gut, placenta	Cortisol, Corticosterone	11β-hydroxysteroid dehydrogenase	Syndrome of apparent mineralocorticoid excess
STAR (STARD1)	*STAR*	8	8p11.2	ZF/ZG/ZR, gonad (L, T)	cholesterol flux within mitochondria	Sterol delivery to P450scc	Lipoid CAH

ZG/ZF/ZR=adrenal zona glomerulosa/fasciculata/reticularis, respectively, L: Leydig cells, T: theca cells, G: granulosa cells, 17OH-preg: 17α-hydroxy pregnenolone, 17OH-prog: 17α-hydroxyprogesterone, DOC: deoxycorticosterone, CAH: congenital adrenal hyperplasia, CMO: corticosterone methyl oxidase

are conjugated as glucuronides, released into the blood, and rapidly cleared into the urine, mostly as pregnanediol glucuronides. On the other hand, P is hydroxylated at carbons 3α, 6β, 16α, 17α, 20α and 21α of the steroid nucleus in the liver and various target tissues (*MacDonald PC et al 1991 and refs therein*). It is also secreted as hydroxylated derivatives by the ovaries and adrenal cortex.

2.3.4 Sex steroid metabolism in the *feto-placental* unit

The placenta plays an important role in metabolism involving the embryo and the maternal compartment. It transports nutrients from the maternal circulation to the fetus, and excretes fetal metabolites crossing into the maternal compartment. In human and primates, placental tissue is known to be capable of producing large amounts of P, E_2 and E_3. Within the syncytiotrophoblast, P is primarily synthesized

from maternal cholesterol through the $\triangle^4$-pathway (Fig. 3). Free cholesterol is mobilized into mitochondria, a rate-limiting step of steroidogenesis. However, STAR is not expressed in the placenta (*Pezzi V et al 2003*) and cholesterol must enter the mitochondria via a STAR-independent pathway (*Bose HS et al 2000*). Cholesterol serves as the substrate for placental CYP11A1 and HSD3B1 to become P (*Pasqualini JR 2005*). The biosynthesis of estrogens during pregnancy involves a complex interplay between the placenta and the fetal adrenal glands. The placenta is capable of carrying out the terminal steps in estrogen synthesis, but is incapable of producing androgens because it does not express CYP17A1 (*Pezzi V et al 2003*). Thus, the immediate androgen precursors of placental estrogens, DHEA and DHEAS, are provided mainly by the fetal adrenal cortex (*Strauss JE 3rd et al 1996*). DHEA is converted to DHEAS by the abundant steroid sulfotransferase activity of the fetal adrenal glands (*Pasqualini JR 2005*). The resulting sulfated compound cannot serve as a substrate for HSD3B2 in the fetal adrenal glands and is secreted into the fetal circulation, where it undergoes 16α-hydroxylation in the fetal liver. Only a small fraction of DHEAS secreted by the adrenal cortex escapes 16α-hydroxylation in the fetal liver. 16α-hydroxy-DHEAS serves as a major substrate for placental sulfatase, HSD3B1, HSD17B1 and CYP19A1 to become E_3. Much smaller amounts of DHEAS reach the placenta without undergoing 16α-hydroxylation in the fetal liver, and are converted by HSD3B1, HSD17B, and CYP19A1 into E_2 or by HSD3B1 and CYP19A1 (Table 2) into E_1 (*Pasqualini JR 2005*). E_3 further travels via the placental circulation to the maternal liver, where it is conjugated to glucuronides, and then secreted into urine.

2.4 Sex steroid target tissues

2.4.1 Ovary

Estrogens have been acknowledged to possess local intrafollicular actions (*Palter SF et al 2001*). Although estrogens are produced in small antral follicles, estrogens are not essential for the growth of follicles to the size equivalent to the preovulatory stage. However, negative selection against subordinate follicles is a result of estrogens produced by a dominant follicle exerting negative feedback upon FSH release and inducing LH receptor expression. Furthermore, the deprivation of adequate FSH stimulation is required for survival of the remaining growing antral follicles (*McGee EA et al 2000 and refs therein*). On the other hand, estrogens appear to protect the growing follicle from androgen-induced atresia because atretic follicles are characterized by a low follicular fluid estrogen milieu. Inhibition of steroidogenic enzymes or anti-estrogen treatment reduced oocyte maturation and fertilization (*Filicori M 1999 and refs therein*). Estrogens also have pleiotropic effects on granulosa cells and the intercellular gap junctions and antrum formation. E_2 actually provides a negative control on FSH and LH secretion during the main part of the cycle (*Chabbert-Buffet N and Bouchard P 2002*). However, in the antrum of the preovulatory follicle, an extremely high level of E_2 is reached. ESR2 (ERβ) is the predominant form in the ovaries (*Drummond AE et al 1999*). Recent studies have assigned distinct roles for each receptor. Esr1 (ERα) mediated action induces ovulation, most likely via an effect on the hypothalamo-pituitary axis, while Esr2 is involved in the stimulation of follicular growth, decreases atresia, and enhances the number of oocytes released following ovulation induction in rodents (*Hegele-Hartung C et al 2004*).

In recent years, *Esr* knockout mice have been generated in an effort to define the role of estrogen action in the ovaries. Female *Esr1*KO mice are acyclic, and infertile. Folliculogenesis is arrested at the antral stage with large follicles becoming cystic and

haemorrhagic (*Lubahn DB et al 1993*). The female *Esr2*KO mice (*Krege JH et al 1998; Emmen JM et al 2005*) have small ovaries, partially arrested follicular development associated with increased numbers of primordial follicles, but significantly fewer numbers of primary and large antral follicles and corpora lutea. Increased atresia of large follicles is evident. The analysis of *Esr1/2*KO (*Couse JF et al 1999c; Dupont S et al 2000*) showed that their ovarian phenotype is distinct from the individual *Esr* knockouts. Thus, it appears that both Esrs have different roles in the maintenance of fertility.

An alternative model to *Esr* depletion is to remove estrogen itself from the ovaries. The *Cyp19a1*KO model has allowed us to define how far follicles can grow in the total absence of estrogens (*Fisher CR et al 1998; Britt KL et al 2000*). These mice were infertile due to an inability to ovulate. The antral follicles appeared morphologically atretic or prematurely luteinized. The phenotype is more severe at older age, containing increasing numbers of morphologically abnormal follicles and haemorraghic cysts (*Britt KL et al 2001*).

Androgens may exert both positive and negative effects on follicular growth and function in a stage-dependent manner. In the early stages of folliculogenesis, androgens appear to promote follicular growth. Administration of androgens leads to decreased number of primordial follicles and enhanced number of follicles at other stages of development (*Steckler T et al 2005*). An Ar antagonist (Casodex), but not a Cyp19a1 inhibitor, inhibited the growth response, indicating the direct stimulatory effect of androgens on mouse follicle growth *in vitro* (*Murray AA et al 1998*). However, androgens have also been reported to inhibit LH receptor expression by granulosa cells and to modulate granulosa cell apoptosis (*Billig H et al 1993*). Treating mice with DHT reduced the number of large follicles and left the mice subfertile (*Nandedkar TD and Munshi SR 1981*). Apart from effects on follicular growth, androgens have been shown to enhance the FSH-mediated differentiation of granulosa cells, as indicated by an increase in P and E_2 production, and to play a role in oocyte maturation (*Vendola K et al 1999*). Despite the apparent roles for androgens in the ovaries, studies on the testicular feminised mouse (Tfm), which lack functional Ar, indicate that Ar is not essential for female fertility. The reproductive lifespan of these mice, however, was reduced due to accelerated aging of the ovaries and they were subfertile (*Lyon MF and Glenister PH 1980*). The development of an *Ar* conditional knockout has further studied on androgen action in the ovaries. Female *Ar* knockout mice have a reduced fertility, evident in fewer litters and reduced numbers of pups. Their ovaries contain normal numbers of follicles, but large antral follicles appeared to have fewer granulosa cells, and reduced numbers of corpora lutea have been observed (*Hu YC et al 2004*).

The ovulatory process is P-dependent, and inhibitors of P synthesis have been shown to block LH-induced ovulation *in vivo* and *in vitro* (*Zalanyi S 2001*). Additionally, the direct effects on granulosa cell function by P have been reported (*Drummond AE 2006*). The importance of Pgr in female reproduction is proven by the infertility of *Pgr* null mice (*Lydon JP et al 1995*). Despite histologically normal ovaries, these mice failed to ovulate even after exogenous stimulation with gonadotrophins (*Lydon JP et al 1995*). Interestingly, *Pgr-A* knockouts were infertile, as a result of a failure to ovulate (*Mulac-Jericevic B et al 2000*), whereas *Pgr-B* knockouts were ovulatory and produced viable offspring (*Mulac-Jericevic B et al 2003*), further highlighting the ability of *Pgr-A* and *Pgr-B* to mediate different actions of P (*Mulac-Jericevic B et al 2004*).

2.4.2 Testis

Spermatogenesis has been thought to be dependent on high local T concentration. Furthermore, *Ar*KO male mice had a female-like appearance, undescended testes and

spermatogenesis arrested at the pachytene spermatocyte stage (*Yeh S et al 2002*). However, the study with *Lhcgr*KO male mice suggested that at least in mouse, spermatogenesis was maintained at a low intratesticular T concentration. In young adult mice, the spermatogenesis arrested at round spermatid, but in old mice even elongated spermatids were identified (*Zhang FP et al 2003*). Ar was detected in Leydig cells, peritublar myoid cells and in Sertoli cells in a stage-dependent manner. The germ cells did not express Ar (*Zhou Q et al 2002*), suggesting that the androgens had an indirect effect on the germ cells through the Sertoli cells. The study using the *hpg* mouse model and morphometric techniques proposed that the most T-sensitive step was the conversion of spermatocytes to spermatids (*Singh J et al 1995*). Several studies showed that the mechanism by which T influences round spermatids involves the production of N-cadherin by the Sertoli cells. N-cadherin was disrupted when T levels declined, and further disturbed the specialized spermatid-Sertoli cell junctions (*McLachlan RI et al 2002*). Furthermore, the concept that T was an essential requirement for the normal formation and maturation of spermatocytes and subsequently the supply of early spermatids had been confirmed by the studies of mutant mice with selective knock out of *Ar* in Sertoli cells. Spermatogenesis was arrested at the late spermatocyte-early spermatids stage (*De Gendat K et al 2004*). The data suggested that the actions of T on Sertoli cells, through their Ars, were crucial for normal meiotic maturation of spermatocytes, the failure of which resulted in depletion of spermatids.

The testes are capable of synthesizing and responding to estrogens throughout all stages of development. The expression of ESR1, ESR2 and CYP19A1 in the adult testes is well characterized (*O'Donnell L et al 2001 and refs therein*). The localization of ESR1, ESR2 and CYP19A1 demonstrates that estrogen action is likely to be important for the Leydig cell, Sertoli cell, and germ cell development and function. In particular, germ cells are capable of local estrogen synthesis and response, via ESR2 which is the predominant, and potentially the only ESR in germ cells, suggesting that the paracrine and intracrine actions of estrogens may be important in male germ cell development. Therefore, the studies provide evidence of a stimulatory role for estrogen in germ cell development including spermatogonial division (*Miura T et al 1999*), germ cell viability and differentiation (*Ebling FJ et al 2000*), acrosome biogenesis, and function of spermatozoa (*Revelli A et al 1998*). Notable roles for estrogens in Leydig cells include the coordinated regulation of progenitor Leydig cell development and the control of adult Leydig cell steroidogenesis in a paracrine manner (*Abney TO 1999 and refs therein*). Estrogens also play a role in testicular descent, at least in part. *In utero* estrogen overexposure causes a decrease in transcription of *INSL3* in fetal Leydig cells, leading to cryptorchidism and deleterious effects on fertility (*Nef S et al 2000*). Moreover, transgenic male mice overexpressing human *CYP19A1* presented a multitude of severe structural and functional alterations in the reproductive organs, such as cryptochidism associated with Leydig cell hyperplasia, dysmorphic seminiferous tubules, and disrupted spermatogenesis (*Li X et al 2001*). Estrogens may participate in the regulation of the FSH-mediated mitogenic activity in Sertoli cell division, yet a negative effect on Sertoli cell maturation (*Sharpe RM et al 1998b*).

Although spermatogenesis was disrupted in the *Esr1*KO mice (*Eddy EM et al 1996*), it seems likely that the spermatogenesis phenotype in the *Esr1*KO mice is primarily due to an indirect effect via efferent ductule dysfunction, rather than a direct effect on spermatogenesis (*Hess RA et al 1997; 2000*). The transplantation of *Esr1*KO germ cells to WT mice depleted of germ cells demonstrated that *Esr1*-deficient germ cells could develop in an environment in which Cyp19a1 and Esrs are present (*Mahato D et al 2000*). This is consistent with the absence of Esr1 expression in the germ cells. *Esr2KO* mice lack any apparent spermatogenic phenotype (*Krege JH et al 1998;*

Dupont S et al 2000). This is surprising as Esr2 appears to be the predominant and more widely expressed Esr in the testes. The absence of spermatogenic disruption in these mice could perhaps be due, in part, to an unknown compensation *in vivo*. The recent study showed that Esr2 selective agonist 3β-diol, but not the potent androgen DHT, was able to stimulate DNA synthesis by spermatogonia in cultures of microdissected stage I segments of rat seminiferous tubules *in vitro*. 3β-diol was reported to selectively bind Esr2 rather than Esr1, but not Ar, suggesting that 3β-diol may exert the stimulatory effects on premitotic DNA synthesis in connection with spermatogenesis via an Esr2-dependent pathway as a growth factor (*Wahlgren A et al 2008 and refs therein*). Three different lines of *Cyp19a1*KO mice have been generated (*Toda K et al 2001; Honda S et al 1998; Robertson KM et al 1999*). However, fertility in these mice is variable and normal spermatogenesis has been observed in these male mice at 14 weeks. Only one of the lines showed decreased fertility after the age of 18 weeks (*Robertson KM et al 1999*), and this was related to the decrease in the numbers of round and elongated spermatids in the testes. The reason for the heterogeneity in the phenotypes of different *Cyp19a1*KO mice is unclear.

2.4.3 Mammary gland

Mammary gland development in humans occurs during fetal life. Further development of the mammary glands becomes hormone dependent and continues at the onset of puberty. The mammary glands only become completely differentiated during pregnancy (*Hennighausen L and Robinson GW 2001; 2005 and refs therein*). Ovariectomy in mice causes regression of the alveoli and lobuloalveolar ducts, and while estrogens alone cause only ductal growth, estrogens and P administration after ovariectomy restore lobuloalveolar development and ductal branching. Moreover, it has been suggested that P has effects on alveolar development that are independent of estrogens. In other studies, direct actions of estrogens on mammary gland end bud proliferation and ductal growth have been reported while the end buds of ductal tips and lateral branching of the ductal tree were markedly suppressed in ovariectomized mice by implanting antiestrogen directly into the mammary glands (*Pepe GJ and Albrecht ED 1995 and refs therein*). Genetic approaches have further indicated that estrogens are required for normal ductal elongation and outgrowth during puberty. By contrast, estrogens are dispensable for pregnancy-mediated alveolar expansion, whereas in the absence of P, alveolar development during pregnancy is completely perturbed (*Hennighausen L and Robinson GW 2001; 2005 and refs therein*).

2.4.4 Epididymis

A role for androgens in the regulation of epididymal function is well known (*Robaire B and Viger RS 1995*). Epididymal histology, intermediate metabolism, ion transport, synthesis and secretion of a number of epididymal proteins and the activity of certain enzymes are under the control of androgens. Furthermore, transport of sperm, acquisition of fertilizing capacity and storage of spermatozoa are dependent on androgens (*Orgebin-Crist MC, 1996*). Androgenic control is mainly mediated by DHT and probably not by T (*Robaire B and Viger RS 1995*). Srd5a enzyme converting T to DHT exhibits a gradient expression of patterns: high levels in the intial segment and decreases in more distal segments (*Viger RS and Robaire B 1996*), while Ar is expressed in all the epididymal regions (*Zhou Q et al 2002*). Many genes are differentially regulated by androgens in different epididymal regions (*Ezer N and Roaire B 2003*). Moreover, the genome-wide profiling of endocrine regulation of genes expressed in the initial segment (IS) and distal caput of mouse epididymides was performed with microarray. The data revealed that of the 15 020 genes expressed in the epididymis, 35% were enriched in one of the two regions. The data, furthermore, showed that 27% of the genes expressed in the IS and/or distal caput epididymidis are

under the regulation of testicular factors present in the duct fluid, while bloodborne androgens can regulate for 14% of them. (*Sipilä P et al 2006*).
Although many studies indicate that estrogens have an essential role for the development and maintenance of the efferent ductules and epididymides (*O'Donnell L et al 2001 and refs therein*), the first major insights into the mechanism of estrogen action in the efferent ductules and epididymides, and requirement for estrogens in male fertility, were gained from the *Esr1*KO mice (*Hess RA et al 1997*). The absence of Esr1 causes defects in efferent ductule development, resulting in disturbed function, particularly in terms of fluid resorption, causing a reduced number of sperm to enter the epididymides. A consequence of the disturbed fluid dynamics causes a buildup of fluid in the testes, resulting in seminiferous epithelial damage and impaired germ cell development. The fact that overexposure to estrogens during neonatal development can also produce similar defects in efferent ductile function highlights the need for a tightly coordinated series estrogen-dependent events in this tissue (*O'Donnell L et al 2001*).

2.4.5 Prostate

Androgens are pivotal regulators of the development, morphogenesis, growth and terminal differentiation of the prostate (*Marker PC et al 2003*). The expression of hundreds of genes encoding prostate proteins is regulated by androgens. Furthermore, transgenic mice overexpressing *AR* in the prostate epithelium present with increased epithelial proliferation and develop prostatic intraepithelial neoplasia (*Stanbrough M et al 2001*), implicating androgens in hyperplastic and neoplastic diseases of the prostate (*Bartsch G et al 2002; Porkka KP and Visakorpi T 2004*). The major androgen of the prostate is DHT (*Steers WD 2001*). Compelling evidence of the critical importance of DHT for the growth of the prostate gland was acquired from studies of male pseudohermaphroditism. These individuals possessed a genetic mutation of the SRD5A2 causing a deficit in its enzymatic activity manifested as ambiguous genitalia at birth, characterized by pseudovaginal perineoscrotal hypospadias with fully differentiated testes and a normal internal male ductal system. Although virilization occurred at puberty, the prostate remained rudimentary (*Steers WD 2001; Andersson S et al 1991*).
Formation of squamous epithelial metaplasia is a classical effect of estrogens on the prostate with decreasing androgen levels and contributes to the genesis of subsequent prostate cancer (*Härkönen PL and Mäkelä SI 2004; Raghow S et al 2002*). Impaired estrogen action can also lead to structural and functional abnormalities in the prostatic epithelium, as has been demonstrated in the *Esr2*KO or *Cyp19a1*KO mice (*Weihua Z et al 2001; McPherson SJ et al 2001*). The effect of estrogens on the prostate may be indirect and mediated by the inhibition of androgen secretion, or direct action mediated via ESRs in the prostate. ESR1 is expressed in the stromal cells of the prostate *(Leav I et al 2001*). The expression of ESR2 has been demonstrated in both the stroma and epithelial cells of the human prostate (*Horvath LG et al 2001*). *Esr2*KO mice develop prostatic hyperplasia spontaneously and have an increase in the expression of the Ar and proliferation markers in the prostate epithelium (*Weihua Z et al 2001*). It was thus proposed that Esr2 is a negative regulator of Ar in the rodent prostate. The expression of ESR2 is decreased in prostate cancer compared to normal tissue (*Leav I et al 2001; Horvath LG et al 2001*). Moreover, the study with *Lhcgr*KO male mice showed that in the presence of sufficient androgenic stimulation, Esr1-mediated signalling promotes epithelial overgrowth, whereas Esr2-mediated signalling protects the prostate against hyperplastic changes (*Savolainen S et al 2007*).

2.5 Physiological function of estrogens and androgens in the bone

The pubertal growth spurt of both sexes is driven primarily by estrogens (*Frank GR 2003*). In women, decreased circulating estrogens after ovariectomy or menopause results in an increase in bone turnover and a negative bone remodeling balance (*Bilezikian JP 2002*), leading to bone loss and an increased fracture risk. Osteoprosis in estrogen deficient females can be prevented or rescued following treatment with estrogens (*Vedi S et al 1996*). Compelling data confirmed that estrogens are also important in the maintance of the male skeleton (*Szulc P 2001*). These observations have shown that age-associated decline in male bone mineral density is more directly related to declining estrogen levels than to declining androgen levels and the control of bone turnover in older men is predominantly under the control of estrogens.

Serum androgen levels in females are positively correlated with bone density at pre- and post-menopause and bone mineral density is decreased in females with androgen-insensitivity syndrome, despite appropriate treatment with estrogen (*Munoz-Torres M et al 1995*). Moreover, the combining T with estrogen in replacement therapy in normal postmenopausal women has been reported to result in higher bone mineral density than by treating the women with estrogen alone (*Tobias JH et al 1999*). Furthermore, T supplementation was found to increase bone mineral density in eugonadal osteoporotic men (*Francis RM 1999*). The effects of androgens on bone growth are manifested particularly by an effect on bone size and males have both larger bones and thicker cortices than females in various species (*Kasra M and Grynpas MD 1995*). In experimental animals, the skeletal effects of castration in the male animal can be prevented by the administration of both T and DHT, indicating that aromatization of androgens to estrogen could not be totally responsible for androgenic skeletal effects (*Sömjen D et al 1994*).

2.6 Activation and inactivation of sex steroids in the peripheral tissues

2.6.1 Intracrinology

An important finding in the field of sex steroids is that a large proportion of androgens and estrogens in men and women are synthesized locally in peripheral target tissues from the inactive adrenal precursors DHEA and DHEA-S (*Labrie F 1991*). Proof of androgen production in the peripheral tissues was obtained by studies showing that approximately 50% of DHT was left in the prostate in men who had their testes removed or had complete blockade of testicular androgen secretion (*Labrie F et al 1996*). Thus, it is expected that 50% of androgens in men, 75% of estrogens in women before the menopause and almost 100% of estrogens after the menopause are synthesized in peripheral hormone-target tissues from abundantly present circulating precursor steroids (*Labrie F et al 2003*). Humans are unique, with some other primates, in having adrenals that secrete large amounts of the precursor steroids DHEA and DHEA-S. Adrenal secretion of DHEA and DHEA-S increases during adrenarche in children at the ages of six–eight years, and maximal values of circulating DHEA-S are reached between the ages of 20 and 30 years, thereafter, followed by an age-dependent decline (*Rainey WE et al 2002*). Transformation of the adrenal precursor steroids of DHEA-S and DHEA into androgens and/or estrogens in peripheral target tissues depends upon the level of expression of the various steroidogenic and metabolizing enzymes in each of these tissues. In fact, steroid sulfatase (STS), several HSDs, CYP19A1 and SRD5As are all presented in peripheral tissues. Genomic studies are in progress to determine and identify all the families of steroidogenic enzymes in the various peripheral target tissues (*Labrie F et al 2003 and refs therein*).

The 70–95% reduction in the formation of DHEA and DHEA-S by the adrenals during aging also results in a dramatic reduction in the formation of androgens and

estrogens in peripheral target tissues. Such a marked decrease in the formation of sex steroids in peripheral tissues could well be involved in the pathogenesis of diseases associated with aging.

The term intracrinology was coined to describe the synthesis of active steroids in peripheral target tissues in which the action is exerted in the same cells where synthesis takes place without release of the active steroids in the extracellular space and general circulation (*Labrie C et al 1988*). This phenomenon is different from autocrine, paracrine and endocrine action. Intracrine action is an efficient mode of hormone action due to the requirement of minimal amounts of biologically active hormones to exert their maximum effects.

2.6.2 The metabolism of steroid hormones in the peripheral target tissues

In the peripheral tissues, including steroid hormone target tissues, HSDs, in addition to CYP19A1 and SRD5As, participate in the processes of synthesis and inactivation of sex steroids (Fig. 4). The cDNA cloning indicates there are multiple HSD17Bs, which show differential tissue specificity in their expression. HSDs belong to at least two distinct protein phylogenies: the SDR family including HSD3Bs, HSD11Bs and most of the HSD17Bs, and the AKR family including AKR1Cs, HSD20A and HSD17B5 (*Penning TM 1997 and refs therein*).

Androgens: The conversion of DHEA to $\triangle^5$-diol is catalyzed by HSD17B1 enzyme (*Peltoketo H et al 1999*). Due to the unidirectional action of HSD3B enzymes, DHEA and $\triangle^5$-diol are converted to $\triangle^4$-dione and T, respectively (*Labrie F et al 2000*). The most important enzymes for T production from $\triangle^4$-dione are HSD17B3 and 5 enzymes (*Geissler WM et al 1994; Dufort I et al 1999*), while the conversion of $\triangle^5$-diol to DHEA is catalyzed by HSD17B2 and 4 enzymes (*Adamski J et al 1995; Labrie F et al 2000*). T oxidation to $\triangle^4$-dione could be catalyzed by HSD17B2, 8 and 9 enzymes (*Andersson S and Moghrabi N, 1997; Fomitcheva J et al 1998; Napoli JL 2001*). The reduction of T at position 5 by SRD5A produces the potent androgen DHT. This is an important synthetic pathway of DHT in the peripheral tissues commencing at DHEA via $\triangle^4$-dione and involving HSD3B and HSD17B5 enzymes (*Labrie F et al 2000*). The HSD3B reaction, albeit with a lower efficiency, can be also mediated by HSD17B2 (*Suzuki T et al 2000*).

DHT may undergo reduction at position 3 to form the weak androgen 3α-diol. Enzymes catalyzing this reaction are HSD17B5, 7 and 10 and AKR1C enzymes (*Penning TM et al 2001; Törn S et al 2003*). The opposite reaction putatively involves reductive and oxidative AKR1Cs, and HSD17B10 enzymes (*Hardy DO et al 2000; Huang XF and Luu-The V 2000; Penning TM et al 2000*). Recently, the possibility of DHT conversion to 3β-diol by HSD3B and HSD17B7 enzymes has been reported (*Penning TM 1997; Luu-The 2001*). 3α-diol is converted to ADT by HSD17B2, 5, 9 and 11 enzymes (*Lin HK et al 1997; Puranen TJ et al 1999; Brereton P et al 2001; Napoli JL 2001*). Another pathway of DHT oxidation at position 17 to A-dione is catalyzed by HSD17B2, 5, 8 and 10 enzymes (*Fomitcheva J et al 1998; Lin HK et al 1997; Puranen TJ et al 1999; Shafqat N et al 2003*).

Estrogens: Unidirectional aromatization of $\triangle^4$-dione and T by CYP19A1 results in the production of E_1 to E_2, respectively (*Simpson ER et al 2002*). Reduction of E_1 to the biologically more potent E_2 is catalyzed by HSD17B1, 5, 7 and 12. The oxidation of E_2 is performed by HSD17B2, 4, 8 and 10 (*Luu-The V et al 2006; Mindnich R et al 2004 and refs therein*).

2.6.3 Sterogenic enzymes in the target tissues

As mentioned above, the synthesis of active androgens and estrogens from DHEA and DHEA-S in the peripheral target tissues is dependent on the level of expression of the various steroidogenic and metabolizing enzymes in each cell type. Elucidation of the

tissue-specific expression of steroidogenic enzymes responsible for the transformation of DHEA and DHEA-S into androgens and estrogens has permitted rapid progress in the area (*Labrie F et al 2003 and refs therein*, Fig. 4).

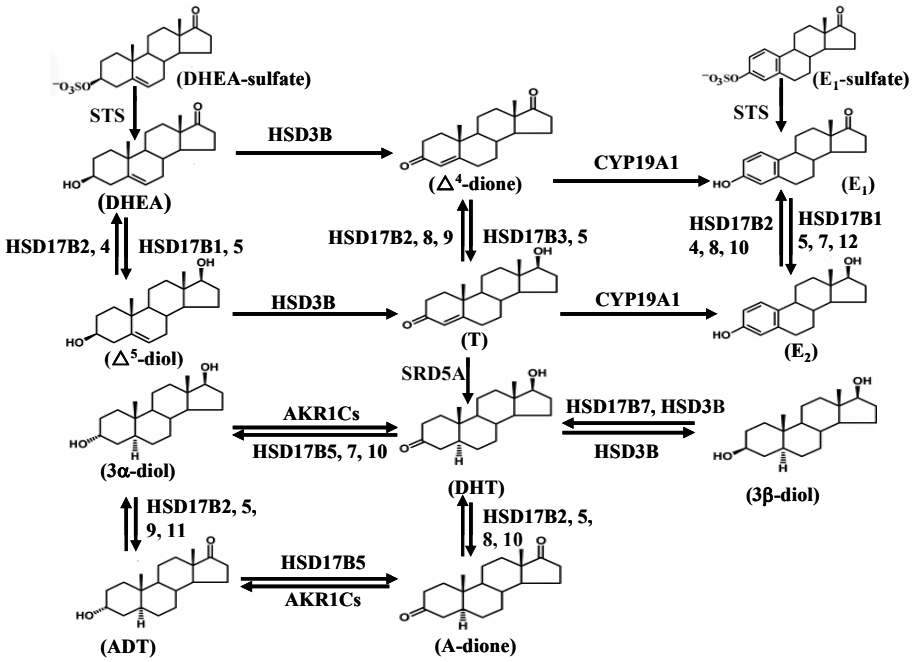


Figure 4. The final steps of estrogen and androgen metabolism in the peripheral tissues.

E_1=Estrone, E_2=Estradiol, T=Testosterone, DHEA=Dehydroepiandrosterone, $\triangle^4$-dione=4-androstene-3, 17-dione, $\triangle^5$-diol=$\triangle^5$-androstene-3β, 17β-diol, 3α-diol=5α-androstane-3α, 17β-diol, 3β-diol=5α-androstane-3β, 17β-diol, DHT=5α-hydrotestosterone, ADT=3α-hydroxy-5α-androstan-17-one, A-dione=5α-androstane-3, 17-dione. This figure was modified from *Mindnich R et al 2004.*

2.6.3.1 Hydroxy-δ-5-steroid dehydrogenase 3β- and steroid δ-isomerase (HSD3Bs)

Conversion of $\triangle^5$-steroids into their $\triangle^4$-congeners, a step required for the production of P, mineralocorticoids, glucocorticoids and sex steroids, consists of two chemical transformations, both performed by the HSD3B enzymes. The first reaction is the oxidation of the 3β-hydroxyl group to ketone, and during this process NAD^+ is converted to NADH. The intermediate $\triangle^5$-3-ketosteroid remains tightly bound to the enzyme and the presence of NADH in the cofactor-binding site activates the enzyme's second activity, the $\triangle^5$-$\triangle^4$-isomerase activity, and the subsequent isomerization of the $\triangle^5$-3-ketosteroid products to yield α, β-unsaturated ketones (*Thomas JL et al 1995*). The majority of steroid hormones contain this functional group. Thus, HSD3B enzymes are required for all steroid hormone biosynthesis. The HSD3Bs also convert 3β-diol to DHT (*Huang XF and Luu-the V 2001*).

Although rodents contain multiple Hsd3b isoforms (*Payne AH et al 1997*), only two active genes have been identified in humans (*Lachance Y et al 1990; 1991*). Both

genes are 7.8kb in length and consist of four exons and three introns. The genes are located in chromosome 1p13.1, and encode for 1.7 kb transcripts in length. HSD3B1 and HSD3B2 enzymes share 93.5% amino acid identity, while the catalytic efficiency of HSD3B1 is 5.9-, 4.5- and 2.8-fold higher than that of HSD3B2 using pregnenolone, DHEA and DHT as substrates, respectively (*Rheaume E et al 1991*). HSD3B1 is expressed in the placenta, liver, brain and some other tissues, whereas HSD3B2 is the principal isoform in the adrenals and gonads (*Rheaume E et al 1991*). The placental isoform is required for P production during pregnancy, which may explain why the deficiency of HSD3B1 has never been described. In contrast, deficiency of HSD3B2 causes a rare form of congenital adrenal hyperplasia known as "HSD3B deficiency" (*Moisan AM et al 1999*). The presence of HSD3B1 in the patients helps to explain why individuals born with severe HSD3B2 deficiency can virilize slightly in utero.

2.6.3.2 Hydroxysteroid (17β) dehydrogenase (HSD17Bs)

HSD17Bs are involved in the activation and inactivation of steroid hormones. The enzymes catalyze reductive and oxidative reactions at the position of C_{17}-, C_{18}- and C_{19}-steroids in which keto groups are converted to hydroxy groups, and vice versa. The enzymes of the HSD17B gene family are responsible for the interconversion of DHEA and $\triangle^5$-diol, $\triangle^4$-dione and T, as well as E_1 and E_2, the interconversion of A-dione and DHT as well as ADT and 3α-diol is controlled by the same enzymes. The HSD17Bs are, therefore, required for the synthesis of all highly active androgens and estrogens as well as for their inactivation (*Labrie F et al 2000*). HSD17B activity is not only distributed in the classical steroidogenic tissues, but also in a large series of peripheral intracrine tissues (*Labrie F et al 1997; Luu-The V 2001, and refs therein*). Several HSD17B enzymes have revealed multifunctionality, being able to regulate the availability of active sex steroids, fatty and bile acids and retinoids (Table 3). Furthermore, HSD17Bs has been shown to be involved in the pathogenesis of human disorders such as various hormone-sensitive cancers (*Gunnarsson C et al 2001; Härkönen P et al 2003*), 46, XY DSD (*Geissler WM et al 1994*), bifunctional enzyme deficiency (*De Launoit Y and Adamski J 1999*), polycystic kidney disease (*Maxwell MM et al 1995*) and Alzheimer's disease (*He XY et al 2002*).

1. HSD17B1. The *HSD17B1* gene is very short (3.2 kb) and consists of 6 exons (*Luu-The V et al 1990*). It is located on chromosome 17 at region q11-q21 (*Luu-The V et al 1989*), which is closely to the susceptibility gene for hereditary breast and ovarian cancer, the *BRCA1* gene on chromosome 17q21 (*Luu-The V et al 1989*). Two human mRNA, 1.3 kb and 2.3 kb (a long 5'-untranslated region) in size, have been detected for *HSD17B1*, whereas 1.3 kb mRNA is expressed in cells producing HSD17B1 protein. The mRNA encodes a protein with a calculated molecular mass of 34 kDa and the expression is subject to hormonal regulation (*Tremblay Y et al 1989*). In the rodent ovaries, both of the rat mRNAs (1.4 and 1.7 kb) are regulated in parallel (*Ghersevich S et al 1994*), and in the mouse only one 1.4 kb signal is typically detected (*Nokelainen P et al 1996*). The HSD17B1 enzyme is a cytosolic protein that exists in a homodimeric form predominantly catalyzing the interconversion of E_1 to E_2 using NAD(H) or NADP(H) as a cofactor (*Ghersevich SA et al 1994*). Human HSD17B1 is expressed in the ovaries and placenta during pregnancy (*Luu-The V et al 1990*), as well as the mammary glands and endometrium (*Maentausta O et al 1991; Poutanen M et al 1992*). In addition, small amounts of mouse Hsd17b1 has detected in the adrenals, uterus, testes, liver, prostate, muscle, heart, spleen, kidneys and lung (*Nokelainen P et al 1996; Sha JA et al 1997*).

2. HSD17B2. The human H*SD17B2* gene is located on chromosome 16 at loci q24.1-q24.2 (*Labrie Y et al 1995*). The gene spans at least 40 kb and contains seven exons. Furthermore, the *HSD17B2* gene encodes two alternatively spliced mRNAs, which give rise to the active HSD17B2 protein and to a truncated 291-residue long

HSD17B2B that has no significant enzymatic activities towards estrogens and androgens (*Labrie Y et al 1995*). A 1.5 kb mRNA for the *HSD17B2* has been identified that encodes a protein with a calculated molecular weight of 42 kDa. HSD17B2 is a membrane-associated protein that contains an amino-terminal type II signal-anchor motif and a carboxyl-terminal endoplasmic reticulum retention motif (*Wu L et al 1993*). HSD17B2 is an oxidative enzyme that catalyzes the conversion of T to $\triangle^4$-dione, E_2 to E_1, DHEA to $\triangle^5$-diol, DHT to A-dione, as well as 3α-diol to ADT (*Wu L et al 1993; Puranen TJ et al 1999*). The enzyme also possesses HSD20A activity toward 20α-dihydroprogesterone and weak HSD3B activity (*Wu L et al 1993; Suzuki T et al 2000*). Northern blot and immunohistological analyses indicated that HSD17B2 is expressed in a wide variety of tissues, including steroid target tissues such as endometrium, breast, uterus, prostate, placenta, pancreas, and in the tissues involved in catabolism and excretion of steroids such as liver, small intestine and kidneys (*Casey ML et al 1994*).

Mouse and rat cDNAs for Hsd17b2 share 82% sequence identity (*Mustonen MV et al 1997*). The rat Hsd17b2 enzyme, 42 kDa in size, shows a hydropathy profile, tissue distribution and catalytic properties similar to those of human enzyme, although two proteins share only 62% sequence homology (*Akinola LA t al 1996*). The expression of Hsd17b2 has also been studied in detail in the mouse tissues (*Mustonen MV et al 1998*).

3. HSD17B3. The human gene for *HSD17B3* contains 11 exons and 10 introns, and spans more than 60 kb of genomic DNA at chromosome 9q22. The reading frame of human *HSD17B3* cDNA encodes a protein with a molecular mass of 34 kDa (*Geissler WM et al 1994*). The mouse Hsd17b3 protein shares 73% and 95% overall identity and similarity with the human protein, respectively (*Sha JA et al 1997*). The primary structures of HSD17B3 enzymes do not contain a putative transmembrane region, but the enzymes have, nevertheless, been suggested to be located in the endoplasmic reticulum because of its short hydrophobic sequences located in N- and C-termini (*Geissler WM et al 1994; Sha JA et al 1997*). HSD17B3 is almost exclusively expressed in the testes (*Geissler WM et al 1994*), but with the RT-PCR method, low levels of mouse enzyme have been found in several tissues (*Sha JA et al 1997*). HSD17B3 predominantly catalyzes the reduction of $\triangle^4$-dione to T, and at lower conversion rates, E_1 to E_2, and DHEA to $\triangle^5$-diol (*Geissler WM et al 1994*). Deficiency of the active enzyme results in 46, XY DSD (*Geissler WM et al 1994*).

4. HSD17B4. Human HSD17B4 is a unique multifunctional enzyme (MFE) consisting of HSD17B-, hydratase- and sterol carrier 2-like domain (*Leenders F et al 1996*). Human *HSD17B4* gene is mapped to Chromosome 5q2. This gene consists of 24 exons, spanning more than 100 kb (*Leenders F et al 1998*). The 2.3 kb mRNA for human *HSD17B4* encodes a protein with a molecular weight of 80 kDa (*Adamski J et al 1995*). HSD17B4 is a peroxisomal protein and is expressed nearly ubiquitously in different adult tissues (*Adamski J et al 1995*). The human, mouse and rat proteins show a close relationship, revealing 85% amino acid similarity, the same multi-domain structure and identical kinetic parameters, suggesting an essential physiological function for the enzyme (*Adamski J et al 1995; Normand T et al 1995; Corton JC et al 1996*).

The N-terminally cleaved 32 kDa fragment and full-length enzyme catalyze dehydrogenase reaction with 3-hydroxyacyl-CoA, and convert E_2 and $\triangle^5$-diol to E_1 and DHEA respectively. The middle domain of the protein catalyzes the 2-enoyl-acyl-CoA hydratase reaction. The C-terminal domain of the protein is similar to sterol carrier protein 2, and facilitates the transfer of 7-dehydrocholesterol and phosphatidylcholine between membranes (*Leenders F et al 1996*). The most important biological functions of HSD17B4 *in vivo* are the metabolism of fatty acids and sterols, evidences of which are also supported by phylogenetic studies (*Breitling R et al*

2001). Deficiency of HSD17B4 leads to severe Zellweger syndrome with the disorders of lipid, fatty acid and sterol metabolism, and neurological disturbances, causing the death of patients usually within the first year of life (*Novikov D et al 1997*).

5. HSD17B5. The synthesis of T from $\triangle^4$-dione in the testes by HSD17B3 provides androgens mainly in men. However, the same enzymatic reaction is catalyzed in the peripheral target tissues and in the ovaries by another enzyme, namely HSD17B5, which represents an additional source of androgens. HSD17B5 belongs to the AKR family, and is also known as AKR1C3. With the other members of AKR family, AKR1C4, AKR1C2 and AKR1C1, HSD17B5 shares 84, 86 and 88% identity, respectively. Both human 1.1 kb cDNA and mouse 1.5 kb cDNA of *Hsd17b5* encode a protein with a predicted molecular mass of 37 kDa (*Lin HK et al 1997; Rheault P et al 1999a*). The human *HSD17B5* gene contains 9 exons within a total length of >15 kb on chromosome 10p14-15 and mouse *Hsd17b5* also contains 9 exons within a total length of 16 kb on chromosome 13a2 (*Rheault P et al 1999a; 1999b; Labrie F et al 2000*). HSD17B5 has been detected in the liver, adrenals, prostate, ovaries and mammary glands (*Luu-The V 2001*). Both human and mouse HSD17B5 catalyze the conversion of $\triangle^4$-dione to T, and additionally possess AKR1C and dihydrodiol dehydrogenase activity to some extent. Human, but not mouse HSD17B5 also converts P to 20α-dihydroprogesterone effectively (*Lin HK et al 1997; Rheault P et al 1999a; Dufort I et al 1999*). Human enzyme activity is highly labile and destroyed upon homogenization of cells or tissue, while mouse enzyme is more stable and is not altered upon homogenization. In the intact cells, human HSD17B5 catalyzes HSD17B reactions several times more efficiently than AKR1C reactions and mouse (*Dufort I et al 1999*).

6. HSD17B6. Rat Hsd17b6 was first described as retinol dehydrogenase (RODH) (*Biswas MG and Russell DW 1997*). The enzyme selectively catalyzes the oxidation of 3α-diol to ADT. However, Hsd17b6 also shows low hydrogenase activity converting DHT to A-dione, and T to $\triangle^4$-dione. Hsd17b6 shares 65% homology with rat RoDH1, and thus, belongs to the retinol dehydrogenase family. Hsd17b6 possesses weak oxidative activity that transforms 3α-diol to DHT, and ADT to A-dione. This enzyme has been discovered also as a 3 (α-β)-hydroxysteroid epimerase (HSE) (*Huang XF and Luu-the V 2000*). Hsd17b6 was shown to catalyze transformation of ADT into epi-androsterone in two steps: the oxidation of ADT to A-dione, followed by the reduction of the latter to epi-androsterone (*Huang XF and Luu-The V 2001*). Northern blot analysis shows high expression of Hsd17b6 in rat liver and prostate with a mRNA size of approximately 2 kb (*Biswas MG and Russell DW 1997*).

7. HSD17B7. Hsd17b7 was cloned from a rat corpus luteum cDNA library and mouse mammary epithelial (HC11) cell cDNA library (*Duan WR et al 1996; Nokelainen P et al 1998*). Mouse cDNA shares 89% identity with the rat, and they catalyze the conversion of E_1 to E_2 (*Nokelainen P et al 1998*). Northern blot analysis showed several mRNAs with multiple sizes, the most strongly expressed mRNAs being 4.6 and 4.3 kb in size in mouse and rat, respectively. Both rat and mouse Hsd17b7 is abundantly expressed in the corpus luteum during the second half of pregnancy. mRNA has also been detected in the liver, placenta, mammary glands, non-pregnant ovaries and kidneys. Using the RT-PCR method, mRNA has also been observed in the mouse brain, testes and small intestine.

A human *HSD17B7* cDNA (*Krazeisen A et al 1999*) encodes a protein of 37 kDa. This enzyme efficiently catalyzes the transformation of E_1 to E_2 and is detected in the ovaries, breast, placenta, testes, prostate and liver. This gene spans 21.8 kb and consists of nine exons (*Krazeisen A et al 1999*). The gene is assigned to human

chromosome 1q23.3 (*Liu H et al 2005*). A second, shorter form of HSD17B7 has also been described (*Liu H et al 2005*). It originates from locus 10p11.2 and has been found to express in the liver, prostate, uterus and placenta by ribonuclease protection assays. The short form of HSD17B7 catalyzes the transformation of E_1 to E_2 and of DHT into 3β-diol. HSD17B7, which produces active estrogens and inactivates active androgens, most probably plays a crucial role in estrogen-sensitive cells and tissues.
The search for homologous proteins and phylogenetic analyses was indicated for an additional role for the enzyme *in vivo* (*Marijanovic Z et al 2003*). The 3-ketosteroid reductase activity of mouse and human HSD17B7 was demonstrated by the conversion of zymosterone to zymosterol, and by a complementation assays in the yeast 3-ketosteroid reductase (Erg27p)-deficient yeast strain (*Marijanovic Z et al 2003*), and in the NS0 cell line (primary host cell line, *Seth G et al 2006*).

8. HSD17B8. Hsd17b8 was first discovered as Ke6 gene in connection with polycystic kidney disease in mouse where it was down-regulated in affected animals (*Aziz N et al 1993*). The enzyme was later classified as HSD17B due to its homology to Hsd17b4 and named Hsd17b8 (*Fomitcheva J et al 1998*). Mouse Hsd17b8 overexpressed in fusion GST efficiently catalyzes the transformation of E_2 to E_1, and to some extent, it also catalyzes oxidative reaction of androgens and the reduction of E_1 to E_2. Mouse Hsd17b8 is mostly found in the liver, kidneys as well as the gonads (*Pelletier G et al 2005; Fomitcheva J et al 1998*).
The role of HSD17B8 in humans is not yet clear. This gene is localized in the HLA region, which is well known to contain genes encoding human major histocompatibility complexs (MHC, *Kikuti YY et al 1997*).

9. HSD17B9. The enzyme Hsd17b9 that was discovered in mouse (*Su J et al 1999*) has 3α-, 17β- and retinol-dehydrogenase activity (*Napoli JL 2001*). There is no human ortholog known.

10. HSD17B10. HSD17B10 was initially identified as short chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD) (*HE XY et al 1998*) and known to catalyze the oxidation of branched and straight fatty acids. This enzyme is a homotetramer with a molecular mass of 108 kDa and located in mitochondria. Its subunit consists of 261 amino acid residues. The human *HSD17B10* gene is organized into six exons, and maps to chromosome Xp11.2. The amino-terminal NAD-binding region of the dehydrogenase is encoded by the first three exons, whereas the other exons encode for the carboxyl-terminal substrate-binding region harboring putative catalytic residues. The enzyme was found to have HSD17B activity oxidating E_2, and oxidative activity, catalyzing the synthesis of DHT from 3α-diol (*He XY et al 1998; 2003*) as well as the ability to oxidize 20β-OH- and 21OH- in C21- steroids. HSD17B10 shows affinity towards amyloid β-peptide (Aβ) (*Yan SD et al 1997*), and seems to play a role in the pathogenesis of Alzheimer's disease. HSD17B10 could increase local Aβ concentrations, facilitating Aβ deposition and promoting neuronal and synaptic loss (*Yang SY et al 2005*).

11. HSD17B11. In search of a new enzyme in glucocorticoid metabolism HSD17B11 was discovered as HSD17B activity due to its homology to other HSD17B family members and due to its ability to convert 17β-hydroxysteroids (*Li KX et al 1998*). Human HSD17B11 displays greatest activity with 3α-diol as the substrate, suggesting that it may be important in androgen metabolism. Enzyme activity was non-saturable with 3α-diol but saturable with retinoids, although retinoids were not metabolized (*Brereton P et al 2001; Chai Z et al 2003*). A more recent study observed strong up-regulation of Hsd17b11 by peroxisome proliferator-activated receptor α (PPARα) agonists in mouse intestine suggesting the involvement in other metabolic pathways, for example, the lipid metabolism (*Motojima K 2004*).

HSD17B11 is not only found in steroidogenic cells but has a broad distribution as it is also detected in the pancreas, kidneys, liver, lung and heart (*Chai Z et al 2003*).

12. HSD17B12. Mammalian HSD17B12 was discovered to be involved in fatty acid elongation (*Moon YA and Horton JD 2003*). Consistent with its function, the enzyme is found ubiquitously with highest levels of expression in tissues that are directly involved in the lipid metabolism, such as the liver, muscle, and kidneys, and also in mouse white and brown adipose tissues (*Moon YA and Horton JD 2003*). HSD17B12 was also found to be highly similar to HSD17B3. In mouse and humans HSD17B3 and 12 share around 40% sequence identity and phylogenetic analyses revealed that HSD17B12 might be an ancestor of HSD17B3 (*Mindnich R et al 2005*). Human HSD17B12 was found to catalyze the transformation of E_1 to E_2 (*Luu-The V et al 2006*).

Table 3 The Different Forms and Properties of Human HSD17B Enzymes

Enzyme type	Gene name	Function	Chromosome	Disease or participation in pathology	Expression pattern
1	HSD17B1	Estradiol synthesis	17q21.2, Φ17q21	Breast and prostate cancer	Wide spread; gonads, breast, placenta, liver
2	HSD17B2	Estradiol and testosterone inactivation	16q23.2	Endometriosis, colon and prostate cancer	Wide spread; prostate, liver intestine
3	HSD17B3	Testosterone synthesis	9q22.32	46, XY DSD	Mainly testis
4	HSD17B4	β-Oxidation of fatty acids, estradiol inactivation, androgen metabolism	5q23.1	D-specific multifunctional protein deficiency, Stiff-man syndrome	Widespread
5	HSD17B5	Testosterone synthesis, detoxification	10p15.1	nk	Liver, prostate
6[a]	HSD17B6[a]	nk	nk	nk	
7	HSD17B7	Cholesterol synthesis, estradiol synthesis	1q23.3, Φ10p11, Φ1q44	nk	Ovary, placenta, liver, brain
8	HSD17B8	Estrogen and androgen inactivation	6p21.3	polycystic kidney disease	Widespread; liver, kidney
9[a]	HSD17B9[a]	nk	nk	nk	
10	HSD17B10	Estrogen and androgen inactivation, b-oxidation of fatty acids, bile acids isomerization	Xp11.22	Isoleucine degradation deficiency, Alzheimer disease	Widespread; liver, CNS, kidney, testis
11	HSD17B11	Estrogen and androgen inactivation	4q22.1	nk	Steroidogenic tissues
12	HSD17B12	3-Ketoacyl-CoA reductase fatty acid synthesis	11p11.2	nk	Liver, muscle kidney

[a] Data for rodent only, nk: not known, Φ: pseudogene, DSD: disorders of sex development, CNS: central nervous system

2.6.3.3 Aldo-keto reductase family 1 (AKR1C)

AKR1Cs are monomeric 37kDa proteins, NAD(P)(H)-dependent, and share a common $(\alpha/\beta)_8$-barrel as a structural motif. Only one known Akr1c appears to be expressed in the rat, while at least four human isoforms exist. Human HSD3A1, 2, 3 and 4 are names for AKR1C4 (*Deyashiki Y et al 1994*), C3 (*Lin HK et al 1997*), C2 (*Deyashiki Y et al 1994; Dufort I et al 1996*) and C1 (*Hara A et al 1996*), respectively (*Jez JM and Penning TM 2001*). The human isoforms share at least 84% amino acid sequence identity, and remarkably AKR1C1 and AKR1C2 differ by only seven amino acids (*Deyashiki Y et al 1994; Dufort I et al 1996*). Progress has been made in characterizing each of the human AKR1C isoforms with respect to their perceived roles in the steroid metabolism in the liver and steroid hormone target tissues. The functional plasticity of these isoforms highlights their ability to modulate the levels of

active androgens, estrogens and progestins. AKR1C4 is catalytically the most efficient, 10-30-fold higher as compared with other isoforms. In the reductive direction, all isoforms inactivate DHT to yield 3α-diol. However, only AKR1C3 reduced $\triangle^4$-dione to produce significant amounts of T. All isoforms reduced E_1 to E_2, and P to 20α-hydroxyprogesterone. In the oxidative direction, only AKR1C2 converted 3α-diol to the active hormone DHT. AKR1C3 preferentially produced ADT and A-dione from 3α-diol, indicative of their associated HSD17B activities. The results obtained with AKR1C3 confirm the previous observations, which showed that this enzyme oxidized 3α-diol to products other than DHT (*Lin HK et al 1997*). AKR1C3 and AKR1C4 oxidize T to $\triangle^4$-dione, and all the isoforms oxidize E_2 to E_1, and 20α-hydroxyprogesterone to P (*Penning TM et al 2000*).
Quantitative RT-PCR indicated that each human AKR1C isoform was expressed in the human liver. AKR1C4 was almost exclusively detected in the liver indicating that it is a liver-specific isoform. One major role of AKR1C4 is to protect the tissue against circulating steroid hormones. However, AKR1C2 is known as human bile acid binding protein in liver (*Hara A et al 1996*). In the human lung, AKR1C isoforms, except AKR1C4, are highly expressed (*Burczynski ME et al 1998*). In the human prostate, the two isoforms most abundantly expressed are AKR1C2 and AKR1C3. Only AKR1C2 forms the active hormone DHT. Thus AKR1C2 may increase the pool of active androgens in the prostate, while AKR1C3 functions as a HSD17B enzyme (*Lin HK et al 1997*) and thus can eliminate DHT. The high expression of AKR1C3 in the human mammary glands could convert $\triangle^4$-dione into T, increasing the pool of steroids that can be aromatized to E_2. In the uterus AKR1C3 and AKR1C2 are dominant, and the latter is more highly expressed. It is postulated that AKR1C2 may be the major contributor to the production of uterine 3α-diol and 20α-hydroxyprogesterone. In the brain, AKR1C1-AKR1C3 is all present. Northern blot analysis detects the presence of AKR1C3 and AKR1C2. Both mRNAs are found to express across many brain regions (*Griffin LD et al 1999*). All isoforms are capable of producing the neuroactive tetrahydrosteroids to regulate the γ-aminobutyric acid type A ($GABA_A$) receptor. The production of the neurosteroids regulated by the AKR1Cs is associated with late luteal phase dysphoria disorder and major unipolar depression (*Griffin LD et al 1999*).

2.6.3.4 Cytochrome P450 family 19 subfamily A polypeptide 1 (CYP19A1)
Conversion of antrogens to estrogens is catalyzed by a microsomal member of the cytochrome P450 superfamily, namely cytochrome P450 family 19 subfamily A polypeptide 1 (the product of the *CYP19A1* gene). The P450 gene superfamily is very large, containing over 3811 members in 711 families, of which CYP19A1 is the sole member of family 19 (*Nelson DR 2006*). This protein is responsible for the binding of C_{19} androgenic steroid substrate and catalyzing the formation of estrogens. In humans, a number of tissues express CYP19A1 and synthesize estrogens. These include the ovaries and testes, the placenta, fetal liver, adipose tissue, bone, and brain (*Simpson ER et al 2002*). The human *CYP19A1* gene expands over a large genomic region in which the size is over 100 kb (*Harada N et al 1990*). The coding region contains 9 exons beginning with exon 2. Upstream of exon 2 are a number of alternative first exon 1s that are spliced into different untranslated regions of the transcript in a tissue-specific fashion. Up to five different transcriptional start sites with individual promoters permit the tissue-specific regulation of expression in diverse tissues (*Simpson ER et al 1993*). The identities of the derived amino acid sequences of CYP19A1 from rat, mouse, chicken, trout, and bovine compared to human sequence are 77%, 81%, 73%, 52% and 86%, respectively (*Simpson ER et al 1994*). The reports of patients with of CYP19A1 deficiency confirm that biologically significant estrogen

synthesis derives entirely from this enzyme (*Conte FA et al 1994; Morishima A et al 1995*).

2.6.3.5 Steroid-5-α-reductase (SRD5A)

SRD5A activity transforms T to DHT, and is responsible for the differentiation of the male external genitalia and prostate as well as virilization of males at puberty. SRD5A however, also has a central role in the development of prostate cancer and benign prostatic hyperplasia. Two types of human SRD5A, SRD5A1 and SRD5A2, have been isolated (*Andersson S et al 1991*). Both *SRD5A1* and *SRD5A2* gene contain five exons (*Jenkins EP et al 1991; Labrie F et al 1992*). The two isoforms are very hydrophobic, 30 kDa microsomal proteins that share 50% identity. The SRD5A1 is limited to the nongenital tissues (liver, scalp and skin), while SRD5A2 remains the predominant enzyme in genital tissues, which explains why deficiency of SRD5A2 is not compensated for SRD5A1 (*Thigpen AE et al 1993*). The human SRD5A2 deficiency results in 46, XY DSD. *Srd5a2* knockout mice do not demonstrate a phenotype similar to that seen in men with SRD5A2 deficiency (*Mahendroo MS 2001*). The role of SRD5A1 in humans is less clear. This enzyme has been ascribed the role of degrading circulating C_{21} steroids in preparation for excretion in the urine. Disruption of *Srd5a1* in mice results in delayed parturition (*Mahendroo MS et al 1996*).

2.7 Overview of vitamin A metabolism and function

2.7.1 Formation and catabolism of vitamin A and its active metabolites

Vitamin A (retinol) is obtained from the diet in the form of retinyl ester or carotenoid. It is either stored as an ester (primarily in the liver) or further metabolized to active compounds (*Blomhoff R et al 1992*). Cells that utilize all-trans retinoic acid (atRA) take up retinol that is then converted to atRA by the activity of specific enzymes. Members of the family of alcohol dehydrogenase (ADH), several SDR, and several cytochrome P450s metabolize retinol to retinaldehyde (*Chen H et al 2000; Napoli JL 1999*). At least five SDRs may play an important role in reducing retinaldehyde to retinol. Furthermore, mammalian aldehyde dehydrogenase (ALDH) family members are able to oxidize all-*trans*-retinaldehyde to atRA *in vitro* (*Grun F et al 2000; Mic FA 2000; Lin M et al 2003*).

atRA is further metabolized to more polar compounds (*Fujii H et al 1997*). CYP26A1 and CYP26B1 play a role in the atRA metabolism. Both enzymes result in the formation of 4-hydroxy-atRA, 4-oxo-atRA, and 18-hydroxy-atRA (*Abu-Abed SS et al 1998; White JA et al 2000*). In addition to atRA, there are several other forms of retinoids that are bioactive. There is evidence for 9-*cis*-specific enzymes that can generate 9-*cis*-RA from 9-*cis*-retinol (*Romert A et al 1998*). In the eye, retinol is oxidized and isomerized to 11-*cis*-retinaldehyde by 11-*cis*-retinol dehydrogenase that is used to generate the visual chromophore in the retinal epithelium (*Palczewski K and Saari JC 1997*).

2.7.2 Retinoid metabolic enzymes and binding proteins

Both the ADH and SDR families have been proposed to play a role in the oxidation of retinol to retinadehyde as well as its reduction back to retinol. Expression of Adh4 begins early in mouse embryonic development, at day 7.5, during the primitive streak stage and locates in the posterior embryonic tissues and in the mesoderm, (*Ang HL and Duester G 1997*). Adh1 is not expressed in the mesonephros and limb buds until day 10.5 of mouse development (*Vonesch JL et al 1994*). On consecutive days of development ADH1 and ADH4 expressions become more widespread in various organs. Adh3 is ubiquitously expressed and Adh2 has a very limited tissue

distribution, being found in the liver and skin (*Duester G 2000*). However, the enzymes are not absolutely essential for embryogenesis, *Adh1* and *Adh4* null mutant mice were viable and fertile (*Deltour L et al 1999a; 1999b*), and *Adh3* null mutant mice were shown to have reduced viability and growth on a vitamin A sufficient diet (*Molotkov A et al 2002a*). Furthermore, *Adh3* and *Adh4* null mutant mice also exhibited severely reduced growth and survival on a Vitamin A deficient (VAD) diet. Both *Adh1* and *Adh3* null mutant mice showed a reduction in RA production upon receiving a large dose of retinol, leading to excessive vitamin A toxicity (*Molotkov A et al 2002b*). Studies on single or double knockout mice indicated that Adh1 provided considerable protection against vitamin A toxicity, whereas Adh4 promoted survival during VAD (*Molotkov A et al 2002c*). The function of individual SDRs in embryogenesis has not yet been tested and thus, much remains to be learned about SDR systems.

Four members of the ALDH family play a role in the generation of retinoic acid and their distribution in embryonic tissue has been examined (*Duester G et al 2003; Lin M et al 2003*). In the mouse embryo, Aldh1a2 is the first retinoid metabolizing enzyme to appear. The early expression of *Aldh1a2* mRNA in the mesoderm adjacent to the node and primitive streak, and in the mesoderm of the headfold stage embryos gives evidence to support a role for retinoic acid in axial patterning of the early embryo (*Haselbeck RJ et al 1999*), whereas its later expression agrees with other studies in which roles for vitamin A in the hindbrain, spinal cord, vertebra, heart, eye, lung, kidney, and gut development have been proposed (*Malpel S et al 2000; Batourina E et al 2001; Wagner E et al 2000*). Furthermore, *Aldh1a2*$^{-/-}$ embryos die in utero before E10.5 (*Niederreither K et al 1999*), thus confirming that Aldh1a2 plays a critical role in the synthesis of retinoic acid early in mouse development. The distribution of Aldh1a1 protein gives support for a role for vitamin A in genitourinary tract development (*Haselbeck RJ et al 1999*). However, *Aldh1a1* deficient mice show no apparent gross abnormalities of defects in survival or growth, and are relatively healthy and fertile (*Fan X et al 2003*). *Aldh1a3* mRNA expression during mouse embryogenesis has been characterized (*Grun F et al 2000; Mic FA et al 2000*). Furthermore, *Aldh1a3* knockout in mouse suppresses RA synthesis and causes malformations restricted to ocular and nasal regions, and the cause of choanal atresia (CA) is responsible for respiratory distress and death at birth (*Dupe V et al 2003*). Aldh1a4 is expressed in adult mouse liver and kidney (*Lin M et al 2003*), but targeted mutation of the gene has not been reported.

Numerous cytochrome P450 enzymes are believed to play a role in embryonic RA oxidation. The expression of *Cyp26a1* mRNA in the developing mouse embryo has been described (*DE Roos K et al 1999; Fujii H et al 1997*). Disruption of the murine *Cyp26a1* gene is embryolethal (*Abu-Abed S et al 2001*). Cyp26b1 mRNA shows a dynamic pattern of expression in the developing hindbrain and limb buds (*Abu-Abed S et al 2002*). Disruption of *Cyp26b1* caused mice to die immediately after birth as a result of respiratory disteress. Furthermore, mice that lack *Cyp26b1* exhibited severe meromelia-like malformations in the limbs (*Yashiro K et al 2004*). Cyp26c1 has recently been cloned and characterized (*Taimi M et al 2004*) and is expressed in the hindbrain, inner ear, first branchial arch and tooth buds.

The retinoid binding proteins (RBP) are likely to play a role in the regulation of the retinoid metabolism as cytoplasmic carriers for the lipophilic retinoids (*Macdonald PN et al 1990; Perez-Castro AV et al 1989; Ruberte E et al 1992*) Plasma RBP4, being extracellular, has been proposed to have a function in the delivery of retinol to the tissues. *Rbp4* null mutant mice are viable and fertile. However, they showed reduced leves of plasma retinol, and markedly impaired ability to mobilize hepatic retinol stores (*Quadro L et al 1999*). RBP1 is believed to promote RA synthesis by presenting retinol to the retinol dehydorgenase (*Napoli JL 1993*). It is dispensable for

normal physiological function with no embryonic lethality in animal fostered with vitamin A–sufficient diet, however, Rbp1 deficiency results in a 50% reduction in retinol ester storage, and a faster rate of retinol turnover under the VAD (*Ghyselinck NB et al 1999*). Mice lacking Rbp2 have reduced hepatic stores of vitamin A, but grow and reproduce normally (*Zhang EX et al 2002*). However, under a VAD diet, there is increased neonatal mortality, indicating the role of Rbp2 in the developing fetus. Since the discovery of Crabp1 and Crabp2 many functions have been proposed to include the protection of RA from oxidation, ligand solubilization and regulation of RA metabolism (*Dong D et al 1999*). However, it should be noted that *Crabp1* null mice demonstrates normal homeostasis (*Gorry P et al 1994*), and *Crabp2* single and *Crabp1/2* double null mutant mice are normal, with the exception of a minor limb abnormality and a slight reduction in postnatal viability (*Lampron C et al 1995*). Thus, the activity of these proteins is not essential to the function of vitamin A in reproduction.

2.7.3 Function of vitamin A and its active metabolites *in vivo*

Clinical findings and experimental approaches have revealed that vitamin A and its active derivatives exert a wide variety of effects on vertebrate embryonic body shaping and organogenesis, tissue homeostasis, cell proliferation, differentiation, and apoptosis (*Morriss-Kay GM and Ward SJ 1999*). Vitamin A is also indispensable throughout postnatal development and adult life for growth, survival, reproduction, vision, and for the homeostasis of numerous tissues (*Corcoran J et al 2002 and refs therein*). In recent years a molecular understanding of the defects has been obtained by analyses of knockouts of retinoid receptors and RA-synthesizing enzymes in the mouse embryo. In addition, vitamin A-deprivation studies in the embryo of rat and mouse models as well as chick and quail models have been developed to investigate the dependency of particular organ systems (*Dickman ED et al 1997; Antipatis C et al 1998; White JC et al 1998*). Under VAD a wide range of abnormalities appear, including defects of the central nervous system, eye, face, dentition, ear, skeleton, urogenital system, lung and cardiovascular system (*Clagett-Dame M and DeLuca HF 2002 and refs therein*).

2.7.4 Role of retinoid signaling in the male and female reproductive tissues

Male: The need for dietary vitamin A or retinol for normal spermatogenesis has been recognized for decades (*Griswold MD et al 1989 and refs therein*). The requirement of vitamin A and its derivatives in the testes has been assessed by examining the effects on spermatogenesis in VAD animals, particulatly in the rat. Germ cells at a stage of spermatids and primary spermatocytes in the VAD rat testes decreased markedly, indicating that the mechanisms responsible for spermatogenesis, completion of spermiation, and differentiation of spermatocytes are extremely sensitive to change in vitamin A status. Additionally, studies support the notion that the degeneration of germ cells occurring in VAD rats was not due to a breakdown of inter-Sertoli cell tight junctions, but instead, was an immediate consequence of the absence of vitamin A (*Chung SS and Wolgemuth DJ 2004a and refs therein*).

In the testes, vitamin A signaling depends on the distribution patterns of RARs, RXRs, retinol- and RA-metabolizing enzymes, and binding proteins. Most of studies have been performed in rat and mouse testes, at different stages of postnatal development and in adults (*Vernet N et al 2006; Livera G et al 2002 and refs therein*). RA represents the vitamin A metabolite that is active in mouse spermatogenesis (*Van Pelt AM and De Rooij DG 1991; Gaemers IC et al 1998*), and acts through binding to Rars or Rxrs (*Chambon P 1996; 2005*). However, only Rara and Rxrb have effects on spermatogenesis (*Lufkin T et al 1993; Kastner P et al 1996*). *Rara* null mutants (*Lufkin T et al 1993; Chung SS et al 2004b; 2005*) display a developmental arrest of

spermatids. However, *Rara*-null testes do not show histological features indicative of a delay in spermatogonia differentiation that is the most notable hallmark of VAD-induced testis degeneration. Moreover, even though Rarg is apparently the sole RA receptor present in spermatogonia, *Rarg*-null mutants do not display spermatogenesis defects, rather, there were abnormalities in the seminal vesicles and prostate (*Lohnes D et al 1993*), suggesting that the delayed, then arrested spermatogonia differentiation observed in the VAD mouse may result from a simultaneous block of Rarg- and Rara-mediated events in spermatogonia and Sertoli cells, respectively. *Rxrb*-deficient males were sterile, had failure of spermatid release in the seminiferous epithelium and abnormal acrosomes (*Kastner P et al 1996*).

Rars, in particular Rara are expressed in all the regions of the epididymides (*Akmal KM et al 1996*), whereas retinoic acid binding protein, lcn5 and lcn8 exhibit a more restricted expression pattern (*Lareyre JJ et al 1998; 2001*). Transgenic mice expressing a dominant negative mutant of *Rara* had reduced fertility due to an abnormally dense ductal fluid that blocked the epididymal lumen (*Costa SL et al 1997*).

Female: Vitamin A is essential for reproduction in the female. However, the relative state of VAD at the time of mating is a critical determinant in reproductive outcome, which includes a spectrum of abnormalities including the failure of reproduction prior to implantation, fetal resorption, and prolonged gestation with or without fetal death (*Clagett-Dame M and Deluca HF 2002 and refs therein*). Retinoids, retinoid receptors, retinoid binding proteins and metabolizing enzymes are expressed in many tissues of the female reproductive tract and placenta, suggesting that these proteins play a role in reproductive function (*Sapin V et al 1997, Dev Dyn 208:199; Vermot J et al 2000*).

Defective RA signaling has previously been implicated in breast cancer development (*Yang LM et al 1999*). In addition, it is well established that administration of retinoids *in vitro* can inhibit proliferation of normal mammary epithelial cells as well as breast cancer cell lines (*Dietze EC et al 2002*). Furthermore, RA is required for proper morphogenesis of the mouse mammary glands in a novel transgenic mouse model system, demonstrating the direct physiological effect of RA signaling *in vivo* (*Wang YA et al 2005*).

2.7.5 Role of retinoid signaling in the skeleton

The importance of retinoid signaling pathway in skeletogenesis has been firmly established from retinoic acid distribution and teratogenicity studies, indicating that this factor coordinates development of the central body axis and the limb axes, as well as the craniofacial skeleton. Much of this coordination of skeleton patterning events is believed to be, at least in part, due to RA's ability to control developmental genes, such as the homeobox genes (*Weston AD et al 2003 and refs therein*). The retinoid receptors, RA-binding proteins and metabolizing enzymes exhibit widespread and dynamic expression patterns throughout skeletal development. Specifically, the expression of RA-degradative enzymes in precartilaginous condensations suggests that a decrease in RA availability, and consequently an increase in Rar-mediated gene repression, may be an important component of chondroblast differentiation. At later stages in the chondrogenic program, RA signaling regulates the transition of growth plate chondrocytes to a hypertrophic phenotype. Moreover, genetic analysis has shown that the absence of the retinoid receptors can lead to deficiencies in cartilage formation while also promoting chondrogenesis at ectopic sites (*Underhill TM and Weston AD 1998 and refs therein*).

2.8 Roles of sex steroids as analyzed by the null mutant mouse models

2.8.1 Estrogen receptor null mutant mice (*Esr1, Esr2* and *Esr1/2*KO)

Female: The female *Esr1*KOs are acyclic, infertile and ovaries are devoid of corpora lutea. Folliculogenesis is arrested at the antral stage with large follicles becoming cystic and haemorrhagic *(Lubahn DB et al 1993*). Administration of a GnRH antagonist to *Esr1*KO mice prevented formation of haemorrhagic cysts (*Couse JF et al 1999a*), indicating that the ovarian phenotype manifests as a consequence of elevated LH levels (*Rosenfeld CS et al 2000*). In the *Esr1*KO, the uterus is hypoplastic and whole uterine weights are approximately half of WT littermates (*Lubahn DB et al 1993*). The luminal and glandular epithelial cells in the *Esr1*KO uterus most often appear healthy, but lack the normal estrogenized morphology (*Couse JF and Korach KS 1999b*). Similar to the uterus, the epithelium of the *Esr1*KO oviduct and vagina usually appears healthy and shows a complete lack of estrogenization. Mammary glands from *Esr1*KO mice showed severe mammary hypoplasia (*Bocchinfuso WP et al 2000*).

Female *Esr2*KOs (*Krege JH et al 1998; Emmen JMA et al 2005*) have small ovaries, partially arrested follicular development. In culture, *Esr2*KO follicle growth is retarded and they produce significantly less estrogens and express less Cyp19a1 than WT follicles (*Emmen JMA et al 2005*). Following ovulation induction, fewer oocytes were released from *Esr2*KO ovaries relative to their WT counterparts, consistent with the reduced numbers of offspring/litter and copora lutea (*Krege JH et al 1998*). In contrast, the uteri and vaginal mucosa of adult *Esr2*KO females appear normal and able to undergo the cyclic changes associated with the ovarian steroid hormone (*Krege JH et al 1998*). *Esr2*KO females of 4-5 months of age exhibit the normal structure and function of the mammary glands and the oviduct (*Couse JF and Korach KS 1999b*).

The generation of *Esr1/2*KO mice by two laboratories (*Couse JF et al 1999c; Dupont S et al 2000*) indicate that these mice are distinct from the individual *Esr* knockouts. These ovaries exhibit follicular trans/re-differentiation with tubular-like structures containing Sertoli-like cells. The phenotype is expressed in the presence of elevated LH levels, similar to that of the *Esr1*KO mouse. Thus it appears that both Esrs have roles to play in the maintenance of fertility. Esr1 actions are important in maintaining a female phenotype in thecal interstitial cells (*Couse JF et al 2006*), while Esr2 appears essential for follicle development and maturation.

Male: *Esr1*KO male mice undergo normal fetal development of both the external and internal structures of the reproductive tract (*Dupont S et al 2000; Eddy EM et al 1996*). Adult *Esr1*KO male mice exhibit several abnormalities and deficiencies. Although FSH is within the normal range, LH and serum T levels are increased. Spermatogenesis is clearly disrupted in *Esr1*KO males (*Eddy EM et al 1996*). The testes of *Esr1*KO are normal at birth and develop normally to puberty, but thereafter they begin to degenerate, and at 5 months of age the testes are atrophic. Luminal fluid appears to accumulate in the seminiferous tubules, rete testes and efferent ductules (*Hess RA et al 1997*). It seems likely that the spermatogenic phenotype in the *Esr1*KO mice is primarily due to an indirect effect of a lack of reabsorption in the epididymides, rather than a direct effect on spermatogenesis (*Hess RA et al 1997; Couse JF and Korach KS 1999b*). The weights of the epididymides and vas deferens were decreased, whereas the size of the accessory sex glands, prostate, and seminal vesicles was increased in *Esr1*KO mice, and this phenotype becomes more pronounced with the age of the animals. In addition to morphological changes in the ventral prostate, the lumina are enlarged and the grandular epithelium flattened (*Couse JF and Korach KS 1999b; Eddy EM et al 1996; Donaldson KM et al 1996*).

*Esr2*KO males appear to be morphologically normal and viable, and demonstrated no observable sexual impairment (*Krege JH et al 1998; Dupont S et al 2000*). Behavioral studies revealed no differences in sexual behavior between *Esr2*KO and WT mice either (*Dupont S et al 2000; Ogawa S et al 1999*). In contrast to *Esr1*KO, the prostates of *Esr2*KO mice frequently show foci of epithelial hyperplasia and disorganization as young as 3 months of age. *Esr1/2*KO mice share some of the phenotypic characteristics of both *Esr1*KO annd *Esr2*KO mice. The testes of the *Esr1/2*KO adult males showed a loss of germ cells in the seminiferous tubules, and a marked dilation of straight tubules and rete testes, similar to *Esr1*KO adult males (*Dupont S et al 2000*).
Studies to discriminate the contributions of Esr1and Esr2 to bone maintenance have been performed. *Esr1*KO and *Esr1/2*KO mice, but not *Esr2*KO mice, have small but partly significant decreases of bone maintenance parameters such as bone-mineral density, bone diameter and length (*Vidal O et al 2000; 1999*). Male *Esr2*KO mice have no major bone abnormalities and female *Esr2*KO mice show slightly elevated bone-mineral density at adult age (*Windahl SH et al 1999*).

2.8.2 Androgen receptor null mutant mice (*Ar*KO)

Female: The development of conditional *Ar*KO mice has been enabled the study of androgen action in the ovaries. Female *Ar*KO mice have longer estrous cycles and reduced fertility. Their ovaries contain normal numbers of follicles. However, in a recent study, the large antral follicles appeared to have fewer granulosa cells, and there were reduced numbers of corpora lutea. Superovulation induction led to the formation of abnormal cumulus-oocyte complexes (*Hu YC et al 2004*). No overt differences in bone phenotype and mass and the morphology of breast were found between female *Ar*KO and WT littermate mice at 8 weeks of age (*Yeh S et al 2002; Kawano H et al 2003*).
Male: *Ar*KO male mice have female-like appearance and body weight. Their genito-anal distance is shorter than in WT male mice, which is similar to female mice. The external genitalia in male *Ar*KO mice show ambiguous or feminized appearance, and the vas deferens, epididymides, seminal vesicles, and prostate are agenesis (*Yeh S et al 2002*). The testes are small in size and cryptorchid locating at the low abdominal area close to the internal inguinal ring. Thus, the testis phenotype is similar to that in Tfm mice (*Goldstein JL and Wilson JD 1972*) or humans with complete androgen-insensitive syndrome (*Ahmed B et al 2000*). Some seminiferous tubules of the testes from *Ar*KO mice contain Sertoli cells only and others contain a few germ cells. In the tubules with germ cells, the spermatogonia are hypoplastic. Occasionally, some spermatocytes at the pachytene stage are found, suggesting spermatogenesis may be arrested at the pachytene spermatocyte stage. The Sertoli cells show fibrillary degeneration. Many pyknotic cells contain apoptotic-like bodies in the tubules. In the interstitial space, Leydig cells are hypertrophic. T concentration in *Ar*KO mice is lower than WT mice. The number and size of subcutaneous adipocytes are changed, but infrarenal adiposytes showed no obvious difference between the WT and *Ar*KO mice, indicating androgens may play a role in adipogenesis (*Yeh S et al 2002*). In addition, histomorphometric analyses of male *Ar*KO mice at the age of 8 weeks showed high bone turnover with increased bone resorption that resulted in reduced trabecular and cortical bone mass without affecting bone shape (*Kawano H et al 2003*).
Sertoli cell-selective *Ar*KO (SC*Ar*KO) mice showed normal external sexual development, and their growth curve was similar to that of WT male littermates. On dissection, the testes were normally located. The size of the testes, however, was reduced to 28.4% of WT littermates at 50 days of age. In contrast with *Ar*KO males, SC*Ar*KO males displayed normal development of the epididymides, ductus deferens,

coagulating gland, seminal vesicles, and prostate. Morphological analysis of SC*Ar*KO testes established that germ cell entry into meiosis appeared normal. The number of Sertoli cell was unchanged, but the numbers of spermatocytes, round spermatids, and elongated spermatids were reduced to 64%, 3%, and 0%, those found in WT mice, respectively (*De Gendt K et al 2004*). These changes were associated with increased germ cell apoptosis, and reduced expression of genes specific for late spermatocytes and spermatid development. The results, thus, indicate that spermatocyte/spermatid development and survival critically depends on androgens. Serum levels of T and LH revealed no significant difference between WT and SC*Ar*KO animals, but FSH levels were 34% higher in SC*Ar*KO males (*De Gendt K et al 2004*).

2.8.3 *Cyp19a1* null mutant mice (*Cyp19a1*KO)

Female: To date, three laboratories have generated *Cyp19a1*KO mice. Female *Cyp19a1*KO mice have undetectable levels of estrogens but exhibit high levels of T, FSH and LH in serum. At 9 weeks of age female *Cyp19a1*KO mice displayed underdeveloped external genitalia and uteri. Development of the mammary glands approximated to that of a prepubertal female (*Fisher CR et al 1998*). Follicle development was arrested at the antral stage rendering these mice infertile due to an inability to ovulate. The antral follicles that were present appeared morphologically atretic or prematurely luteinized. The phenotype exacerbated with age, the ovarian interstitium becoming increasingly diffuse and containing an increasing number of morphologically abnormal follicles and haemorraghic cysts (*Fisher CR et al 1998; Britt KL et al 2000*). Secondary and antral follicles become less common in the ovaries and eventually the number of primary follicles also decreased (*Britt KL et al 2000; 2001*). Light microscopy identified the abnormal follicles as seminiferous tubule-like structures filled with Sertoli-like somatic cells, apparently arising from the trans/re-differentiation of granulosa cells (*Britt KL et al 2001; 2004*). Cells morphologically resembling testicular Leydig cells were present within the interstitial regions of *Cyp19a1*KO ovaries (*Toda K et al 2001a*). Adult female *Cyp19a1*KO mice showed osteopenia in the lumbar spine and were characterized by significant decreases in trabecular bone volume and trabecular thickness (*Oz OK et al 2000*).

Male: The *Cyp19a1*KO males from each of the three laboratories all showed defects in sexual behavior with reduced mounting frequency and increased latency between the mounts. However, fertility in the KO males was variable. All mice showed normal spermatogenesis at 14 weeks of age (*Toda K et al 2001a; 2001b; Honda S et al 1998; Robertson KM et al 1999*). One line of *Cyp19a1*KO mice showed progressive disruptions of spermatogenesis until 1 year of age, while at that age all animals showed evidence of spermatogenic disruption. The disruption of spermatogenesis was related to the decrease in the numbers of round and elongated spermatids in the testes. Sperm from *Cyp19a1*KO had a significant reduction in motility. This was associated with Leydig cell hyperplasia (*Robertson KM et al 1999*). In contrast, another *Cyp19a1*KO mouse line showed no disruptions in spermatogenesis at 14 weeks to 10 months of age, although there was a significant reduction in seminiferous epithelial height in these animals (*Toda K et al 2001b*). Testicular histology in older mice from a third line of *Cyp19a1*KO mice has not been described (*Honda S et al 1998*). The reason for the heterogeneity in the phenotypes of different *Cyp19a1*KO mice is unclear. Diet can provide variable levels of phytoestrogens that may contribute to the heterogeneity. Another source of variation could be in the extent of *in utero* exposure of male *Cyp19a1*KO pups to estrogen from the maternal circulation. Adult male *Cyp19a1*KO mice also showed osteopenia, and femur length showed decreased growth (*Oz OK et al 2000*).

2.8.4 Progesterone receptor null mutant mice (*Pgr*KO)

The importance of PGR in female reproduction is underscored by the infertility of *Pgr* null female mice (*Lydon JP et al 1995*). Despite histologically normal ovaries, these mice failed to ovulate even after exogenous stimulation with gonadotrophins, as indicated by the presence of unruptured preovulatory follicles in the ovaries and an absence of oocytes in the oviduct. Female infertility in *Pgr*KO mice was associated with defective uterine implantation and a lack of decidualization of uterine stromal cells in response to P (*Lydon JP et al 1995*). The ductal architecture of adult *Pgr*KO mammary glands was similar to that of an age-matched WT virgin. However, the adult *Pgr*KO mammary glands failed to develop the typical pregnancy-associated epithelial ductal morphogenesis that consists of extensive side-branching with interductal lobuloalveolar development (*lydon JP et al 2000*). Furthermore, *Pgr-A*KO females were infertile, the result of a failure to ovulate and uterine implantation, whereas *Pgr-B*KO females were ovulatory and produced viable offspring. However, in the *Pgr-A*KO female mice, ductal branching and alveolar budding was observed upon stimulation with estrogen and P, showing no adverse effect in development if only *Pgr-A* was deleted (*Mulac-Jericevic B et al 2000*), and the *Pgr-B* isoform is required to carry out the proliferative effects of P on mammary epithelial cells (*Mulac-Jericevic B 2003*). These studies further highlight the capacity of *Pgr-A* and *Pgr-B* to mediate different actions of P (*Mulac-jericevic B and Conneely OM 2004*).

2.9 Function of retinoid receptors from genetic dissections in null mutant mice

Diversity in the control of gene expression by retinoid signals is generated from complexity at different levels of the signaling pathway. A major source of diversity originates from the existence of two families of retinoid receptors, three RAR and RXR isotypes (*Chambon P 1996 and refs therein*). The multiple RAR and RXR receptors selectively control expression of distinct RA-target genes (*Chambon P 1996*), while, the importance of RAR and RXR in vitamin A function has been clearly demonstrated by the generation of null mutant mice for these receptors (*Mark M et al 2006*).

2.9.1 Single null mutants of *Rar* genes

*Rarb*KO mice showed postnatal growth retardation but were normal at adulthood. Disruption of *Rara* results in early postnatal lethality and testis degeneration, but embryonic development is normal (*Lufkin T et al 1993*). Knockout of the *Rarg* gene resulted in early postnatal lethality, male sterility due to squamous metaplasia of the seminal vesicle and prostate gland epithelia, and the developmental changes of the axial skeletal and craniofacial defects (*Lohnes D et al 1993*). *Rar*KO mice display some aspects of the fetal and postnatal VAD syndromes, as well as some additional congenital malformations (Table 4).

2.9.2 Compound null mutants of *Rar* genes

Rar double null mutants die in utero or at birth owe to fetal VAD-induced syndrome (Table 4). *Rar* double mutant mice display severely disrupted development, and almost all abnormalities of vitamin A deprivation are recapitulated by the different combinations of *Rar* double mutants (*Kastner P et al 1995*), ranging from agenesis of the Harderian gland to skeletal defects of the skull, face, vertebrae, and limbs (*Ghyselinck NB et al 1997; Lohnes D et al 1994; Mendelsohn C et al 1994; Mark M et al 2006 and refs therein*).

2.9.3 RXRA is the main RXR isoform and RXRA/RAR heterodimers are involved in embryogenesis

Rxra-null mutants display thinner and less compact layer of the ventricular myocardium, and die by cardiac failure around E14.5 (*Kastner P et al 1997; 1994*). In addition to heart defects, all *Rxra*-null fetuses display a characteristic ocular syndrome associating with a persistent and hyperplastic vitreous body (*Kastner P et al 1994*). Importantly, the fact that mice lacking both Rxrb and Rxrg do not display any obvious morphogenetic defects except for male sterility of *Rxrb* null mutants, even when additionally lacking one allele of *Rxra*, clearly indicates that Rxra is functionally the most important Rxr during morphogenesis of the embryo (*Kastner P et al 1996; Krezel W et al 1996*). *Rar* and *Rxra* loss-of-function mutations support the conclusion that various RXRA/RARs heterodimers are the functional units transducing RA signals during embryogenesis *in vivo* (*Ghyselinck NB et al 1998*).

Table 4. Abnormalities or Abnormalities Absent from the Fetal Vitamin A Deficiency (VAD) Syndrome Present in Single and Compound *Rar*-Null Mutants

Abnormalities or abnormalities absent from the VAD syndrome	Abnormalities in *Rar*-null mutants
Respiratory system defects	
• Agenesis or hypoplasia of the lung	*Rara/Rarb*
Hypoplasia of the ventricular myocardium	*Rara/Rarg*
Heart outflow tract defects	
• Persistent truncus arteriosus	*Rara/Rarb, Rara/Rarg*
• High interventricular septal defect	*Rara/Rarb, Rara/Rarg*
• Abnormal great arteries derived from aortic arches	*Rara/Rarb, Rara/Rarg, Rarb/Rarg*
Kidney hypoplasia and agenesis	*Rara/Rarb, Rara/Rarg*
Ureteral defects	
• Agenesis and ectopia	*Rara/Rarb, Rara/Rarg, Rarb/Rarg*
Genital tract defects	
• Agenesis of the oviduct and uterus	*Rara/Rarb, Rara/Rarg*
• Agenesis of the cranial vagina	*Rara/Rarb, Rara/Rarg*
• Agenesis or dysplasia of the vas deferens	*Rara/Rarg*
• Agenesis of the seminal vesicles	*Rara/Rarg*
• Male sterility	*Rara, g*
Skeletal defects	
• Agenesis or multiple malformations of cranial	*Rara/Rarg*
skeletal elements and limb bones	*Rara/Rarg*
• Homeotic transformation and malformations	*Rara, b, g, Rara/Rarg*
of cervical vertebrae	*Rara/Rarb, Rarb/Rarg,*
• Reappearence of atavistic skeletal elements	*Rara, Rara/Rarg,*
• Pila antotica and ptrerygoquadrate cartilage	*Rara/Rarb*
Ocular defects	
• Coloboma of the retina and optic disc	*Rara/Rarg, Rarb/Rarg*
• Persistence et hyperplasia of the primary	*Rara/Rarb, Rarb/Rarg,*
vitreous body (PHPV)	*Rara/Rarg*
• Hypoplasia of the conjunctival sac	*Rarb/Rarg, Rara/Rarg*
• Thickening of the corneal stroma	*Rarb/Rarg, Rara/Rarg*
• Ventral rotation of the lens	*Rarb/Rarg*
• Shortening of the ventral retina	*Rarb/Rarg*
Other defects	
• Webbed digits	*Rara, g, Rarb/Rarg*
• Abnormal laryngeal cartilages and tracheal rings	*Rarg, Rara/Rarg, Rara/Rarb, Rarb/Rarg*
• Agenesis of the anal canal	*Rara/Rarb*

2.10 Pathophysiological actions of estrogens and androgens in the human

Naturally occurring mutations in humans, which render them devoid of estrogen or resistant to its actions, can provide us with information specific to the role of sex hormones. Such mutations are extremely rare. Only a single case has been reported of a mutation in *ESR1* (*Smith EP et al 1994*). Similar reports of human estrogen deficiency were also lacking in the clinical literature until recently. However, five cases of *CYP19A1* deficiency, a lack of estrogen synthesis have been reported (*Carani C et al 1997; Conte FA et al 1994; Shozu M et al 1991; Morishima A et al 1995; Mullis PE et al 1997*). All three reports of females homozygous for inactivating mutations in the *CYP19A1* gene describe 46, XX DSD, the presence of internal female reproductive structures but ambiguous external genitalia. This phenotype is due to the lack of CYP19A1 activity in the fetus and placental unit during gestation, leading to the accumulation of androgens, which in turn elicit masculinizing effects on the fetus. In all three cases, development of the internal structures of the female reproductive tract did not appear to be altered by a lack of estrogen action (*Conte FA et al 1994; Morishima A et al 1995; Mullis PE et al 1997*). Serum levels of androgens, androgen precursors, FSH, and LH were elevated in the CYP19A1-deficient patients as early as infancy and continued to be elevated as the females approached puberty. At 12–14 years of age, secondary development of the breast, a pubertal growth spurt, and menarche were all absent, but virilization of the external genitalia was reported. In all cases, hyperstimulation of the ovaries and the development of multifollicular cysts were illustrated, even as early as 2 years of age (*Conte FA et al 1994; Morishima A et al 1995; Mullis PE et al 1997*). The ovarian pathology exhibited was similar to that observed in the *Esr1*KO females and was compatible with a diagnosis of PCOS (*Morishima A et al 1995*). Estrogens and P replacement therapy alleviated the above phenotypes.

The clinical syndromes exhibited by the two reported human male cases of CYP19A1 deficiency (*Carani C et al 1997; Morishima A et al 1995*) and the single known human inactivation of the *ESR1* gene (*Smith EP et al 1994*) are strikingly similar. All three patients exhibited a normal onset of puberty and no gender-identity disorders, as well as normal external genitalia and prostate size. The patient lacking functional ESR1 exhibited a testicular volume and sperm density within the normal range for men of his age (*Smith EP et al 1994*). A normal testicular volume was also reported in one of CYP19A1-deficient patients (*Morishima A et al 1995*). However, small testes and severe oligozoospermia and infertility were reported in the other male lacking CYP19A1 (*Carani C et al 1997*), although the presence of similar findings in a brother with a normal *CYP19A1* gene suggested other possible familial factors for this finding. Serum levels of androgens, FSH, and LH were all consistently elevated in the CYP19A1-deficient males (*Carani C et al 1997; Morishima A et al 1995*), as well as E_1 and E_2 in the ESR1-deficient male (*Smith EP et al 1994*).

The most overt phenotypes in all patients lacking sufficient estrogen action involved the skeleton. Females homozygous for mutations of the *CYP19A1* gene displayed a delayed in bone age, a significant decrease in bone mineral density of the lumbar spine, and absence of the pubertal growth spurt (*Conte FA et al 1994; Mullis PE et al 1997*). Both the ESR1-deficient male and the two males lacking CYP19A1 were evaluated as adults, and exhibited strikingly similar skeletal phenotypes. All three were characterized by tall stature with continued slow linear growth, a low upper/lower body segment ratio, unfused epiphyses, bone age of 14–15 years, and decreased bone density (*Smith EP et al 1994; Carani C et al 1997; Morishima A et al 1995*).

On the basis of these observations, the inhibition of estrogen action by aromatase inhibitors (AIs) could decelerate the process of growth plate fusion, and thus AIs may be used therapeutically to increase adult height. Testolactone, a nonselective steroidal

AI, has been used successfully as an adjunct to antiandrogen and gonadotropin-releasing hormone analogue (GnRHa), therapy for children with familial male-limited precocious puberty (FMPP) and congenital adrenal hyperplasia (CAH), and with some success in girls with McCune-Albright syndrome (*Shulman DI, 2008 and refs therein*). The boys with delayed puberty treated with letrozole, a selective nonsteroidal AI, + testosterone delayed bone maturation and good growth response and achieved an increase in predicted adult height. Aromatase inhibitors may thus provide a new and effective treatment modality for growth disorders of various etiologies (*Dunkel L 2006 and refs therein*).

Almost 500 germ line mutations for the *AR* gene causing an androgen insensitivity syndrome are known in the literature (*Gottlieb B et al 2004*). Phenotypes of *AR* mutations can be grouped into three classes: complete, partial, and mild androgen insensitivity. Complete androgen insensitivity syndrome (CAIS) is characterized by normal female external genitals, the absence of the uterus and the presence of testes. This kind of phenotype is either detected in early childhood due to the presence of labioscrotal or inguinal testes or quite often it is revealed at puberty with primary amenorrhoea (*Ahmed SF et al 2000*). In partial androgen insensitivity syndrome (PAIS), external genitalia are ambiguous and therefore PAIS is evident at the time of birth. Mild androgen insensitivity syndrome (MAIS) is not easily detected, and its phenotype may be, for example, infertility (*Yong EL et al 2003*).

3. AIMS OF THE PRESENT STUDY

Although HSD17B2 has been suggested to protect sex steroid target tissues from excessive sex hormone influence, the physiological function of HSD17B2 *in vivo* is only superficially known. To gain further insight, transgenic mice ubiquitously expressing human *HSD17B2* under the CMV-enhanced chicken β-actin promoter were generated. These mice provide a novel tool to study the physiological function of the HSD17B2 *in vivo*, particularly to identify the metabolic pathways regulated by HSD17B2 activity.

The specific aims of the study were:

1. To construct a transgenic mouse model ubiquitously expressing the human *HSD17B2* (HSD17B2TG mice) (I).
2. To characterize the structure and function of the testes, epididymides and prostate in the HSD17B2TG male mice (I).
3. To analyze the bone phenotype, body growth and pubertal development in the HSD17B2TG male mice (II).
4. To study the structure and function of the mammary glands and ovaries in the HSD17B2TG female mice (III).
5. To study the putative opposite roles of HSD17B1 and HSD17B2 in the sex steroid metabolism in the HSD17B1/B2 double transgenic mice (III)

4 MATERIALS AND METHODS

4.1 Experimental animals (I-III)

WT FVB/N mice were used as controls (I-III). However, in the measurement of body growth the non-TG littermates of the same litter were used in order to standardize the nutritional state (I, III). Non-TG littermates were also used in the studies aimed at rescuing the testis phenotype observed in the HSD17B2 TG males (I).

HSD17B2TG females were used as breeders, and were crossed with WT FVB/N males to maintain the colony, while the bi-TG (HSD17B1-HSD17B2TG) mice were generated from crossbreeding HSD17B1TG males with HSD17B2TG females (I-III).

The animals were handled in accordance with the institutional animal care policies of the University of Turku and with appropriate permissions. The mice were housed one to four per cage with commercial soy-free mouse chow and tap water *ad libitum*, in controlled conditions of light and temperature (I-III).

4.2 Production of transgenic mouse lines (I)

A 1.4 kb full-length Sol I/ Not I fragment of human *HSD17B2* was excised from an original vector, and ligated to the pCR® 4Blunt-TOPO vector with the TOPO® Shotgun Subcloning Kit. Moreover, the EcoR I fragment excised from the pCR® 4Blunt-TOPO vector was ligated to the pCAGGS vector under the chicken β-actin promoter (I).

HEK-293 cells were transiently transfected with the pCAGGS-*hHSD17B2* construct using the lipofectamine method. After overnight the cells were incubated with 2 ml of serum-free medium containing 200 nM [^{3}H]-E_2 (2×10^5 cpm/ml) at 37° C for 1, 2, 4, 8 and 24 h. The amount of E_2 converted to E_1 was analyzed by separating E_1 and E_2 using a high performance liquid chromatography system (HPLC) (I).

The 3.6 kb long fragment containing the transgene (pCAGGS-*hHSD17B2*) was released from the vector bone by digestion with Sol I and Hind III enzymes (I). The DNA fragment was isolated and purified, and then diluted in TE buffer to 2 ng/μl concentration (I).

Transgenic founder mice were generated in the genetic background of the FVB/N strain by microinjection of the DNA fragment into the pronuclei of fertilized oocytes using standard techniques (I).

4.3 Genotyping (I-III)

For routine genotyping of the HSD17B2TG and bi-TG mice, PCR analyses were carried out using DNA extracted from ear biopsies (I-III).

For the genotyping of bi-TG mice, in addition to PCR analysis of the human *HSD17B2* transgene, one DNA fragment (240 bp) was also amplified using the primer specific for human *HSD17B1* (III).

4.4 The preparation of skeleton samples (II)

At the age of 26 days the young male mice were given intraperitoneal (ip) injections of oxytetracycline and calcein five days and two days before the mice were euthanized. The same injections for the adult mice at the age of two and six months were given eight days and two days before euthanasia (II).

The left tibiae and femora were stored in 96% ethanol at 4°C for histomorphometric study, and the right tibiae and femora were stored at –20°C for bone density measurement (II).

4.5 Growth and fertility studies (I-III)

Growth of the HSD17B2TG (lines 141 and 011) and WT mice was analyzed (at least 6 mice/group) by weighing the mice twice a month. In the HSD17B2TG-141 mouse line, eye opening was analyzed and compared with WT mice (I).

For analyzing the onset of male and female puberty, HSD17B2TG and WT males of the same litters were examined daily for balanopreputialseparation (II).

HSD17B2TG and WT females of the same litters were examined daily for vaginal opening (III).

The duration of the estrous cycle was determined in female mice by analyzing the vaginal smears daily (III).

For testing fertility, both HSD17B2TG male and female mice were bred with WT FVB/N mice at the age of 2 months separately. Continuous mating was carried out to analyze the fertility of the HSD17B2TG mice up to twelve months of age (I, III).

4.6 HSD17B activity measurements *in vivo* and *in vitro* (I)

HSD17B activity was determined in HSD17B2TG mouse embryos (E14.5) *in vitro* using the method described (*Tseng L and Gurpide E, 1974*), with minor modifications. Protein concentrations were determined from the embryo homogenates in 10 mM KH_2PO_4, pH 7.5, 1 mM EDTA, 0.02% NaN_3, and different amounts of total proteins (10-200 µg) were mixed with [^{3}H]-E_2 (5×10^5 cpm) to a final concentration of 6.3 nM. The reaction was started at 37°C by addition of 50 µl of NAD+ (Sigma-Aldrich, MO, USA, final concentration 1.4 mM). After 60 min incubation the steroids were extracted by ether extraction, evaporated to dryness and dissolved in 150 µl acetonitrile-water (48:52, vol:vol). The amount of E_2 converted to E_1 was analyzed by separating the E_1 and E_2 using HPLC system.

HSD17B activity was measured *in vivo* with two-three month old HSD17B2TG-141 male mice. [^{3}H]-E_2 in a final concentration of 5.6 µg/kg was used as substrate. An *i.v.* injection of 2.5 µl/g body weight was given via the tail vein. The blood was collected by cardiac puncture after 18 minutes. The amount of E_2 converted to E_1 was analyzed as described above in the HSD17B activity measurements *in vitro* (I).

4.7 Morphological and histological analysis (I, III**)**

HSD17B2TG, bi-TG and WT mice were sacrificed at the age of four and six months. The blood was allowed to coagulate overnight and centrifuged to separate serum. The serum samples were stored at -70°C until analyzed (I, II, III).

For morphological and histological analyses organs were removed. The tissues were fixed in 4% paraformaldehyde (PFA). The testes were also fixed in Bouin's solution. The five-µm thick sections were stained with Harris hematoxylin and eosin for macroscopic analyses (I, III).

For the whole mount preparations, the inguinal mammary glands were dissected out and fixed, and stained overnight at room temperature (III).

4.8 Immunohistochemical analyses (I, III)

For immunohistochemistry, the five-µm thick paraffin sections were cut from the 4% PFA-fixed testis and mammary glands. The sections were incubated with one of the following antibodies in PBS containing 3% BSA at +4˚C (I, III): 1) a rabbit polyclonal antibody for androgen receptor, 2) a rabbit polyclonal antibody for cleaved caspase-3, 3) a rat monoclonal antibody for mouse Ki-67 antigen, 4) a mouse monoclonal antibody for human estrogen receptor 2, 5) a mouse monoclonal antibody for human estrogen receptor 1, 6) a rabbit polyclonal antibody for human progesterone receptor. The primary antibodies were detected by biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-rat IgG followed by incubation with avidin-biotin-peroxidase complex and

the colour was developed with DAB as a substrate or by using the DAKO Envision+® system-HRP (I, III).

4.9 Hormone measurements (I-III)

Serum FSH and LH were measured by immunofluorometric assays (I, III).
Serum T was measured from diethyl ether extracts of serum using a radio immunoassay (RIA). Tissue T was determined from diethyl ether extracts of the tissue homogenates (I, II).
Concentrations of serum E_2 and P were measured after extracting the steroids by diethyl ether using E_2 and P Delfia Kits (III).
The prolactin (Prl) level was measured with RIA using a mouse Prl antibody.
Serum osteocalcin and insulin-like growth factor 1 (Igf1) levels were measured with commercially available ELISA kits according to the manufacturer's instructions (II).

4.10 RNA analysis (I-III)

Total RNA was isolated from various tissues and primary osteoblasts with RNeasy Mini Kit (I, II, III).
Mammary glands and brain RNA were isolated using the RNeasy Lipid Tissue Mini Kit (I, III).
Prostate RNA was extracted and isolated from homogenates by applying the cesium chloride (CsCl)-ultracentrifuge method (I).
Primary osteoblasts were obtained from the calvaria (frontal and parietal bones of skull) of two or three-day-old neonatal mice (II).
One μg of total RNA was treated with DNase I (I-III).
RT-PCR and Quantitative (q) RT-PCR analyses were used to detect tissue distribution of human *HSD17B2* transgene and to compare human *HSD17B2* transgene expression in the HSD17B2TG-141 and HSD17B2TG-011 lines for a selected amount of tissues, respectively (I).
RT-PCR was performed to analyze the stage-dependent genes in the osteoblasts (II).
qRT-PCR analysis was performed to analyze androgen-dependent genes in the epididymides, prostate, and specific genes in the development of mammary glands (I, III).
qRT-PCR analysis was performed to analyze retinoic acid target genes in the testes, and estrogen and P target genes of mammary glands transplanted WT mammary epithelium (I. III).
All qRT-PCR data were normalized to the expression of mouse ribosomal protein L19 (*Rpl19*). While Esr1 and Pgr target genes of mammary glands transplanted in the WT mammary epithelium were normalized to the average expression of *Rpl19* and peptidylprolyl isomerase A (*Ppia*) (I, III).

4.11 pQCT measurements and bone histomorphometry (II)

Bone density was measured and analysed from the right tibiae and femora by pQCT scanner (II).
The left tibiae and femora were dehydrated, degreased, and then embedded into methylmethacrylate. Four and eight μm thick longitudinal undecalcified sections from the tibia and 80 μm thick cross sections of the femur were prepared, and stained using the Masson-Goldner-trichrome method. Furthermore, the histomorphometric data was measured (II).
The calculation of the growth rate of growth plate is based on the double fluorescence labelling. The distance (μm) between the two fluorescence labeling lines divided by labeling time period (days) is defined as the growth rate of growth plate (II).

The eight and 80 μm unstained sections were used to measure the dynamic parameters of bone formation (II).

4.12 4-[(E)-2-(5, 6, 7, 8-Tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB) treatment (I)

HSD17B2TG and WT males of the same litters were treated with vehicle solution or with a retinoic acid receptor agonist, TTNPB, 8 mice per group. The mice were treated with 1.5 μg TTNPB/kg body weight twice a week from the age of 20 days until the age of 4 months. The testes were fixed in Bouin's solution and the five-μm thickness sections were stained with Harris hematoxylin and eosin. All the seminiferous tubules in three independent sections from one testis of all the mice were evaluated under the microscope (I)

4.13 Mammary gland transplantations (III)

The endogenous epithelium of both the 4th mammary glands of 21day-old FVB/N female mice was surgically removed (III).

A small piece from the adult donor HSD17B2TG female mammary glands was inserted into the hosts (III).

The mice were sacrificed at the age of 5 months and the transplants were divided into three parts and examined with whole mount, histological analysis and RNA analyses (III).

4.14. Statistical analysis (I-III**)**

Statistical analyses were performed using the SigmaStat program.

All data were analyzed by t-test or Mann-Whitney rank sum test, and by one-way repeated measures analysis of variance (ANOVA) followed by the Holm-Sidak test or Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Dunn's test. Significance was set as $p<0.05$ and the values were presented as mean ± standard deviation (SD) or standard error of the mean (SEM).

5. RESULTS

5.1 Generation of mice expressing human HSD17B2 (I)

The *pCAGGS-hHSD17B2* construct was transiently transfected in the HEK-293 cells and measured to convert E_2 to E_1 up to 24 hrs. With the 0.4 nmol $E_2/10^5$ cells, most of E_2 was converted to E_1 in the 17HSD17B2–expressing cells within four hrs, while an opposite reaction was not detected, indicating that human HSD17B2 under the chicken β-actin promoter catalyzed oxidative HSD17B activity in cultured cells (*Wu L et al 1993; Puranen TJ et al 1999*).

Fifteen HSD17B2 founder mice were obtained (seven females and eight males). Two independent founders were selected for further studies by their growth retardation phenotype and the genomic southern blot analysis showing the integration of several copies of transgene (HSD17B2TG-141 mouse line with a higher copy number of transgene and HSD17B2TG-011 mouse line with a lower copy number of transgene). Other mouse lines did not present any obvious phenotype. At the age of four months, qRT-PCR analyses were carried out in RNA extracted from the heart, kidneys, liver and testes. A different level of transgene expression was indicated in HSD17B2TG-141 and HSD17B2TG-011 mice. Furthermore, RT-PCR analysis in HSD17B2TG-141 and HSD17B2TG-011 indicated ubiquitous transgene expression in various mouse tissues.

Table 5. Oxidative HSD17B Activity in HSD17B2TG Fetuses *in vitro*

Embryo number	Protein (µg)	E2->E1 conversion (%)
HSD17B2TG founder fetuses [*] [a]		
1370/16	100	23.9
1371/10	50	2
1457/10	50	58.1
1458/4	50	79.6
1470/2	50	5.4
1470/3	50	52.4
1473/5	50	45.4
6 HSD17B2TG founder fetuses did not show detectable activity		
HSD17B2TG-141 fetuses [*]		
1738/1	200	0
1738/4	200	0
Wild type fetuses [*]		
1457/1	200	0
1457/2	200	0
1461/4	200	0

[*] All fetuses were collected at E14.5 age. [a] HSD17B2TG founder fetuses were generated by microinjection of the DNA into the fertilized oocytes and then transferred into recipient female mice.

The activity of the HSD17B2 transgene in the HSD17B2TG-141 mice was measured to convert E_2 to E_1 *in vivo* by injecting [^{3}H]-E_2 into the tail vein. A slightly increased oxidative HSD17B-activity could be detected in the mice as compared with wild type ($p < 0.05$). Similar results were observed by measuring the conversion of E2 to E1 in tissue slices *ex vivo*, or in tissue homogenates *in vitro* ($p > 0.05$). However, HSD17B2TG-011 did not show any oxidative HSD17B-activity. Thus, it was important that more founder TG mice were generated and analyzed at fetal age. 700

microinjected oocytes were transferred into 25 recipient females, and 158 fetuses were collected at E14.5, out of which 13 TG fetuses were identified. Seven HSD17B2TG fetuses presented with detectable HSD17B-activity, while six TG fetuses did not. Similarly, the HSD17B-activity in the fetuses of the HSD17B2TG-141 mouse line was below the detection limit (Table 5). The results confirmed that the transgene possesses HSD17B activity in TG mice, and suggested a reduced survival rate for mice with high HSD17B2 activity. Furthermore, some founder fetal mice with high HSD17B2 activity displayed the delay of the limb development (Fig. 5).

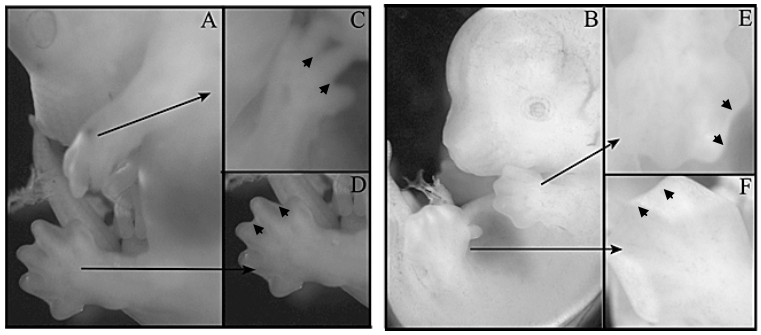


Figure 5. (A) Wild-type (WT) and (B) HSD17B2TG fetuses at E14.5.
HSD17B2TG fetus with high HSD17B activity displayed delayed limb development, as compared with WT fetus. C and D are higher magnification of A; E and F are higher magnification of B. The *arrowheads* in the C, D, E and F further showed the different phenotype of limb development between WT and HSD17B2TG fetuses.

5.2 Body growth and reproductive organ weights (I-III)
Evaluation of the HSD17B2TG-141 and HSD17B2TG-011 mouse lines revealed that the growth of HSD17B2TG mice was slower during postnatal development and young adulthood compared with WT littermates. The differences in body weight became significant at the age of one month for males and at the age of 14 days for females. At the age of one month the body weight of HSD17B2TG-141 and HSD17B2TG-011 males was 40-50% reduced as compared with WT males ($p<0.001$), and at the age of one and two months the female body weight of two mouse lines was 20-30% reduced as compared with WT females ($p<0.001$). However, the life span of HSD17B2TG mice was normal. The body weight in the HSD17B2TG-141 mouse line increased very slowly after puberty, as compared with WT and HSD17B2TG-011. Up to the age of 6 months, HSD17B2TG-141 females and HSD17B2TG-011 mouse line had a normal body weight.
A significant reduction in organ weight (($p<0.001$) was detected only for the testes and epididymides in the HSD17B2TG-141 males at the age of four and six months. Both male and female HSD17B2TG-141 mice also displayed delayed eye opening. At the age of 13.5 days, 75% (82 out of 109) of the WT mice had both eyes opened, whereas only 6% (4 out of 64) of the HSD17B2TG mice did. In some HSD17B2TG mice one of the eyes showed a squinting phenotype several days after eye opening. All the WT mice had both eyes opened at the age of 15.5 days, whereas HSD17B2TG mice were at the age of 25.5 days.
Furthermore, the data indicated that both HSD17B2TG-141 male and female mice presented with delayed onset of puberty. The day of the balanopreputial separation was 29.0 days ± 0.7 in WT males and 34.9 days ±1.7 in HSD17B2TG males. Vaginal opening was detected in WT female littermates at the age of 26 days (26.2 days ±1.7),

whereas the onset of puberty was observed in the HSD17B2TG female mice 6 days later (32.9 days ±3.0, $p<0.001$), measured by the age of vaginal opening daily.

5.3 Breeding and fertility (I, III**)**

The fertility of the HSD17B2TG males was analyzed up to six months of age. The fertility of the HSD17B2TG-011 males was proven to be normal, whereas 50% of the HSD17B2TG-141 males were sub-fertile and 50% infertile at the age of two months, and at about three months of age all the HSD17B2TG males became infertile.

The litter size for HSD17B2TG-141 females was significantly smaller (5.1±0.8 pups) than that for WT females (9.7±1.1 pups). Abnormal estrous cycle in HSD17B2TG-141 females was observed at the age of 8-12 weeks. Average duration of the cycle in TG mice was 8.7±1.6 days, and in the WT mice 5.3±0.6 days ($p<0.001$). Especially the proestrous and diestrous stages were significantly longer in HSD17B2TG-141 females. Due to the abnormal estrous cycle, there was a 1-1.5 month long delay in the first pregnancy for HSD17B2TG females. At the age of 6 months, a number (15%) of HSD17B2TG-141 females became infertile, and at the age of 8 months most of HSD17B2TG-141 females were infertile. The histological appearance of the ovaries was, however, apparently normal in 4-month-old HSD17B2TG-141 females.

5.4 Circulating hormone concentrations (I-III)

The mean serum and intratesticular T concentrations tended to be lower in the HSD17B2TG males as compared with WT males at 26 days of age. However, no difference was found in serum LH, FSH and P concentrations, or in intratesticular and serum T concentrations among WT, HSD17B2TG-141 and HSD17B2TG-011 males analyzed at adulthood. For example, at the age of four months the serum T values were 2.5±2.6 ng/ml for WT, 1.4±1.4 ng/ml for HSD17B2TG-141 and 2.2±1.3 ng/ml for HSD17B2TG-011 male mice. No significant changes were observed in intratissular T concentrations between HSD17B2TG-141 and WT males measured in the prostate, kidneys, heart, muscle and brain at four months of age.

No significant difference was found in E_2 levels between adult HSD17B2TG-141 and WT females, while the P concentration was significantly elevated in the HSD17B2TG females (HSD17B2TG: 9.1 ± 3.2 nmol/l; WT: 4.6 ± 1.7 nmol/l, $p<0.01$). Interestingly, serum LH and prolactin levels were also increased four-fold (LH, HSD17B2TG: 1.2 ± 0.7 ng/ml; WT: 0.3 ± 0.3 ng/ml, $p<0.01$ and Prolactin, HSD17B2TG: 414.0 ± 177.8 ng/ml; WT: 97.7 ± 90.7 ng/ml, $p<0.001$). In addition, no significant increase was found for FSH levels in the HSD17B2TG females as compared with age-matched WT mice. High LH levels and the reduced litter size together with the prolonged estrous cycle indicate a defect in the luteinization and ovulation of the HSD17B2TG females.

5.5 Osteocalcin and Igf1 concentrations in the serum (II**)**

As the growth hormone GH/IGF1 axis plays an important role in the overall skeletal size and development. Serum Igf1 concentration was measured in HSD17B2TG males at the age of 26 days, and found to be 54% lower than in WT males ($p<0.001$), whereas, the serum Igf1 concentration in the HSD17B2TG at the age of two months was found to be higher than in WT males ($p<0.05$). The serum Igf1 levels in HSD17B2TG males also correlated well with skeleton parameters and postnatal growth retardation. Serum osteocalcin concentration is a marker for bone formation. Igf1 can regulate the osteocalcin level. In the HSD17B2TG males, the osteocalcin levels were decreased by 44% ($p<0.001$) at the age of 26 days but was not significantly different at the older age groups, as compared with WT littermates.

5.6. Expression of androgen-dependent genes in the prostate and the epididymides (I)

Although the HSD17B2TG-141 males were infertile, the prostate and seminal vesicle size and their histology were normal, indicating proper androgen action. Despite the normal histological appearance, an androgen action was further confirmed by analyzing the mRNA expression of the androgen dependent genes in the prostate. The analysis of qRT-PCR showed four out of five gene expressions were not significantly different between the HSD17B2TG and WT mice, while *Srd5a2* showed slightly reduced expression in HSD17B2TG mice compared with WT mice. However, due to the disrupted spermatogenesis in the HSD17B2TG males, the *lcn8*, *lcn5* and *Rnase 9* mRNA expression in the epididymides was significantly different between HSD17B2TG and WT mice. Especially, *lcn8* and *lcn5* mRNAs were markedly down-regulated. In the epididymides, the gene expressions are regulated by both the testicular factors from testes and circular hormones.

5.7 Histology of the testis (I)

Germ cell degeneration caused reduced testicular weight, and this also resulted in the infertility. At the age of six months, seminiferous tubules were severely atrophic and mostly contained only Sertoli cells. Spermatogenesis was more extensively disrupted in older males compared with those at the age of four months, indicating a progressive degenerative process. This was in line with the observed progression of the subfertility to infertility in 50% of the TG males. Histological analysis of the spermatogenetic cycles showed that full spermatogenesis was first established. The cellular contents of the seminiferous epithelium varied in different cross-sections of seminiferous tubules. The changes were not specific for defined stages of the seminiferous epithelial cycle. Between the ages of four and six months, the degeneration proceeded fast and Sertoli cell-only histology ensued. In many seminiferous tubules, the early phases of spermatogenesis (spermatogonia and spermatocytes) were missing, while spermatids were still present, suggesting a block in proliferation.

Immunohistochemical staining for an apoptotic marker (cleaved caspase-3) indicated that there was no significant difference in the apoptosis rate in the germ cells between HSD17B2TG-141 and WT males at the age of two months. However, this did not exclude the possibility of an increased apoptotic rate at other time points. The HSD17B2TG testes expressed AR normally in the Sertoli, Leydig and myoid cells. In WT mice, AR staining was strongest at stage VII-VIII, whereas in HSD17B2TG staining was more even in all stages, suggesting that stage specificity typically observed in mice may depend on the presence of germ cells

5.8 Rescue of the testis phenotype (I)

TTNPB, as a highly effective synthetic retinoid acid analog having a high affinity for all RAR isoforms but not for RXRs, is not metabolized by HSD17B2. To investigate whether HSD17B2 was involved in the metabolism of retinoids and whether the testis phenotype was due to the lack of retinoid action HSD17B2TG males were treated with TTNPB for 3.5 months. Although the TTNPB treatment did not result in normal spermatogenesis completely, the seminiferous tubule damage was significantly reduced. The amount of tubules with very severe and severe damage was reduced from 39.6±12.9% and 34.1±3.7% in placebo treated mice to 14.6±10.7% and 19.6±7.1% in TTNPB treated mice, respectively, whereas, the amount of moderately or mildly damaged tubules increased, and the amount of normal tubules increased from 1.3±1.3% to 6.5±4.3% with TTNPB treatment. Furthermore, the expression of *Rbp1*, *Rara* and *Stra8* (three RA-target genes) in the testes of HSD17B2TG males increased significantly after the TTNPB treatment. As a control experiment, the non-

transgenic littermates treated with TTNPB and placebo did not affect spermatogenesis.

5.9 Bone growth and dimensions (II)
Tibia and femur length of HSD17B2TG males were significantly shorter as compared with those of the WT animals, both the ash weight of tibia and femur were also much lower in the HSD17B2TG males at the age of 26 days. However, at the age of two and six months, the differences in tibia and femur length between the TG and WT animals were obviously decreased, and the differences of the bone ash weights disappeared.

5.10 Peripheral quantitative computerized tomography (II)
At the prepubertal age of the HSD17B2TG mice, the density of both trabecular and cortical bones was significantly lower in the HSD17B2TG than in the WT animals. The bone tissue area, cortical bone area and polar moment of inertia of the HSD17B2TG mice were also much smaller as compared with the WT males. Interestingly, at the adulthood most parameters in the HSD17B2TG mice normalized to the level of the WT males. However, some of the parameters were still statistically different between the HSD17B2TG and the WT mice. The only exception was that the density of trabecular bone in section 1 of the proximal tibia was significantly higher in the HSD17B2TG males as compared with that of the WT mice at the age of six months.

5.11 Bone histology and morphometry (II**)**
Histomorphometric data of the proximal tibia showed that in young mice the percentage of trabecular bone volume and trabecular bone thickness was significantly decreased in the HSD17B2TG males. In adult HSD17B2TG males trabecular bone volume return to the normal level, but the difference was maintained in trabecular bone thickness between the HSD17B2TG and the WT animals. However, the trabecular bone growth was not different between the HSD17B2TG and the WT at an adult age. At the age of 26 days, the thickness of the growth plate of proximal tibia was reduced in the HSD17B2TG males as compared with WT controls, but at adult age the growth plate thickness was identical**.** The growth rate of the growth plate was slower in the HSD17B2TG male mice at the age of 26 days. However, at the age of two months the growth rate of the growth plate in the HSD17B2TG male mice was found to be faster than in the WT males.
HSD17B2 expression in the males induced a thinner femoral shaft in young 26-day old mice that was normalized at the age of two and six months. The obviously decreased bone formation rate at the periosteum after puberty in the HSD17B2TG males returned to the normal level at adulthood. At the endocortical surface, the bone formation rate was much higher in the HSD17B2TG males than in the WT nimals in all three age groups.

5.12 RT-PCR analysis of the osteoblast-specific gene expression (II)
The primary osteoblasts were isolated from the calvaria of neonatal HSD17B2TG mice (two or three day old). The human *HSD17B2* transgene expression was identified in the primary osteoblast obtained from the HSD17B2TG mice. To investigate if the bone phenotype of HSD17B2TG at prepubertal age is associated with the osteoblast differentiation, RT-PCR analysis was used to analyze the expression of osteoblast-specific genes. Three of the marker genes of osteoblast differentiation (Runx2, osteopontin and osteocalcin) were expressed at a lower level, and one (collagen type 1a) was expressed normally in the primary osteoblasts isolated from the HSD17B2TG mice.

5.13 Mammary gland hyperplasia (III)

The whole mount analysis showed a substantial lobuloalveolar development in virgin HSD17B2TG mice at the age of four months. Histological examination of the mammary glands of the HSD17B2TG mice further revealed considerable alveolar hyperplasia throughout the gland epithelium, with a large number of vacuoles filled with lipid and milk-like secretion, resembling the pre-lactating mammary gland epithelium.

Immunohistochemical staining indicated that hyperplasia of epithelial cells in the HSD17B2TG females was associated with an observable increase in Mki67 (Ki-67) expression. Furthermore, qRT-PCR analyse confirmed the increased expression of pregnancy-induced differentiation markers such as *Wap*, *Csn2*, *Expi* and *Lalba* ($p<0.001$). The data also indicated that a ductal epithelial marker, *Slc12a2,* was unchangeable but a marker for the secretory function of lactating females, *Slc34a2,* was expressed at a high level ($p<0.001$). The high expression of downstream target genes of Prl and Pgr, *Stat5a* and a *Ccnd1*, also indicated the pregnancy-like phenotype of mammary gland in the HSD17B2TG females. While the expression of the *Cdkn1b* gene, a member of the Cip/Kip family that inhibits cyclin and cyclin-dependent kinase (CDK) complexes, was decreased.

5. 14 Mammary gland transplantation (III)

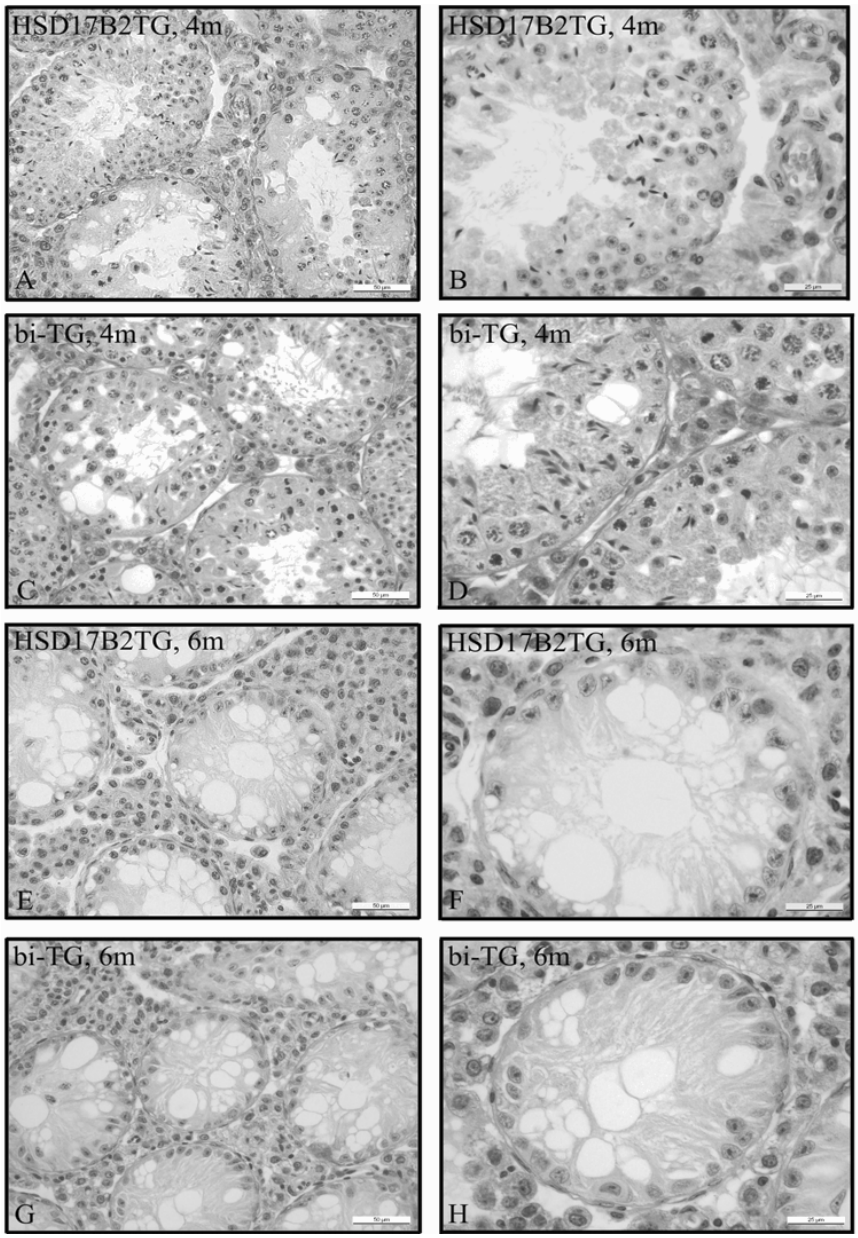
To determine if mammary gland phenotype was associated with the endocrine status of the HSD17B2TG females, HSD17B2TG mammary glands were transplanted into WT females. The results revealed that the pregnancy like phenotype disappeared in the TG mammary gland transplanted into a WT host. Thus, the enforced local expression of HSD17B2 in the mammary gland epithelium had no marked effect on ductal morphology, indicating that the macroscopic phenotype observed was caused by the the systemic hormone alteration.

By immunohistochemistry, Esr1 and Pgr were detected in the WT and HSD17B2TG mammary gland epithelium and in the TG epithelium transplanted into the WT host. Furthermore, we validated the local HSD17B2 activity for the putative modulation of estrogen action by analyzing the expression of a set of estrogen target genes in the TG mammary gland transplanted into a WT host. A significant down-regulation was obtained for *Areg*, *C-fos* and *Gata3*, while *Esr1* was slightly up-regulated. These data suggest a reduced estrogen action in the HSD17B2TG transplanted mammary glands as compared with the WT mammary glands. Some of the Pgr target genes (*Msx2* and *Wnt4*) were significantly down-regulated in the TG mammary gland, whereas *Pgr* itself was not significantly altered.

5.15 Bi-transgenic mice (HSD17B1-HSD17B2TG) (III)

HSD17B1 and HSD17B2 have opposite activities *in vivo*. We speculated that the phenotypes observed in the HSD17B2TG females could be rescued by the expression of HSD17B1. Bi-TG (HSD17B1TG-HSD17B2TG) mice in which the transgenes were expressed under the same ubiquitous promoter were generated. Our data showed that several primary phenotypes observed in both single TG females were not efficiently rescued in the bi-TG mice. The growth retardation observed in the HSD17B2TG females was persistent also in the bi-TG mice with high HSD17B1 activity. Bi-TG females showed lack of vaginal opening, improper separation vagina and urethra, suppressed nipple development and ovarian benign serous cystadenomas, similar to that previously reported for HSD17B1TG females. However, androgen-dependent increase in the anogenital distance and reduced uterus weight of HSD17B1TG females were partially rescued in bi-TG mice. The data collectively indicate that HSD17B2 modestly inactivates androgen and estrogen action *in vivo*.

But the main phenotypes of the HSD17B2TG mice, such as growth retardation, are sex hormone independent.
Like HSD17B2TG males, Bi-TG males also showed growth retardation and severely disrupted spermatogenesis (Fig. 6). Histological analysis showed that at the age of six months, seminiferous tubules were severely atrophic and contained mostly only Sertoli cells (Fig. 6G and 6H).



Figture 6. Histology of the testes of HSD17B2TG and bi-TG mice.
A and B: four-month-old HSD17B2TG mice, C and D: four-month-old bi-TG mice, E and F: six-month-old HSD17B2TG mice, G and H: six-month-old bi-TG mice. Similar degeneration of the seminiferous epithelium was detected in the HSD17B2TG and bi-TG mice (A-H).

6 DISCUSSIONS

6.1 Growth retardation and delayed puberty

One of the most notable findings in the HSD17B2TG mice was the postnatal growth retardation. Interestingly, the phenotype observed did not mimic the phenotypes of mice deficient in estrogen or androgen action. The independency of the phenotype from sex steroid action was supported by the findings, showing that the expression of HSD17B1, with the ability to activate E_1 to E_2 and $\triangle^4$-dione to T (*Saloniemi T et al 2007*), was not able to rescue the body size in the bi-TG mice expressing both HSD17B2 and HSD17B1. Previous data have also shown that body growth and puberty onset were normal in *Esr1* (*Vidal O et al 2000; Sims NA et al 2002*), *Esr2* (*Windahl SH et al 2001; 1999*) and *Cyp19a1* deficient male mice (*Jones ME et al 2000*), while the *Ar* deficient male mice have a female-like appearance and reduced body weight (*Yeh S et al 2002*). Similarly, *Esr* and *Cyp19a1* deficient females were different from those of the HSD17B2TG females. For example, in *Esr1* deficient females, the changes of body weights were observed in mice only after eight weeks of age and in those mice the body weight was not decreased (*Vidal O, 1999*; *Lindberg MK 2001*). In the *Esr2* and *Cyp19a1* deficient female mice, the increases of body weight were reported at 11-12 weeks (*Windahl SH et al 1999; Jones ME et al 2000*), whereas, *Ar* deficient female mice exhibited no phenotypic abnormality up to eight weeks (*Kawano H et al 2003*).

The lack of inappropriate androgen action in HSD17B2TG males, the delayed eye opening, the growth retardation and the delayed puberty HSD17B2TG mice, together with the lack of correlation between HSD17B2 expression and the hormonal status in female rat (*Akinola LA et al 1997*) prompted us to seek for other molecular mechnism putatively affected by HSD17B2 expression. Interestingly, *HSD17B2* is a close homolog for a number of retinoic acid metabolizing enzymes (*Baker ME 2001*). Thus, it is interesting to note that some of the phenotypes observed, such as the growth retardation in HSD17B2TG mice resembles the phenotype of *Rar* deficient mice (*Lohnes D et al 1993; Lufkin T et al 1993; Ghyselinck NB et al 1997*). For example, the *Rara* and *Rarg* deficient mice are not embryonically lethal, and no obvious malformations or lesions were macroscopically or histologically detected. However, they exhibited a slower growth rate and postnatal lethality. Those mice that survived till the age of two months, however, were similar in size compared with their WT littermates. Furthermore, the *Rarb* mutants are growth-deficient, but have normal longevity. Of the *Rxr* mutant mice, heterozygous mice with the disrupted *Rxra* allele and the *Rxrb KO* mice exhibited a slower growth rate or postnatal lethality (*Kastner P et al 1994; 1996*). The various *Rar* double mutant mice died in utero or at birth, and *Rar/Rxr* compound and various *Rxr* double mutant mice exhibited the similar phenotypes observed in single *Rar* and *Rxr* mutants (*Kastner P et al 1997; 1994; Lohnes D et al 1994; Mendelsohn C et al 1994; Krezel W et al 1996*).

6.2 Infertility of the HSD17B2TG male mice

The HSD17B2TG-141 males were infertile, but no significant changes were found in the reproductive endocrine parameters. The disrupted spermatogenesis resulted in testes and epididymides smaller in size. Our data suggested that the germ cell degradation at different developing stages was not associated with germ cell apoptosis dependent on cleaved caspase-3. At the age of six months, the seminiferous epithelium of the testes was atrophic, with the presence of the mostly Sertoli cells only. Interestingly, similar changes have been observed in the testes of *Rara* deficient male mice (*Lufkin T et al 1993*) and vitamin A deficient male mice (*Gaemers IC et al 1996; 1998; Schrans-Stassen BH et al 1999*). Most frequently, the early stages of spermatogenetic cell differentiation (spermatogonia and primary spermatocytes) were

affected in the *Rara* deficient male mice, similar to that observed in HSD17B2TG males. Although infertility is also associated with *Esr1* and *Ar* knockout male mice, the reproductive phenotypes in these mice are different from those of the HSD17B2 TG males. In the *Ar* null males (*Yeh S et al 2002*), the external genitalia are ambiguous or show feminized appearance, including small and cryptorchid testes. *Gnrh1* (hpg, gonadotropin releasing hormone), *Ar* and *LH receptor* null males (LuRKO mice; with highly reduced testosterone production) show that the stages prior to meiotic division are not androgen-dependent (*Yeh S et al 2002; Singh J et al 1995; Zhang FP et al 2001; 2003*). In the *Esr1* null males the testis phenotype with dilated seminiferous tubules and the spermatogenetic failure is due to fluid build-up and the impaired efferent ductules, resulting in rete testis (*Couse JF and Korach KS 1999b; Eddy EM et al 1996*), whereas, in *Esr2* deficient mice the testis phenotype and fertility are normal (*Krege JH et al 1998*). These types of defects were not observed in the HSD17B2TG male mice.
The epididymides of HSD17B2TG was histologically normal, but mRNA expression for two members of the lipocalin superfamily, *lcn8* (mouse epididymal protein) and *lcn5* (murine epididymal retinoic acid binding protein, *Fouchecourt S et al 2003; Lareyre JJ et al 1998*), were markedly down-regulated. The biological significance of lipocalins in the epididymides is uncertain but *lcn8* and *lcn5* have been suggested to be involved in retinoid trafficking in the specific region of epididymides (*Ong DE et al 2000; Orgebin-Crist MC et al 2002*). There is evidence that retinoids also play an important role in regulating gene expression in the epididymides (*Astraudo CA et al 1995*). However, the epididymal genes are under complex regulation by androgens, other testicular factors and by the presence of germ cells in the epididymides, the putative regulation of *lcn5* and *lcn8* by retinoids remains to be clarified.

6.3 Delayed eye opening of the HSD17B2TG mice

The HSD17B2TG males and females displayed delayed eye opening, and in some HSD17B2TG mice the eyes had a squinting phenotype several days after the normal age of the eye opening. A similar phenotype has been reported in vitamin A deficient mice (*Gaemers IC et al 1998; Smith JE 1990*), whereas it is not reported in *Esr1* (*Lubahn DB et al 1993*), *Esr2* (*Krege JH et al 1998*) or *Ar* deficient mice (*Yeh S et al 2002*). It is known that the eyes are the most sensitive organ to retinol deprivation, and it is often the first site of malformations in vitamin A deficient fetuses (*Grondona JM et al 1996; Lohnes DM et al 1994*). Moreover, various retinoid receptor mutant mice (*Lohnes DM et al 1994; Kastner P et al 1994*) have implicated the role of retinoid signaling at most steps of prenatal eye morphogenesis and eye development.

6.4 Rescue of the testis phenotype

Vitamin A induces early cessation of spermatogenesis while the only remaining germ cells are the undifferentiated spermatogonia. Administrating a high dose RA to VAD animals is sufficient to restore and synchronize spermatogenesis in the seminiferous tubules (*Gaemers IC et al 1996; 1998; Schrans-Stassen BH et al 1999*). To determine whether HSD17B2 enzyme participates in the metabolism of retinoids, a rescuing experiment was performed with TTNPB. Interestingly, TTNPB efficiently reduced the damage of the seminiferous tubules observed in the HSD17B2TG males, suggesting that the HSD17B2 enzyme might have a role in the action of retinoids (Table 6).

Table 6. Similar Phenotypes between HSD17B2TG and Various Null Mutants Related to Retinoid Receptors and Metabolic Enzymes

Abnormalities	HSD17B2TG	Various RAR null mutants
body growth	growth retardation delayed puberty	growth retardation
eye defects	delayed eye opening, squinting eye	severe ocular defects, e.g. agenesis of the lens
male sterility	disrupted spermatogenesis germ cell degradation and Sertoli cell left	defective spermatogenesis, testis degeneration, squamous metaplasia of prostate and seminal vesicle epithelia
female reproduction	subfertility	reduced reproduction or infertility
mammary gland	hyperplasia	hyperplasia
skeletal development	delayed skeletal and limb development	multiple cranial-cervical of skeletal defects, limb defects

6.5 Subfertility of the HSD17B2TG female mice

Despite a normal serum E_2 concentration in the HSD17B2TG females compared with WT females, HSD17B2TG females revealed abnormal estrous cycle and were subfertile. However, the elevated serum P concentration which could be converted from 20α-dihydroprogesterone by the HSD17B2 would be the main reason for subfertility in HSD17B2TG females. Furthermore, the metabolism of P to 20α-dihydroprogesterone by AKR1C18 (20α-HSD) could be prohibited. The AKR1C18 expression in granulosa cells is widely involved in the regulation of P level during the estrous cycle and in pregnancy *(Piekorz RP et al 2005; Akinola LA et al 1997*). After oocyte release in response to LH, granulosa cells initiate the formation of a corpus luteum and the cells begin to express AKR1C18. In the absence of coital stimulation, AKR1C18 activity reduces the P level, the granulosa cells undergo apoptosis and the corpus luteum regresses (*Amsterdam A et al 1999*). With coital stimulation, prolactin stimulation of granulosa cells suppresses the expression of AKR1C18 and allows the development of a secretory corpus luteum producing the P required for the initial stages of implantation and maintenance of pregnancy (*Albarracin CT et al 1994*). At term, the ovarian release of P is terminated, and AKR1C18 contributes to the rapid elimination of P (*Akinola LA et al 1997)*. Our data support the theory that expression of HSD17B2 would counteract AKR1C18 activity and stabilize the corpora lutea, resulting in a higher P concentration and increased length of the estrous cycle.

6.6 Characterization of the mammary glands in the HSD17B2TG female mice

The high circulating P concentration together with the elevated concentration of PRL, in the presence of adequate amount of E_2, is obviously the cause of the pregnancy-like phenotype observed in the mammary gland epithelium of HSD17B2TG females. Interestingly, the local expression of the transgene was not able to cause the morphological alterations in the epithelium of mammary gland. However, similar to that observed for certain androgen dependent genes in the males, the expressions of several estrogen dependent genes in the mammary glands were significantly down-regulated by the local expression of HSD17B2 in HSD17B2TG mammary glands transplanted in WT host. Thus, the data suggest a modulatory role for HSD17B2 in estrogen target gene expression, at least in the mammary glands.

6.7 HSD17B1 and HSD17B2 action on different pathways

The studies *in vitro* have revealed that the main difference between HSD17B1 and HSD17B2 is the direction of their enzymetic activities. Human HSD17B2 predominantly catalyzes oxidation of E_2 to E_1 and T to $\triangle^4$-dione, while human HSD17B1 catalyzes the opposite reaction to HSD17B2. Recently, HSD17B1 has shown to activate E_1 to E_2 and $\triangle^4$-dione to T also *in vivo* (*Saloniemi T et al 2007*). To study if the phenotype observed in the HSD17B2TG mice could be rescued by the expression of HSD17B1, the bi-TG (HSD17B1-HSD17B2TG) mice were generated under the same promoter. However, although the bi-TG mice showed features of both HSD17B1TG and HSD17B2TG mice, the phenotypes observed in the single TG mice were not rescued in the bi-TG. These mice were masculinized similar to the HSD17B1TG mice, but were growth retardation at prepubetal and young adult age, and had a marked lobulo-alveolar development similar to the HSD17B2TG mice. This suggests that certain phenotypic characteristics in the two HSD17B1TG and HSD17B2TG lines are caused by different signalling pathways. The most notable changes in the HSD17B1TG mice are well explained by alterations in sex steroid action, whereas in the HSD17B2TG mice the explanation is weaker. HSD17B2 could act on other metabolic pathways, although the data indicate that the enzyme is also capable of modulating sex steroid action at the target tissues.

6.8 The vital role of the GH/IGF1 axis in skeletal formation and pubertal growth

The GH/IGF1 axis has an important role in skeletal maturation, pubertal growth and bone mass accrual (*Christoforidis A et al 2005*). The effect of GH and IGF1 on bone mass is due to their stimulatory action on osteoblast proliferation and differentiation, and collagen synthesis, leading to increased bone formation (*Ohlsson C et al 1998*). Before puberty, GH and IGF1 are essential for maintaining bone growth. GH and IGF1 promote the synthesis of protein, inhibit the formation of fat and carbohydrate, and are necessary for the prolifration of cartilage cells at the epiphyseal plate permitting linear growth. The onset of puberty in the human is marked by an increase in the amplitude of gonadotropins, further resulting in the elevations of gonadal sex steroidal production. In turn, sex steroids stimulate the secretion of GH and IGF1 (*Terasawa E and Fernandez DL 2001*). During puberty, in addition to a dramatic activation of the GH/IGF1 axis, sex steroid hormones play an important role. Although sex steroids and the GH/IGF1 axis exert independent effects on growth, the *interaction* of sex steroid hormones with GH/IGF1 potentiates their effect on bone gorwth (*Rogol AD et al 2002*; *saggese G et al 2002*). Thus, GH, IGF1 and sex steroids act coordinatedly to support the pubertal growth spurt (*Giustina A and Veldhuis JD 1998; Rogol AD et al 2002*). In the present TG mouse model with ubiquitous expression of HSD17B2, the cooperative action of Igf1 and sex steroids is disrupted, resulting in a lower circulating Igf1 and T concentrations at prepubertal period, and thus, the delay in both the appearance of the typical measure of the male puberty (balanopreputial separation) and the bone development. Furthermore, the conversion of the highly active 17-hydroxy steroids (T, E_1) to less potent 17-keto forms ($\triangle^4$-dione, E_2) catalyzed locally by the transgene is likely to further reduce the androgen and estrogen action in the bone. Thus, the local inactivation of sex steroids in the bone together with the reduced circulating concentration of Igf1 and T, is likely the cause of the bone phenotype observed.

GH and IGF1 are known to increase serum osteocalcin (*Ohlsson C et al 1998*), and thus, the decreased serum osteocalcin levels may be caused by the drop in serum Igf1 levels in the HSD17B2TG males at prepubertal age. The formation of IGFs, their receptors, and their binding proteins in skeletal tissues is regulated by many hormones, including GH and sex steroids. The knockout studies have also confirmed that both Igf1 and Igf2 are essential for normal prenatal growth, as mice deficient in

either Igf1 or Igf2 were only 60% of normal size at birth (*Baker J et al 1993*). Thereafter, the role of Igf1 becomes predominant, and both endocrine and paracrine action in the bone have been well documented for Igf1. The endocrine production of IGF-I from the liver is highly dependent on GH action, and similar to HSD17B2TG mice, GHR null mice exhibit severe postnatal growth retardation, associated with markedly reduced Igf1 concentrations. However, these mice have normal T concentrations (*Zhou Y et al 1997*). Interestingly, in the GHR knockout mice, the femoral length and the growth plate width were not significantly shorter at the age of 2 weeks, but significant differences were observed from three week onwards (*Sims N et al 2000*). Thus, the reduced bone growth in the HSD17B2TG mice was initiated at younger age as compared with the GHR null mice. However, similar to HSD17B2TG mice, the trabecular bone remodeling was markedly reduced in GHR null mice, and reduced osteoblast surface in GHR null mice indicated an impaired osteoblast proliferation or life span (*Wang J et al 1999*). Like GHR and Igf1 null mice, HSD17B2TG male mice showed a reduced width of the growth plate and abnormal skeletal metabolism at pre-pubertal age.

Igf1 stimulates the differentiation and maturation of osteoblasts that mediate the bone formation of the mammalian skeleton, and are specialized for the production of extracellular matrix and for the mineralization process (*Ohlsson C et al 1998; Saggese G et al 2002*). Accordingly, we observed reduced expression of Runx2, osteopontin and osteocalcin in the osteoblasts of the HSD17B2TG mice as compared with the WT controls, thus, indicating an inappropriate osteoblast differentiation, affecting the skeletal development in the HSD17B2TG mice. The presence of *HSD17B2* transgene in the osteoblasts also provides its possibility of local direct effects on the bone.

6.9 The role of sex steroids in skeletal formation and metabolism

HSD17B2 inactivates both estrogens and androgens, but although several previous studies elucidated the dysfunction of skeletal development in the *Esr1*, *Esr2*, *Esr12*, *Ar* and *Cyp19a1* deficient male mice, their bone phenotypes were different from those of the HSD17B2TG males. In the adult *Esr1* and *Esr12* knockout males (*Vidal O et al 2000; Sims NA et al 2002*), there were minor skeletal abnormalities with reduced longitudinal bone growth and reduced bone mineral density. However, no skeletal defects or altered bone growth or remodeling were detected at birth, postnatal age or at puberty while the bone changes were observed in mice at 10 weeks of age. In the *Esr2* knockout male mice, no bone abnormalities were displayed at any age (*Windahl SH et al 1999; 2001*). The *Cyp19a1* deficient mice presented with osteopenia in the lumbar spine and were characterized by a significant decrease in trabecular bone volume and trabecular thickness. The low bone turnover in *Cyp19a1* KO males indicated the presence of age-related osteopenia. The *Cyp19a1* KO males also demonstrated significantly reduced longitudinal femur growth compared with WT littermates. However, all skeletal abnormalities were reported in 46-65 day old mice and no bone changes were noted in newborns or during pubertal age (*Oz OK et al 2000; 2001*). Although *Ar* KO male mice showed growth retardation compared with WT male littermates, the growth curve and external reproductive organs were indistinguishable from those of WT female littermates up to eight weeks of age, while internal genitalia were agenesis. Serum gonadal androgen levels were markedly reduced (*Kawano H et al 2003; Yeh S et al 2002*). X-ray and histomorphometric analysis revealed severe osteopenia of the femora and tibiae in eight week old male mice. The bone shape and length were not affected by *Ar* inactivation while the bone loss in the *Ar* deficient males was caused by high bone turnover with increased bone resorption. No skeletal phenotypes have been reported at prepubertal or pubertal age (*Kawano H et al 2003*). The single knockout mouse models for the sex steroid receptors (*Esr1, Esr2, Ar*) do not well mimic the phenotype observed in the present

study, indicating that that the inactivation of both androgens and estrogens simultaneously by HSD17B2, together with the reduced Igf1 concentration results into more severe delay in bone development at prepubertal period.

6.10 The role of retinoic acid in the skeleton

In the present study, we observed a severely disrupted spermatogenesis in the HSD17B2TG males that were partially rescued by a synthetic retinoid analogue (TTNPB). In addition, the testis phenotype and growth retardation were similar to those observed in the *Rar* and *Rxr* deficient models and vitamin A deficient male mice. We can not exclude the possibility that the bone phenotype observed in the HSD17B2TG mice would be caused by a metabolic role of the HSD17B2 that is independent of its ability to inactivate the sex steroids. Widespread and dynamic expression of the Rars and Rxrs throughout skeletal development indicates that these receptors are important regulators of this process (*Weston AD et al 2003*). Furthermore, various *Rar* and *Rxr* deficient models exhibited abnormalities of the axial skeleton and appendicular skeleton including webbed digits (*Lohnes D et al 1994*; *Underhill TM and Weston AD 1998*). Due to high sensitivity of skeleton to retinoids, skeletal abnormalities in the *Rar* and *Rxr* deficients have not been reported to rescue successfully. However, it has been suggested that the retinoid action on skeletal development could be mediated by changes in the IGF/IGFBP axis (*Gabbitas B and Canalis E 1997*).

7 CONCLUSIONS

To investigate the physiological function of HSD17B2 *in vivo*, transgenic mice ubiquitously expressing human HSD17B2 were generated (HSD17B2TG). This model gave an excellent possibility to study the consequences of HSD17B2 expression *in vivo* in detail.

HSD17B2TG male and female mice displayed growth retardation and showed delayed eye opening, and resulted in delayed puberty.

HSD17B2TG males presented with disrupted spermatogenesis with Sertoli cells-only phenotype in adult mice. Disrupted spermatogenesis was evident in the presence of normal hormone concentrations. Proper androgen action in the target tissues was confirmed by the normal histological appearance of the prostate and the epididymis, and quantitative RT-PCR analysis. The disrupted spermatogenesis could be rescued partially by a RAR agonist.

Ubiquitous human HSD17B2 expression inhibits skeletal development in male mice at prepubertal age. The disturbances in skeletal development partially resulted from growth retardation. These changes were, at least partially, explained by lower serum Igf1, osteocalcin and T concentrations, and were normalized at adult age.

HSD17B2TG females showed subfertility, disrupted estrous cycle with ovary dysfunction and lobulo-alveolar development in the mammary glands. The mammary gland phenotype could be rescued by transplanting the HSD17B2TG mammary gland into a WT host.

Mild reduction in both E_2-dependent genes in mammary gland transplants in wild-type host, and T/DHT-dependent genes in the prostate were observed, suggesting that HSD17B2 modulates estrogen and androgen actions *in vivo*.

HSD17B1 is mainly involved in the sex steroid metabolism. However, the phenotypes reported for HSD17B2TG mice can not be rescued by HSD17B1 in the bi-TG mice expressing human HSD17B1 and HSD17B2, suggesting that HSD17B1 and HSD17B2 primarily act on different signaling pathways *in vivo*, and HSD17B2 is primarily involved in pathways other than sex steroids but modulates the actions of estrogens and androgens.

A RAR agonist (TTNPB) could rescue the disrupted spermatogenesis partially. However, the molecular mechanism for disrupted spermatogenesis by HSD17B2 is still unclear completely. It will be important to investigate further the signaling pathway acted by HSD17B2 *in vivo*. HSD17B2TG females showed normal histological appearance of ovary, whereas the ovary dysfunction was evident. It promotes us to study the phenotype of HSD17B2TG ovaries at the molecular level. In the endometrium, HSD17B2 activity is considered to be critical for normal endometrial growth and differentiation. Thus, HSD17B2TG females can be used to study the endometrial development *in vivo*.

8 ACKNOWLEDGEMENTS

The thesis work was carried out at the Department of Physiology, Institute of Biomedicine, University of Turku.
I am very grateful to Professor Ilpo Huhtaniemi, MD, PhD, former chairman of the Department of Physiology, for giving me the opportunity to work at the Department and for providing me with excellent research facilities and scientific atmosphere.
I wish to express my deepest gratitude to my supervisor, Professor Matti Poutanen, PhD, and to my collaborators, Professor Jorma Toppari, MD, PhD and Professor Kalervo H Väänänen, MD, PhD. Thank you, Matti, for giving me the opportunity to work in the Department of Physiology and for guiding me patiently to switch my research field from immunology to endocrinology. You have been a great supervisor. I am astonished at your always-enthusiastic attitude towards science, tremendous experience, and profound knowledge in the field of reproductive physiology and endocrinology. I am grateful for your careful guidance, never-ending support, encouragement, faith and understanding throughout these years. Without these, it would not have been possible for me to accomplish this thesis. Professor Jorma Toppari, I especially thank you for generously offering your enormous knowledge, invaluable and educational comments, and your enthusiastic and supportive attitude towards to me. Your rich knowledge in reproductive biology and endocrinology and expertise on spermatogenesis has been important in completing this study. Professor Kalervo H Väänänen is warmly acknowledged for sharing his knowledge in bone biology and bone morphology. I am deeply impressed by your great personality, helpful advice and inspiring scientific discussions.
I am thankful to the reviewers of my thesis, docent Päivi Miettinen, MD, PhD, and docent Jukka Hakkola, PhD, for their comments and criticism, which helped to improve the quality of this work. Michael Nelson PhD is acknowledged for the revision of the language of the thesis. I express my sincere thanks to Professor Olle Söder for being the opponent.
I am indebted to my co-authors Zhiqi Peng MD, PhD, Tarja Lamminen PhD, Pia Rantakari MSc, Yi Sun MD, Aino Rönnblad MD and Taija Saloniemi MSc, for their contribution to this thesis.
Docent Fuping Zhang MD, PhD, one of my best friends, is thanked for helping me with constructive suggestions and supportive advice. His friendship and unfailing encouragement have been and will be remembered.
My warm thanks to our “steroid team” members, Kaisa Huhtinen MSc, Heli Koivuniemi MSc, Jenni Paviala MSc and Päivi Järvensivu MSc for their helpful suggestions and comments.
All the technical personnel of the lab are acknowledged for their important help during this study. I especially want to thank Ms Tarja Laiho and Taina Kirjonen, who always had time for my samples, Ms Hannele Rekola for the help with an uncountable number of DNA samples and Johanna Lahtinen, Jonna Palmu and Taija Leinonen for the high-quality histological samples. Ms Nina Messner BSc, Ms Heli Niittymäki, Ms Erja Mäntysalo and Katri Hovirinta are acknowledged for their help with various experiments with mice. Without their help many experiments would have been much more difficult to do. Finally, I thank all the personnel of the animal department taking care of my mice.
I am deeply grateful to all the present and former colleagues and personnel of the Department of Physiology. You have created a friendly atmosphere at the department. I shall miss that feeling in the lab, as well as those days I spent there with you. I would especially like to acknowledge: Docent Pirjo Pakarinen, PhD, and Tuula Hämäläinen, PhD, for their expert guidance and scientific advice in the daily laboratory work; my office roommates: Tomi Pakarainen, MD, PhD, Ramin

Lindqvist, MSc, Tassos Damdimopoulos, PhD, Susanna Vuorenoja, MD, for chatting together and sharing their happiness; Min Jiang MD, PhD, and Yang Yang Wang MD, PhD, for sharing helpful suggestions during these years. Thanks to Leena Strauss, PhD, Sonia Bourguiba, PhD, Pasi Koskimies, PhD, Noora Kotaja, PhD, Antti Perheentupa, MD, PhD, Petteri Ahtiainen MD, Nafis Rahman, MD, PhD, Adolfo Rivero, PhD, Helena Virtanen, MD, PhD, Ari Pujianto PhD, Heikki Turunen, MSc, Mirja Nurmio MSc, Annika Adamsson, MSc, Juho Mäkelä MSc, Veronika Mamaeva, MD, Nimisha Desai, MSc. I would also like to thank our secretary Ms Anneli Vesa for her help with office affairs.

I would like to express my special thanks to all my Chinese friends and their families in Turku, for family companing and sharing happiness together in our leisure time. The friendship, help and encouragement of each other are important and indispensable.

I want to thank my parents and my sister deeply for their never-ending love and encouragement and support during these years. Finally, I devote my heartiest thanks to my wife. You have shared all my good and bad moments in the research field and outside it during these years. Your encouragement and support has been essential for me, and without you, I do not believe that I would have been able to finish this thesis, and also to my lovely son for so much happiness and challenges that he brings to me.

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