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ROLE OF HUMAN HYDROXYSTEROID (17β) DEHYDROGENASE TYPE 1 (HSD17B1) IN STEROID-DEPENDENT DISEASES IN FEMALES – NOVEL INDICATIONS FOR HSD17B1 INHIBITORS

Phenotypic analysis of transgenic mice overexpressing human HSD17B1

by

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ABSTRACT

Taija Saloniemi: Role of human hydroxysteroid (17β) dehydrogenase type 1 (HSD17B1) in steroid-dependent diseases in females –novel indications for HSD17B1 inhibitors. Phenotypic analysis of transgenic mice overexpressing human HSD17B1.
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Hormone-dependent diseases, *e.g.* cancers, rank high in mortality in the modern world, and thus, there is an urgent need for new drugs to treat these diseases. Although the diseases are clearly hormone-dependent, changes in circulating hormone concentrations do not explain all the pathological processes observed in the diseased tissues. A more inclusive explanation is provided by intracrinology – a regulation of hormone concentrations at the target tissue level. This is mediated by the expression of a pattern of steroid-activating and -inactivating enzymes in steroid target tissues, thus enabling a concentration gradient between the blood circulation and the tissue.

Hydroxysteroid (17β) dehydrogenases (HSD17Bs) form a family of enzymes that catalyze the conversion between low active 17-ketosteroids and highly active 17β-hydroxysteroids. HSD17B1 converts low active estrogen (E1) to highly active estradiol (E2) with high catalytic efficiency, and altered HSD17B1 expression has been associated with several hormone-dependent diseases, including breast cancer, endometriosis, endometrial hyperplasia and cancer, and ovarian epithelial cancer. Because of its putative role in E2 biosynthesis in ovaries and peripheral target tissues, HSD17B1 is considered to be a promising drug target for estrogen-dependent diseases.

A few studies have indicated that the enzyme also has androgenic activity, but they have been ignored. In the present study, transgenic mice overexpressing human HSD17B1 (HSD17B1TG mice) were used to study the effects of the enzyme *in vivo*. Firstly, the substrate specificity of human HSD17B1 was determined *in vivo*. The results indicated that human HSD17B1 has significant androgenic activity in female mice *in vivo*, which resulted in increased fetal testosterone concentration and female disorder of sexual development appearing as masculinized phenotype (increased anogenital distance, lack of nipples, lack of vaginal opening, combination of vagina with urethra, enlarged Wolffian duct remnants in the mesovarium and enlarged female prostate). Fetal androgen exposure has been linked to polycystic ovary syndrome (PCOS) and metabolic syndrome during adulthood in experimental animals and humans, but the genes involved in PCOS are largely unknown. A putative mechanism to accumulate androgens during fetal life by HSD17B1 overexpression was shown in the present study.

Furthermore, as a result of prenatal androgen exposure locally in the ovaries, HSD17B1TG females developed ovarian benign serous cystadenomas in adulthood. These benign lesions are precursors of low-grade ovarian serous tumors. Ovarian cancer ranks fifth in mortality of all female cancers in Finland, and most of the ovarian cancers arise from the surface epithelium. The formation of the lesions was prevented by prenatal antiandrogen treatment and by transplanting wild type (WT) ovaries prepubertally into HSD17B1TG females. The results obtained in our non-clinical TG mouse model, together with a literature analysis, suggest that HSD17B1 has a role in ovarian epithelial carcinogenesis, and especially in the development of serous tumors. The role of androgens in ovarian carcinogenesis is considered controversial, but the present study provides further evidence for the androgen hypothesis. Moreover, it directly links HSD17B1-induced prenatal androgen exposure to ovarian epithelial carcinogenesis in mice.

As expected, significant estrogenic activity was also detected for human HSD17B1. HSD17B1TG mice had enhanced peripheral conversion of E1 to E2 in a variety of target tissues, including the uterus. Furthermore, this activity was significantly decreased by treatments with specific HSD17B1 inhibitors. As a result, several estrogen-dependent disorders were found in HSD17B1TG females. Here we report that HSD17B1TG mice invariably developed endometrial hyperplasia and failed to ovulate in adulthood. As in humans, endometrial hyperplasia in HSD17B1TG females was reversible upon ovulation induction, triggering a rise in circulating progesterone levels, and in response to exogenous progestins. Remarkably, treatment with a HSD17B1 inhibitor failed to restore ovulation, yet completely reversed the hyperplastic morphology of epithelial cells in the glandular compartment. We also demonstrate that HSD17B1 is expressed in normal human endometrium, hyperplasia, and cancer. Collectively, our non-clinical data and literature analysis suggest that HSD17B1 inhibition could be one of several possible approaches to decrease endometrial estrogen production in endometrial hyperplasia and cancer.

HSD17B1 expression has been found in bones of humans and rats. The non-clinical data in the present study suggest that human HSD17B1 is likely to have an important role in the regulation of bone formation, strength and length during reproductive years in female mice. Bone density in HSD17B1TG females was highly increased in femurs, but in lesser amounts also in tibias. Especially the tibia growth plate, but not other regions of bone, was susceptible to respond to HSD17B1 inhibition by increasing bone length, whereas the inhibitors did not affect bone density. Therefore, HSD17B1 inhibitors could be safer than aromatase inhibitors in regard to bone in the treatment of breast cancer and endometriosis. Furthermore, diseases related to improper growth, are a promising new indication for HSD17B1 inhibitors.

Key words: *hydroxysteroid (17β) dehydrogenase 1, transgenic mice, inhibitor, estrogen, androgen, steroidogenesis, sexual development, endometrium, ovary, bone, hyperplasia, adenoma, longitudinal growth*

TIIVISTELMÄ

Taija Saloniemi: Ihmisen hydroksisteroidi (17β) dehydrogenaasi 1 (HSD17B1) -entsyymin merkitys naisten steroidiriippuvaisissa sairauksissa – uusien tauti-indikaatioiden etsintä HSD17B1-estäjälääkkeille. Ihmisen HSD17B1:ä ylituottavien naarashiirien ilmiasun tutkimus.
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Hormoniriippuvaiset sairaudet, erityisesti syövät, aiheuttavat korkeaa kuolleisuutta länsimaissa, ja tarve uusille lääkkeille on suuri. Vaikka sairaus olisi selvästi hormoniriippuvainen, muutokset verenkierron hormonipitoisuuksissa eivät useinkaan selitä sairauden kulkua. Intrakrinologia tarkoittaa hormonipitoisuuden säätelyä paikallisesti kohdekudoksessa ja tämä mekanismi on usein häiriintynyt hormoniriippuvaisissa sairauksissa kerryttäen hormoneja kohdekudokseen.

Hydroksisteroidi (17β) dehydrogenaasit (HSD17Bt) ovat entsyymejä, jotka katalysoivat inaktiivisten 17-ketosteroidien muuntumista aktiivisiksi 17β-hydroksisteroideiksi. HSD17B1 voimistaa estrogeenivaikutusta muuttamalla tehokkaasti verenkierron mukana tulevaa inaktiivista estronia (E1) aktiiviseksi estrogeeniksi estradioliksi (E2) ja HSD17B1:n on havaittu ilmentyvän useissa hormoniriippuvaisissa sairauksissa, kuten rintasyövässä, endometrioosissa, kohtusyövässä, ja munasarjan pintaepiteelin syövässä. Koska HSD17B1:llä uskotaan olevan merkittävä vaikutus munasarjan ja perifeeristen kudosten E2:n biosynteesissä, sitä pidetään lupaavana lääkekohteena estrogeenivasteisten sairauksien hoitoon.

Joissakin tutkimuksissa on osoitettu, että HSD17B1:llä on myös androgeenistä aktiivisuutta, mutta nämä tutkimukset on jätetty huomiotta. Tässä tutkimuksessa käytimme siirtogeenisiä hiiriä, jotka ylituottavat ihmisen HSD17B1-entsyymiä (HSD17B1TG-hiiret) tutkiaksemme HSD17B1:n ilmentymisen vaikutuksia. Aluksi määritimme entsyymin substrattispesifisyyden *in vivo*. Tuloksemme osoittivat, että ihmisen HSD17B1:llä on merkittävää androgeeneja aktivoivaa vaikutusta, mikä johti lisääntyneeseen testosteronipitoisuuteen sikiökaudella ja naarashiirien maskulinisaatioon, jonka oireita olivat pidentynyt anogenitaaliväli, nisien kehittymättömyys, avautumaton vagina, vaginan yhtyminen virtsaputken kanssa, laajentuneet Wolffin tiehyeen jäänteet mesovariumissa ja laajentuneet Skenen parauretraalirauhaset. Sikiökautinen androgeenialtistus on yhdistetty polykystiseen munasarjaoireyhtymään (PCOS) ja metaboliseen oireyhtymään, mutta PCOS:aa aiheuttavia geenejä ei edellenkään tunneta. Tässä tutkimuksessa osoitimme mekanismin, jossa HSD17B1:n ylituotto johtaa androgeenien kertymiseen sikiökaudella.

Paikallisen sikiökautisen androgeenialtistuksen seurauksena HSD17B1TG-hiiret kehittivät aikuisiällä munasarjan hyvälaatuisia serosaalisia kystadenoomia. Nämä hyvälaatuiset muutokset ovat munasarjan pintaepiteelin karsinoomien esiasteita. Munasarjasyöpä on Suomessa viidenneksi tappavin kaikista naisten syövistä ja valtaosa syövistä kehittyy juuri pintaepiteelistä. Leesioiden muodostuminen HSD17B1TG-hiirissä estettiin sikiökautisella antiandrogeenikäsittelyllä ja siirtämällä villityypin munasarjat HSD17B1TG naaraisiin ennen murrosikää. Nämä tulokset yhdessä kirjallisuusanalyysin kanssa viittaavat siihen, että HSD17B1:llä on merkitystä munasarjan pintaepiteelin karsinogeneesissä ja erityisesti serosaalisten kasvainten kehityksessä. Androgeenien merkitys munasarjan karsinogeneesissä on epäselvä. Tutkimustuloksemme tukevat androgeenihypoteesia munasarjasyövän aiheuttajana. Lisäksi osoitimme yhtymäkohdan HSD17B1:n aiheuttaman sikiökautisen maskulisaation ja munasarjan pintaepiteelin karsinogeneesin välillä hiiressä.

Kuten odotettiin, HSD17B1:llä oli myös merkittävää estrogeenista aktiivisuutta *in vivo*, jota voitiin vähentää HSD17B1-estäjillä eli inhibiittoreilla. Lisääntynyt HSD17B1-aktiivisuus johti E2:n kertymiseen useisiin kohdekudoksiin, mukaanlukien kohtuun, ja aiheutti kohdun limakalvon liikakasvua eli endometriumin hyperplasiaa. Kuten ihmisissä, endometriumin hyperplasia HSD17B1TG-naaraissa saatiin korjattua indusoimalla ovulaatio ja antamalla progesteronia. Vaikka HSD17B1-inhibiittorikäsittely ei korjannut ovulaatiota, se korjasi täysin rauhasten morfologian, mutta korjaava vaikutus oli heikompi luminaalisessa epiteelissä. Osoitimme myös, että ihmisen HSD17B1 ilmentyy ihmisen normaalissa endometriumissa ja lisäksi endometriumin hyperplasiassa ja kohdunrungonsyövässä. Yhteenvedettynä tuloksemme ja kirjallisuusanalyysi viittaavat siihen, että HSD17B1-aktiivisuuden esto on yksi monista tavoista vähentää endometriumin estrogeenituotantoa hyperplasiassa ja syövässä.

HSD17B1:n on havaittu ilmentyvän ihmisen ja rotan luussa. Tämän tutkimuksen tulokset osoittivat että HSD17B1-aktiivisuudella on merkitystä luun muodostumisessa, vahvuudessa ja pituudessa lisääntymisikäisissä naarashiirissä. Luun tiheys oli voimakkaasti lisääntynyt reisiluussa, ja vähemmän sääriluussa. Erityisesti sääriluun kasvulevy, mutta eivät muut tutkitut alueet, oli herkkä HSD17B1-inhibitiolle lisäten luun pituutta, kun taas inhibiittorit eivät vaikuttaneet luun tiheyteen. Siten HSD17B1-inhibiittorit voisivat olla aromataasi-inhibiittoreita turvallisempia luun suhteen rintasyövän ja endometrioosin hoidossa. Lisäksi luun kasvuhäiriöt ovat lupaava uusi tauti-indikaatio HSD17B1-inhibiittoreille.

Avainsanat: *hydroksisteroidi (17β) dehydrogenaasi 1, siirtogeeninen hiiri, inhibiittori, estrogeeni, androgeeni, steroidogeneesi, sukupuolielinten kehitys, kohdun limakalvo, munasarja, luu, hyperplasia, adenooma, pituuskasvu*

TABLE OF CONTENTS

ABBREVIATIONS **8**

LIST OF ORIGINAL PUBLICATIONS **10**

1 INTRODUCTION **11**

2 REVIEW OF THE LITERATURE **12**

2.1 ESTROGENS AND ANDROGENS 12

2.1.1 NUCLEAR RECEPTORS 12

2.1.2 ESTROGEN RECEPTORS 13

2.1.2.1 Nuclear ESRs 1 and 2 14

2.1.2.2 Membrane ESRs 15

2.1.3 ANDROGEN RECEPTORS 17

2.1.4 PHYSIOLOGICAL EFFECTS 17

2.1.5 BIOSYNTHESIS AND REGULATION 18

2.1.5.1 Sex steroid biosynthesis 18

2.1.5.2 Regulation of sex steroid biosynthesis 19

2.2 ROLE OF ESTROGENS AND ANDROGENS DURING EMBRYONIC DEVELOPMENT 20

2.2.1 BIPOTENTIAL GONAD 20

2.2.2 MALE SEXUAL DIFFERENTIATION 21

2.2.2.1 Development of male gonad (testis) 21

2.2.2.2 Development of other male primary sexual characteristics 22

2.2.3 FEMALE SEXUAL DIFFERENTIATION 22

2.2.3.1 Development of female gonad (ovary) 22

2.2.3.2 Development of other female primary sexual characteristics 23

2.2.4 DISEASES RELATED TO IMPROPER SEX STEROID ACTION DURING DEVELOPMENT 24

2.2.4.1 Disorders of sexual development 24

2.2.4.2 Polycystic ovary syndrome 24

2.2.4.3 Testicular dysgenesis syndrome 25

2.3 HYDROXYSTEROID (17β) DEHYDROGENASE TYPE 1 25

2.3.1 HSD17Bs 25

2.3.2 HSD17B1 25

2.3.2.1 Gene 25

2.3.2.2 Protein 26

2.3.2.3 Physiological role 28

2.3.2.4 Tissue distribution and regulation of expression 29

2.3.2.5 Other HSD17Bs with estrogen reductase activity 32

2.4 ROLE OF HSD17B1 IN TISSUE-SPECIFIC REGULATION OF ESTROGEN ACTION 32

2.4.1 PLACENTA 32

2.4.1.1 Human placental steroid biosynthesis 32

2.4.1.2 Role of HSD17B1 in placental steroid biosynthesis 33

2.4.1.3 Rodent placenta 33

2.4.2 OVARY 33
2.4.2.1 Folliculogenesis 33
2.4.2.2 Role of HSD17B1 in ovarian estrogen biosynthesis 35
2.4.2.3 Role of estrogen in the ovaries and disturbances of estrogen action....35
2.4.3 ENDOMETRIUM....37
2.4.3.1 Menstrual cycle....37
2.4.3.2 Role of HSD17B1 in endometrial estrogen biosynthesis 37
2.4.3.3 Role of estrogen in the endometrium and its disorders 39
2.4.3.3.1 Endometrial carcinoma 40
2.4.3.3.2 Endometrium of PCOS patients....41
2.4.3.3.3 Endometriosis 42
2.4.4 BREAST....42
2.4.4.1 Role of estrogen in the breast and its disorders 43
2.4.4.2 Role of HSD17B1 in local estrogen biosynthesis in the breast .43
2.4.5 BONE....44
2.4.5.1 Bone function....44
2.4.5.2 Role of estrogen in normal and diseased bone 44
2.4.5.3 Role of local estrogen biosynthesis in bone 45
2.5 HSD17B1 AS A DRUG TARGET 46
2.5.1 INHIBITORS OF LOCAL ESTROGEN BIOSYNTHESIS....46
3 AIMS OF THE STUDY....48
4 MATERIALS AND METHODS....49
4.1 TRANSGENIC MICE (I-V)....49
4.1.1 HSD17B1TG MICE (I-II, IV-V) AND MMTV-HSD17B1TG MICE (III) .49
4.1.2 BI-TG MICE 49
4.1.3 ANIMAL EXPERIMENTATION (I-V)....50
4.2 HORMONE DETERMINATIONS (I-II)....50
4.3 RT-PCR (I, III, V)....50
4.3.1 QUANTITATIVE RT-PCR (I) 50
4.3.2 SEMI-QUANTITATIVE RT-PCR (I, III, V)....51
4.4 ACTIVITY MEASUREMENTS ***IN VITRO*** (I)....52
4.5 ACTIVITY MEASUREMENTS ***IN VIVO*** (I, III, IV) 52
4.5.1 DETERMINATION OF SUBSTRATE SPECIFICITY AND INHIBITION OF HSD17B1 ACTIVITY IN HSD17B1TG AND MMTV-HSD17B1TG MICE (I, III) 52
4.5.2 DETERMINATION OF E2 ACCUMULATION IN HSD17B1TG TISSUES (IV) 52
4.6 UTERUS WEIGHT TEST (IV)....53
4.7 PRENATAL FLUTAMIDE TREATMENT (I, II)....53
4.8 OVARY TRANSPLANTATIONS (I)....53
4.9 SUPEROVULATION TREATMENT (IV)....54
4.10 PHENOTYPE RESCUING INHIBITOR TREATMENTS (IV-V)....54

4.11 PROGESTIN TREATMENT (IV) 54
4.12 MOUSE HISTOLOGY (I-II, IV) 55
4.13 IMMUNOHISTOCHEMISTRY (I-II) 55
4.13.1 FETAL MICE (I-II) 55
4.13.2 HUMAN ENDOMETRIUM 56
4.14 PERIPHERAL QUANTITATIVE COMPUTATIVE TOMOGRAPHY (V) 57
4.15 STATISTICS (I-V) 57
5 RESULTS 58
5.1 GENERATION OF HSD17B1TG MICE (I) 58
5.2 HORMONAL STATUS OF HSD17B1TG MICE (I) 58
5.3 SUBSTRATE SPECIFICITY OF HUMAN HSD17B1 ***IN VIVO*** (I, III-IV) 59
5.4 ANDROGEN-DEPENDENT DISORDERS IN HSD17B1TG FEMALES (I-II) 61
5.4.1 FEMALE DSD (I-II) 61
5.4.2 OVARIAN BENIGN SEROUS CYSTADENOMAS (I) 61
5.5 ESTROGEN-DEPENDENT DISORDERS IN HSD17B1TG MICE AND THEIR TREATMENT WITH HSD17B1 INHIBITORS (IV-V) 62
5.5.1 ENDOMETRIAL HYPERPLASIA (IV) 62
5.5.1.1 Characterization of endometrial phenotype of HSD17B1TG females 62
5.5.1.2 Effect of various rescuing treatments on endometrial hyperplasia 62
5.5.1.3 Expression of HSD17B1 in normal and malignant human endometrium 63
5.5.2 BONE QUALITY AND LENGTH (V) 64
5.5.2.1 Characterization of bone phenotype in HSD17B1TG females 64
5.5.2.2 Effect of HSD17B1 inhibitors on bone 65
5.5.2.3 Effect of HSD17B2 overexpression on HSD17B1TG bone 65
6 DISCUSSION 67
6.1 SUBSTRATE SPECIFICITY OF HUMAN HSD17B1 ***IN VIVO*** 67
6.2 ROLE OF HUMAN HSD17B1 IN ANDROGEN-DEPENDENT FEMALE DISORDERS 68
6.2.1 FETAL ANDROGEN ACCUMULATION 68
6.2.2 OVARIAN CARCINOGENESIS 70
6.3 ROLE OF HUMAN HSD17B1 IN ESTROGEN-DEPENDENT DISEASES AND AS DRUG TARGET 71
6.3.1 ENDOMETRIAL HYPERPLASIA AS A DISEASE INDICATION FOR HSD17B1 INHIBITORS 71
6.3.2 LOCAL ESTROGEN BIOSYNTHESIS AND ITS PREVENTION IN BONE 73
7 SUMMARY AND CONCLUSIONS 75
8 ACKNOWLEDGEMENTS 77
9 REFERENCES 80
ORIGINAL PUBLICATIONS 97

ABBREVIATIONS

ACTH	adrenocorticotrophin
A-dione	androstenedione
AF	activation function
AGD	anogenital distance
AIS	androgen insensitivity syndrome
AKR	aldo-keto reductase
AKT	v-akt murine thymoma viral oncogene homolog 1
AMH	anti-Müllerian hormone
AP-1	activator protein
AR	androgen receptor
ARE	androgen response element
ArKO	aromatase knockout
Arom+	aromatase transgenic mice
BMC	bone mineral content
BMD	bone mineral density
BSA	bovine serum albumin
αERKO	estrogen receptor 1 knockout
αβERKO	estrogen receptor 1 and 2 knockout
βERKO	estrogen receptor 2 knockout
CAH	congenital adrenal hyperplasia
CAIS	complete androgen insensitivity syndrome
cAMP	cyclic adenosinemonophosphate
DAB	3,3'-diaminobenzidine
DAX1	dosage sensitive sex reversal adrenal hypoplasia congenital critical region of the x chromosome gene 1
DES	diethylstilbestrol
DHEA(S)	dehydroepiandrosterone (sulfate)
DHH	Desert hedgehog
DHT	dihydrotestosterone
DPN	2,3-bis(4-hydroxyphenyl) propionitrile
DSD	disorder of sexual development
E	embryonic day
E1	estrone
E2	estradiol
E3	estriol
E4	estetrol
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMX2	empty spiracles homolog
ERE	estrogen response element
ESR	estrogen receptor
EST	sulfotransferase family 1E, estrogen-preferring, member 1
FGF	fibroblast growth factor
Figla, Figα	folliculogenesis-specific basic helix-loop-helix
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
FOXL2	Forkhead box L2
FOXO1	forkhead box O subclass transcription factor 1
FSH	follicle-stimulating hormone
FST	follistatin
GnRH	gonadotropin-releasing hormone
GPR30	estrogen-binging G protein-coupled estrogen receptor 1
GRE	glucocorticoid response element
hCG	human chorionic gonadotrophin
HE	hematoxylin-eosin
HRAS	Harvey rat sarcoma viral oncogene homolog
HRT	hormone replacement therapy
HSD17B	hydroxysteroid (17β) dehydrogenase
HSD3B	hydroxysteroid (3β) dehydrogenase
HPGA	hypothalamus-pituitary-gonadal axis

HPLC	high performance liquid chromatography
IGF	insulin-like growth factor
IGF1R	insulin-like growth factor 1 receptor
INSL3	insulin-like 3
IL	interleukin
i.p.	intraperitoneal
i.v.	*in venum*
JUN	jun oncogene
KO	knockout
LDL	low-density lipoprotein
LH	luteinizing hormone
LHX9	lim homeobox protein
MAPK	mitogen-activated protein kinase
MMTV	mouse mammary tumor virus
MPA	medroxyprogesterone acetate
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
NAD	nicotinamide adenine dinucleotide
NGS	normal goat serum
NO	nitric oxide
NR	nuclear receptor
P	progesterone
PAIS	partial androgen insensitivity syndrome
PBS	phosphate-buffered saline
PCOS	polycystic ovary syndrome
PEG	polyethylene glycol
PI3K	phosphoinositide-3-kinase
PK	protein kinase
PMA	phorphol-12-myristate-13-acetate
PMI	polar moment of inertia
PMSG	pregnant mare serum gonadotropin
POR	P450 oxidoreductase
PPT	propylpyratzole triol
pQCT	peripheral quantitative computative tomography
PRE	progesterone response element
PTEN	phosphatase and tensin homolog
RRE	retinoic adic response element
SARM	selective androgen receptor modulator
s.c.	subcutaneously
SDR	short-chain dehydrogenase/reductase
SEM	standard error of the mean
SERM	selective estrogen receptor modulator
SF1	steroidogenic factor 1
SHBG	sex hormone binding globulin
SMA	smooth muscle actin
SOX9	Sry-related HMMG box
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
SRY	sex-determining region of Y
SSI	strength strain index
StAR	steroidogenic acute regulatory protein
STS	steroid sulfatase
T	testosterone
TBS	tris-buffered saline
TMA	tissue microarray
TLDU	terminal ductal lobular unit
TEB	terminal end bud
TGFα	transforming growth factor α
TNFα	tumor necrosis factor α
TG	transgenic
WNT	wingless-related MMTV integration site
WT	wild type
WT1	Wilms tumor 1

LIST OF ORIGINAL PUBLICATIONS

I. **Saloniemi T**, Lamminen T, Huhtinen K, Welsh M, Saunders P, Kujari H, Poutanen M: Activation of androgens by hydroxysteroid (17β) dehydrogenase 1 *in vivo* as a cause of prenatal masculinization and ovarian benign serous cystadenomas. *Mol Endocrinol. 2007 Nov;21(11):2627-36.*

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V. **Saloniemi T***, Vääräniemi J*, Järvensivu P, Koivuniemi H, Väänänen K, Poutanen M: Effect of novel hydroxysteroid (17β) dehydrogenase 1 (HSD17B1) inhibitors on enhanced long bone mineral density and decreased tibia length induced by overexpression of human HSD17B1 in transgenic mice. *Manuscript. *Equal contribution.*

1 INTRODUCTION

Hormone-dependent diseases affect the reproductive heath of large numbers of women. For example breast and uterine corpus cancers are the leaders in the incidence of all cancers in women. These diseases rank high in mortality, with breast cancer leading, ovarian cancer being fifth, and corpus uterine cancer 11th (Finnish Cancer Registry). Furthermore, endometriosis affects up to 10% of women and is a major cause of pelvic pain and infertility (Eskenazi and Warner. 1997, Dassen et al. 2007). Although these diseases are clearly hormone-dependent, changes in circulating hormone concentrations do not explain all pathological processes observed in the diseased tissues. A more inclusive explanation is provided by intracrinology – a regulation of hormone concentrations at the target tissue level. This is mediated by the expression of a certain pattern of steroid-activating and -inactivating enzymes in steroid target tissues, thus enabling a concentration gradient between the circulation and the tissue. The importance of intracrinology in normal tissue is demonstrated by the important role of aromatase enzyme (P450aromatase; CYP19A1, Cytochrome P450, family 19, subfamily A, polypeptide 1), that converts androgens to estrogens, in estrogen-dependent epiphyseal closure of male bone, that stops the growth after puberty. Consequently, lack of aromatase results in tall stature in affected men (Simpson. 2000). Furthermore, aromatase has been shown to play a role in locally enhanced estrogen production in breast cancer, inducing estrogen-mediated growth and proliferation of cancer cells (Harada. 1999). A good example of a succesful intracrinology-based treatment strategy is provided by aromatase inhibitors, generally used in the treatment of breast cancer. However, resistance to these drugs is almost universally reached (Urruticoechea. 2007), and the treatment results in bone loss (Folkestad et al. 2009). Recently, the role of hydroxysteroid (17β) dehydrogenase type 1 (HSD17B1), converting weak estrogen estrone (E1) to highly active estradiol (E2), in the intracrinology of breast cancer and endometriosis has been emphasized, and the clinical relevance has become apparent (Poutanen et al. 1992, Miettinen et al. 2000, Oduwole et al. 2004, Smuc et al. 2007, Gunnarsson et al. 2008). HSD17B1 inhibitors are new drugs designed for the treatment of endometriosis and breast cancer. Moreover, human HSD17B1 expression has been found to positively correlate with the malignancy of ovarian serous carcinomas (Sasano et al. 1996), and its expression has been detected in several pathological conditions of the endometrium, such as endometrial hyperplasia (Smuc et al. 2006), endometrial cancer (Maentausta et al. 1992, Smuc et al. 2006), and in the endometrium of polycystic ovary syndrome (PCOS) patients (Leon et al. 2008, Bacallao et al. 2008). Human HSD17B1 is also expressed in bone (Sasano et al. 1997) as well as in the malignant pituitary, especially in prolactinomas (Green et al. 1999). Thus, similarly to aromatase, human HSD17B1 is involved in the intracrinology of several normal and diseased tissues.

The objective of the present study was to further analyze the role of human HSD17B1 in different sex hormone -dependent diseases using a non-clinical mouse model. The approach chosen was a phenotypic analysis of transgenic (TG) mice overexpressing human HSD17B1. The hormone-dependent diseases induced in these mice were studied in detail to understand the role of human HSD17B1 in the various human disease -like symptoms observed, and ultimately, to evaluate these diseases as novel target indications for HSD17B1 inhibitors.

2 REVIEW OF THE LITERATURE

2.1 ESTROGENS AND ANDROGENS

Estrogens and androgens are sex steroids conventionally described as female and male hormones, respectively, although both have physiological roles in males and females. There are four naturally occurring estrogens: estrone (E1), estradiol (E2), estriol (E3) and estetrol (E4). E2 is the most active estrogen and is predominant during the reproductive years. E1 is a precursor and metabolite of E2, and has weak estrogenic activity. After menopause, E1 becomes the predominant estrogen, and it is synthesized in the adipose tissue and skin from adrenal precursors. E3 is synthesized from E1 and E2, and is produced in large quantities by the placenta during pregnancy (Coelingh Bennink. 2004). E4 is only produced by the fetal liver, and its significance is unclear (Coelingh Bennink et al. 2008). In addition to naturally occurring estrogens, there are various exogenous and/or synthetic steroidal and non-steroidal compounds that can act as estrogens, when coming into contact with the human body. These include, for example, ethinylestradiol and conjugated equine estrogens, and selective estrogen receptor modulators (SERMs), used in contraception and hormone replacement therapy (HRT), respectively, dietary phytoestrogens, and a number of other synthetic agents, including pesticides and plasticizers (Sharpe. 2001, Toppari et al. 1996, Coelingh Bennink. 2004). Figure 1 presents the three most important endogenous estrogens.

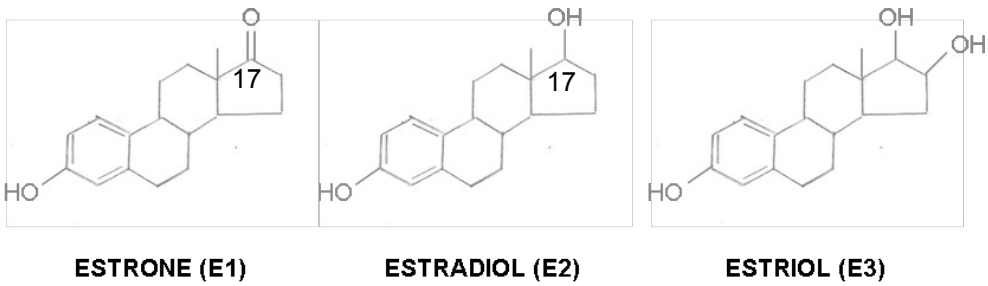


FIGURE 1. Structure of endogenous estrogens. 17-hydroxy group is important for the activity of estrogens. The reduction of the 17-keto group of E1 by HSD17B1 produces E2, the most potent biological estrogen.

Testosterone (T) and 5α-dihydrotestosterone (DHT) are the most active biological androgens. Also their precursors, dehydroepiandrosterone (DHEA)(S) produced by the adrenal cortex, androstenedione (A-dione), which is a precursor of T, and the T metabolites 3α-androstanediol and 3β-androstanediol, are weak androgens (Roy et al. 2008, Beck et al. 2008).

2.1.1 NUCLEAR RECEPTORS

Steroid hormones are lipid-soluble compounds that are able to pass through the plasma membrane of cells by diffusion, and signal mainly via nuclear receptors (NRs). Natural ligands for NRs are generally lipophilic in nature and include steroid hormones, bile acids, fatty acids, thyroid hormones, certain vitamins, and prostaglandins. Steroids

can be further divided into five main classes called androgens, estrogens, progestins, glucocorticoids and mineralocorticoids. In humans, 48 members of the NR family have been identified, but the ligand for several of them is unknown, and these receptors are referred to as orphan receptors (McEwan. 2009). Structurally, NRs are all characterized by a C-terminal ligand-binding domain, a centrally located DNA-binding domain, an N- terminal transactivating domain and the ability to regulate transcription (McEwan. 2009, Gronemeyer et al. 2004). In the absence of ligands, NRs are maintained in a latent, highly ligand-binding conformation by co-chaperone proteins. Upon ligand binding, the receptors dimerize, which leads to dissociation of the co-chaperone molecules and exposure of co-regulator interaction surfaces. The ligand-bound NRs then act as transcription factors by binding to regulatory elements of DNA and regulate gene expression (Pratt and Toft. 1997). Once bound to promoter or enhancer elements, NRs activate transcription via activation function (AF) 1 and/or AF2 by the recruitment of proteins or protein complexes with enzymatic activities that permit opening of the chromatin structure, and components of general transcription machinery resulting in the formation of the preinitiation complex. Alternatively, NRs can also repress transcription. This is mediated by ligands which can expose inactivative co-regulator binding sites, and thus, induce individual responses (McEwan. 2009).

An additional level of regulation of NR-mediated responses is obtained by tissue-selective action of NR ligands. One way to explain this is by the expression level of various co-regulators in different cell types. Examples of this mechanism are provided by the action of the SERMs, tamoxifen and raloxifen. Tamoxifen is an estrogen receptor (ESR) agonist in the endometrium and an antagonist in the breast, whereas raloxifen is an antagonist in both tissues. Tamoxifen recruits co-activators on ESR-regulated promoter in the endometrium, while co-repressors are recruited in the breast. Raloxifen recruits co-repressors in both tissues (Shang and Brown. 2002). Similar mechanisms are likely to take place in the action of other selective nuclear receptor modulators, for example, selective androgen receptor modulators (SARMs). Interestingly, in addition to NR co-regulator mechanisms, SARMs have recently been shown to activate inhibitory and proliferative intracellular signaling pathways depending on an antagonistic or agonistic effect (Narayanan et al. 2008). Lastly, NRs are subject to a number of post-translational modifications, including phosphorylation, acetylation, sumoylation, ubiquitination and glycosylation, which further modulate the receptor activity (McEwan. 2009).

2.1.2 ESTROGEN RECEPTORS

Biological effects of estrogens are mediated via ESRs. Currently, three different types of ESRs have been characterized, two of them (ESR1 and ESR2) being NRs. ESR1 (66 Kda, 595 amino acids) (White et al. 1987) was first reported in 1986 (Green et al. 1986, Greene et al. 1986). Ten years later, another form designated to ESR2 (54 Kda, 530 amino acids) (Moore et al. 1998), was reported (Kuiper et al. 1996, Mosselman et al. 1996, Ogawa et al. 1998). Several splice variants of both receptors have been found in normal and cancerous tissue, but their significances are yet unclear (Matthews and Gustafsson. 2003, Pearce and Jordan. 2004). The third form of ESRs has not yet been identified but studies have indicated that it is a membrane-bound receptor.

2.1.2.1 Nuclear ESRs 1 and 2

Similar to other NRs, ESR1 and 2 contain six functional domains: the N-terminal A/B domain possesses ligand-independent AF1 and an interaction surface for co-regulators, and demonstrates only 17% identity between human ESR1 and 2. The central C domain mediates DNA-binding through two zinc finger structures and has the highest homology between ESR1 and 2 (97%). The D domain exhibits approximately 30% identity between ESR1 and 2, and acts as a flexible hinge containing a nuclear localization signal. The C-terminal E/F domains are responsible for ligand binding, have ligand-dependent AF2 and another interaction surface for co-regulators. The E domain possesses 60% homology between ESR1 and 2, while the F-domain is unique to ESRs, contributing to the transactivation capacity of ESR1 and 2, and shares 18% homology between the ESRs (Enmark et al. 1997, Couse and Korach. 1999). In spite of the total homology of 47%, these two receptors are encoded by different genes located in different chromosomes (Enmark et al. 1997). The domain organization of ESR1 is shown in Figure 2.

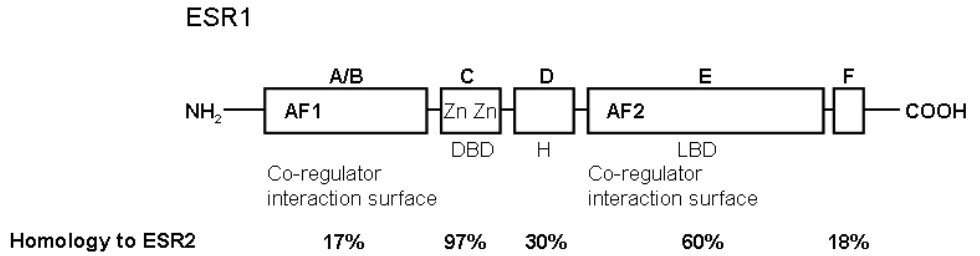


FIGURE 2. Domain organization of ESR1. A/B domain contains ligand-independent AF1 and a co-regulator interaction surface. C domain is a DNA-binding domain (DBD). D domain contains a flexible hinge region. The E/F domain is a ligand-binding domain (LBD) and contains ligand-dependent AF2 and another interaction surface for co-regulators.

Like other NRs, ESR1 and 2 are transcription factors that dimerize upon ligand binding and bind to DNA to regulate transcription. The receptors have been shown to form both homodimers and heterodimers so that ESR1 prefers the formation of homodimers, whereas ESR2 preferentially heterodimerizes (Pace et al. 1997, Ogawa et al. 1998, Couse and Korach. 1999). The activated receptors bind to estrogen response elements (EREs) of DNA and regulate transcription of the ERE-mediated genes via interaction with co-regulators and basal transcription machinery (Couse and Korach. 1999). Several variants of ERE sequences binding the ESRs with different affinities have been reported (Driscoll et al. 1998, Couse and Korach. 1999). The ligand-dependent transcriptional regulation of ERS1 is mediated via two separate activation domains, AF1 and AF2 (Tora et al. 1989, Couse and Korach. 1999), but ESR2 lacks AF1 activity (Hall and McDonnell. 1999). The ligand-dependent transcriptional regulation is called classical ESR signaling. The ESRs can also regulate gene expression indirectly, without binding to ERE, by interacting with other transcription factors, for example, the activator protein 1 (AP-1) complex (Sukovich et al. 1994, Webb et al. 1995, Webb et al. 1999, Couse and Korach. 1999). This is called crosstalk (Gottlicher et al. 1998). Finally, the ESRs can be activated in the absence of ligands by phosphorylation (Weigel and Zhang. 1998).

Despite similarities, several studies indicate that there are also subtype-specific actions of the ESRs. Although both bind E2 with the same affinity, the responses obtained by certain

ligands differ depending on the receptor subtype. For example, propylpyratzole triol (PPT) is specific to ESR1 (Stauffer et al. 2000), while genistein and 2,3-bis(4-hydroxyphenyl) propionitrile (DPN) are ESR2-specific agonists (Meyers et al. 2001, Escande et al. 2006). Furthermore, the selective agonist activity of tamoxifen seems to be specific for ESR1 (Tremblay et al. 1997, Watanabe et al. 1997). ESR1 and 2 have been shown to signal in opposite ways when complexed with E2 from the AP1 site. Furthermore, tamoxifen and raloxifen were potent transcriptional activators of ESR2 at the AP1 site (Paech et al. 1997). ESR1 and 2 were also reported to have an opposite effect on cell proliferation in a mouse mammary cell line (Helguero et al. 2005). Finally, it has been suggested that although the receptors bind to the same DNA regions, they are also likely to have individual target genes in addition to overlapping ones (Matthews and Gustafsson. 2003).

The tissue distributions of ESR1 and 2 partially overlap, but they also possess a receptor-specific expression pattern. Furthermore, even when expressed in the same tissue, the receptors often localize to different cell types. For example, in the ovaries, ESR1 is expressed in the theca cells, whereas ESR2 is expressed in the granulosa cells (Saunders et al. 2000). Mouse Esr1 is expressed at least in the ovary, uterus, oviduct, mammary gland, pituitary, hypothalamus, olfactory bulb, cortex, heart, aorta, liver, kidney, lung, skeletal muscle, testis, prostate, epididymis (Couse and Korach. 1999) and bone (Bland. 2000). Esr2 expression has been detected at least in ovary, uterus, hypothalamus, cortex, lung, testis, prostate, epididymis (Couse and Korach. 1999) and bone (Bland. 2000). Studies in knockout (KO) mice have indicated divergent physiological roles for these receptors, highlighting the role of Esr1 in the classical estrogen target tissues (uterus, vagina, oviduct, ovary and mammary gland) (Couse and Korach. 1999), but also in the male reproductive system (testis, epididymis, vas deference and prostate) (Couse and Korach. 1999, Chen et al. 2009). However, Esr1 also has significance outside the reproductive system, for example in bone, cardiovascular system, central nervous system (Couse and Korach. 1999), pituitary (Ogasawara et al. 2009), fat, energy metabolism (Couse and Korach. 1999, Ropero et al. 2008), kidney (Lane. 2008), and immune system (Islander et al. 2003). The role of Esr2 is emphasized outside the reproductive system, affecting the physiology of lung (Morani et al. 2006), bone (Chagin et al. 2004), skeletal muscle (Glenmark et al. 2004), bladder (Imamov et al. 2007), cardiovascular system (Rubanyi et al. 2002), central nervous system (especially behavior) (Morissette et al. 2008, Tomihara et al. 2009), energy metabolism (Ropero et al. 2008, Foryst-Ludwig et al. 2008), spleen (Zhang et al. 2004) and immune system (Islander et al. 2003). Esr2 also affects the female reproductive system including the ovary (Couse and Korach. 1999), uterus (Wada-Hiraike et al. 2006) and mammary gland (Forster et al. 2002), but defects observed in ESR2 KO (βERKO) mice are milder than in ESR1 KO (αERKO) mice. ESR2-induced defects have also been reported in the male reproductive system, including testis (Gould et al. 2007) and prostate (Imamov et al. 2004).

2.1.2.2 Membrane ESRs

The genomic effects of estrogens described in the previous sections are relatively slow, but estrogens have also been reported to induce rapid cell signaling via a membrane-associated receptor. The E2-binding membrane receptor triggering rapid generation of cyclic adenosinemonophosphate (cAMP) was first described already in 1977 (Pietras

and Szego. 1977). Thereafter, studies have indicated that these membrane-initiated signaling pathways include the second messengers Ca^2+, cAMP and nitric oxide (NO), activation of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 receptor (IGF1R), and activation of protein/lipid kinases, for example, phosphoinositide-3-kinase (PI3K), v-akt murine thymoma viral oncogene homolog 1 (AKT), mitogen-activated protein kinase (MAPK) family members, v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian) (SRC) family members and protein kinase A and C (PKA and PKC) (Prossnitz et al. 2007). At present, several membrane ESR candidates have been suggested: 1) there is evidence that the nuclear ESRs, ESR1 and ESR2, accumulate in the plasma membrane and signal via MAPK/ERK pathways upon estrogen stimulation (Razandi et al. 2003, Razandi et al. 2004, Micevych et al. 2009). 2) Studies have indicated that rapid estrogen signaling is mediated through G-proteins, and recently, an estrogen-binging G-protein -coupled estrogen receptor 1 (GPER1, GPR30) has been described (Filardo et al. 2000, Filardo et al. 2002, Filardo. 2002), and shown to signal via ERK, PI3K, Ca^2+ and cAMP in response to E2 and tamoxifen stimulation (Filardo et al. 2000, Filardo et al. 2002, Filardo. 2002, Revankar et al. 2005, Prossnitz et al. 2007). Exceptional among G-proteins, GPR30 is located intracellularly in the endoplasmic reticulum (Revankar et al. 2005, Prossnitz et al. 2007). 3) A plasma membrane -associated ESR referred to as ER-X (Toran-Allerand et al. 2002), and 4) STX-binding protein coupled to PLC-PKC-PKA pathway and with unknown subcellular localization (Qiu et al. 2003, Qiu et al. 2006) have also been reported as candidates for membrane ESR. Thus, it seems that several different membranous receptors mediate estrogen signaling, further increasing the complexity of estrogen signaling. A summary of ESRs is shown in Table 1.

Table 1. Estrogen receptors.

	ESR1	**ESR2**	**MEMBRANE ESR**	**REFERENCES**
Identified	1986	1996	Not identified, several candidates	(Green et al. 1986, Greene et al. 1986, Kuiper et al. 1996, Mosselman et al. 1996, Filardo et al. 2000, Filardo. 2002, Filardo et al. 2002, Toran-Allerand et al. 2002, Razandi et al. 2003, Qiu et al. 2003, Razandi et al. 2004, Qiu et al. 2006, Micevych et al. 2009)
Length	595 amino acids	530 amino acids	Unknown	(White et al. 1987, Moore et al. 1998)
Domains	A/B, C, D, E/F	A/B, C, D, E/F	Unknown	(Enmark et al. 1997, Couse and Korach. 1999)
AFs	AF1, AF2	AF2	Unknown	(Enmark et al. 1997, Couse and Korach. 1999, Hall and McDonnell. 1999)
Signaling	-Classical -Cross-talk -Ligand-independent activation by phosphorylation -Indirect regulation of gene expression via other transcription factors		Ca2+, cAMP, NO, EGFR, IGF1R, PI3K, AKT, MAPK-members, SRC-members, PKA, PKC	(Sukovich et al. 1994, Webb et al. 1995, Gottlicher et al. 1998, Weigel and Zhang. 1998, Couse and Korach. 1999, Webb et al. 1999, Prossnitz et al. 2007)

2.1.3 ANDROGEN RECEPTORS

At present, one functional NR is known for androgens. Similar to ESR1 and 2, androgen receptor (AR) has a structure typical to NRs, composed of N-terminal domain, DNA-binding domain, hinge region and ligand-binding domain. Human AR is composed of 918 amino acids and forms a protein of 110 kDa (Brinkmann et al. 1989). The N-terminal domain accounts for most transcriptional activation of AR, is mainly involved in co-regulator recruitment and contains transcriptional activation regions AF1 and AF5. The ligand-binding motif contains strictly ligand-dependent AF2, which functionally interacts with intermediary factors and nuclear co-regulators (Gobinet et al. 2002). Upon androgen binding, the activated AR dissociates from its cytoplasmic chaperone proteins, undergoes a conformational change including phosphorylation, translocates to the nucleus, homodimerizes and binds to androgen response elements (AREs) of DNA, and regulates transcription (Centenera et al. 2008). Similar to other NRs, AR recruits various co-regulators and interacts with basal transcription machinery to regulate transcription (Gobinet et al. 2002, Centenera et al. 2008, Narayanan et al. 2008).

AR is widely distributed among reproductive and non-reproductive tissues including the prostate and seminal vesicles, male and female external genitalia, skin, testes, ovary, cartilage, sebaceous glands, hair follicles, sweat glands, cardiac muscle, skeletal muscle and smooth muscle, bone, gastrointestinal vesicular cells, thyroid follicular cells, adrenal cortex, liver, pineal, and numerous brain cortical and subcortical regions. Lack of AR in humans results in androgen insensitivity syndrome (AIS) which may be either complete (CAIS) or partial (PAIS), with symptoms including complete feminization of the male external genitalia or undervirilization, respectively (Matsumoto et al. 2008, Kerkhofs et al. 2009). Furthermore, recent studies in KO mice have indicated a role for androgens in the female folliculogenesis and mammary gland development (Shiina et al. 2006).

Like estrogens, androgens also induce rapid, membrane-initiated cell signaling. The pathways activated are very similar to estrogen-activated non-genomic signaling pathways, but it is unclear whether the receptor is the classical AR accumulating in the membranes or a new, yet unidentified membrane receptor (Foradori et al. 2008).

2.1.4 PHYSIOLOGICAL EFFECTS

Estrogens are involved in various physiological processes. At puberty, estrogens promote the growth and development of oviducts, uterus, vagina and external genitalia. After sexual maturation, estrogens regulate the menstrual cycle together with other hormones (Johnson et al. 1998). Estrogens act on the trophism of the urethral and vaginal mucosa, muscle tone, connective tissue of the genitourinary tract, and ductal elongation and epithelial proliferation of the breast (Delmanto et al. 2008), stimulate vaginal epithelial proliferation (Johnson et al. 1998) and protect against vaginal infections (Heinemann and Reid. 2005). During pregnancy, E2 contributes to uterine growth, placental development, parturition, and the development of the mammary gland (Coelingh Bennink. 2004). Estrogens conserve bone mass (Hughes et al. 1996, Manolagas. 2000), promote, maintain, and control the typical distribution of body fat and adipose tissue metabolism (Pallottini et al. 2008), and have an important effect on vascular physiology via increasing endothelial vasodilator function and promoting angiogenesis (Miller

and Duckles. 2008). In the brain, estrogens are neuroprotective and have a cognition-preserving function, protecting from Parkinson's and Alzheimer diseases (Green and Simpkins. 2000), and affect sexual behavior (Kudwa and Rissman. 2003, Imwalle et al. 2005). In males, estrogens are important in the regulation of bone mineral density and length (Simpson. 2000).

Androgens are needed for normal differentiation of male internal and external genitalia during fetal development (Kaufman and Bard. 1999, Winters. 1999, Welsh et al. 2008). During puberty, androgens are required for the development of male secondary sexual characteristics, stimulation of sexual behavior and function, and initiation of sperm production. In adult males, androgens maintain muscle mass and strength, fat distribution, bone mass, erythropoiesis, male hair pattern, libido, potency, and spermatogenesis (Winters. 1999). Androgens also have important functions in females. Androgens assist normal vaginal development (Drews. 2007). They also promote early follicular growth and proliferation of granulosa cells in the ovary and the development of the mammary gland, but the AR-mediated effect is not required for fertility, at least in mice (Drummond. 2006, Shiina et al. 2006, Matsumoto et al. 2008). However, androgens positively affect female sexual function, and furthermore, serve as estrogen precursors (Palacios. 2007).

2.1.5 BIOSYNTHESIS AND REGULATION

2.1.5.1 Sex steroid biosynthesis

Sex steroid biosynthesis *de novo* starts from cholesterol, which is mainly derived from serum low density lipoprotein (LDL) (Faust et al. 1977, Kovanen et al. 1980), but steroid-synthesizing cells can also synthesize cholesterol from acetyl coenzyme A (Rainey et al. 1986). Cholesterol is converted to pregnenolene by cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1, cytochrome P450, family 11, subfamily A, polypeptide 1). Pregnenolone can be further converted to 17-hydroxypregnenolone by cytochrome 17α-hydroxylase (CYP17A1, cytochrome P450, family 17, subfamily A, polypeptide 1) or to progesterone (P) by hydroxysteroid (3β) dehydrogenase 2 (HSD3B2). 17-hydroxypregnenolone can be converted to weak androgen androstenedione (A-dione), via the formation of DHEA by the enzymes CYP17A1 and HSD3B2. Alternatively, A-dione can be produced from P via 17-hydroxyprogesterone by CYP17A1. A-dione is an essential precursor for the last steps of estrogen and androgen biosynthesis. A-dione is converted to high-active T by HSD17Bs. Both A-dione and T can be further converted to estrogens by aromatase (Thompson and Siiteri. 1974). Weak estrogen E1 formed from A-dione is further converted to highly active E2 by HSD17B1 (Poutanen et al. 1993, Ghersevich et al. 1994c). Alternatively, T can be further converted to DHT, which is an important androgen especially in the prostate, via 5α-reduction (Luu-The et al. 2008).

In humans, steroid biosynthetic tissues include the ovaries, testes and adrenals. These tissues are capable of sex steroid biosynthesis *de novo*. In normal non-pregnant women of reproductive age, the majority of plasma E2 is produced by the ovaries, whereas plasma E1 is produced by extragonadal (adipose, skin) aromatization of A-dione to T, about half of the A-dione being of adrenal and half of ovarian source (Risch. 1998). Also adrenals express aromatase and are, therefore, capable of estrogen production (Moreau

et al. 2009). Circulating estrogens and androgens are bound to plasma albumin and sex hormone binding globulin (SHBG), leaving some 2-3% in a free form (Risch. 1998). In mice, commonly used as animal models in biomedical research, the adrenals lack Cyp17a1 expression, and consequently, mouse adrenals do not synthesize sex steroids (Keeney et al. 1995). In addition to biosynthesis *de novo*, especially humans and primates can synthesize sex steroids from circulating steroid precursors, such as E1 and A-dione, in extragonadal steroid target tissues. The ability to modulate steroid responses in the target cells at the prereceptor level is called **intracrinology** (Labrie et al. 2000, Labrie et al. 2001). Ovarian sex steroid biosynthesis is presented in Figure 3.

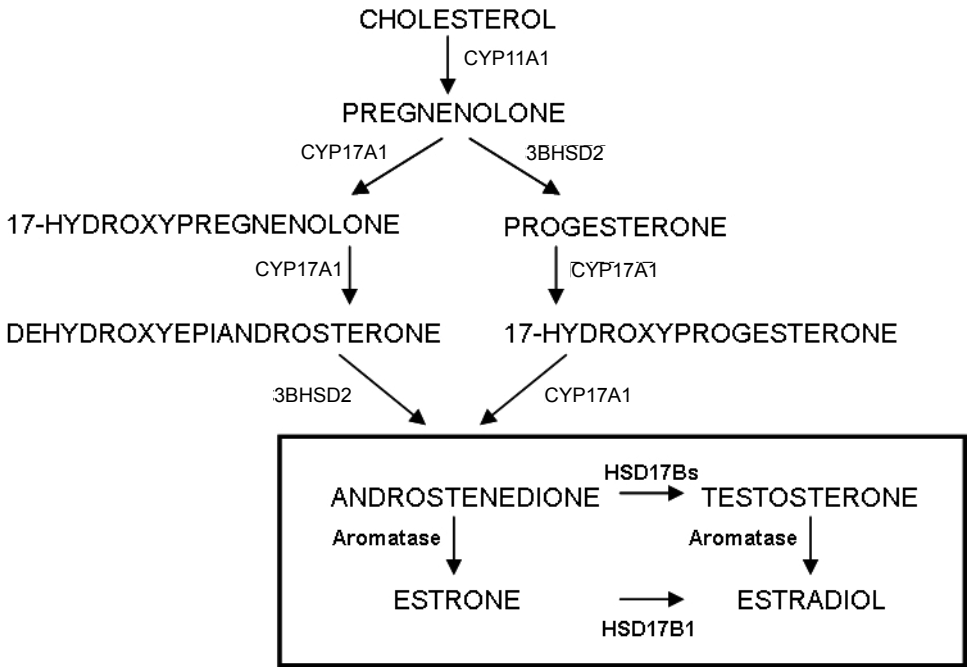


FIGURE 3. Ovarian sex steroid biosynthesis. Sex steroid biosynthesis *de novo* begins from cholesterol and via intermediates and P formation results in the formation of low active steroid precursors A-dione and E1, which are further converted to their active forms T and E2, respectively. The last steps of androgen and estrogen biosynthesis (highlighted by black box) can also take place in steroid target tissues expressing various enzymes able to catalyze the conversion between active and inactive steroid forms. This kind of prereceptor regulation is called intracrinology.

2.1.5.2 Regulation of sex steroid biosynthesis

The regulation of sex steroid biosynthesis is controlled via a feedback system called hypothalamus-pituitary-gonadal axis (HPGA), the regulation taking place on several levels. Steroid biosynthesis in the gonads is stimulated by two gonadothropins secreted from the anterior pituitary: luteinizing hormone (LH) stimulates androgen biosynthesis from the cholesterol in testicular Leydig cells (Choi and Cooke. 1990, Cooke et al. 1992, Pakarainen et al. 2005) and ovarian theca cells (Armstrong and Papkoff. 1976), while follicle-stimulating hormone (FSH) stimulates estrogen biosynthesis from androgenic precursors in females by upregulating aromatase activity (Dorrington et al. 1975, Erickson and Hsueh. 1978, Garzo and Dorrington. 1984). In males, FSH indirectly stimulates

spermatogenesis via the effects on testicular Sertoli cells (Walker and Cheng. 2005). The secretion of gonadotropins is further controlled by gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus. Additionally, the HPGA is affected by negative and positive feedback systems and two ovarian hormones called inhibin and activin. The function of HPGA differs in males and females and is programmed during embryonic development (Robinson. 2006). However, sex steroid biosynthesis in the adrenals is independent of gonadotropins, and is mainly regulated by adrenocorticotrophin (ACTH). Figure 4 presents the female HPGA.

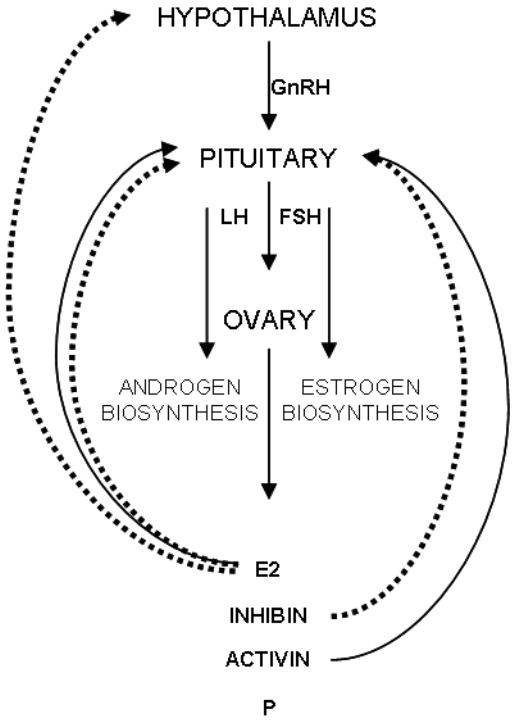


FIGURE 4. Regulation of sex steroid biosynthesis in females. E2 exerts a negative feedback effect on hypothalamus and pituitary gland, thus inhibiting ovarian E2 biosynthesis. Preceding ovulation, increasing E2 concentration triggers LH surge via positive feedback. Inhibin prevents FSH production from the pituitary, whereas activin stimulates FSH production. —— positive feedback, - - - - - negative feedback.

2.2 ROLE OF ESTROGENS AND ANDROGENS DURING EMBRYONIC DEVELOPMENT

2.2.1 BIPOTENTIAL GONAD

Sexual development in males and females begins with the migration of primordial germ cells to the urogenital ridge from the wall of the yolk sac at week four of gestation (embryonic day (E) 7-7.5 in mice) (Sariola et al. 2003, Kaufman and Bard. 1999). A mammalian gonad forms within the developing urogenital system, which itself derives from the intermediate mesoderm that runs the length of the embryo on either side of the

midline (Swain and Lovell-Badge. 1999). At this stage, the gonad is bipotential, thus it has the capability to differentiate either to the male or female pathway. As indicated by mouse studies, several factors including Wilms tumor 1 (Wt1) (Kreidberg et al. 1993), steroidogenic factor 1 (Sf1) (Luo et al. 1994), Lim homeobox protein (Lhx9) (Birk et al. 2000) and empty spiracles homolog (Emx2) (Miyamoto et al. 1997) are required for the formation of the indifferent gonad and, without any of these factors, gonadal development fails in both sexes. In addition to these, low levels of Sry-related HMMG box (Sox9) and dosage sensitive sex reversal adrenal hypoplasia congenital critical region of the x chromosome gene 1 (Dax1), which are essential genes for male and female differentiation, respectively, have been detected in the indifferent gonad (Swain et al. 1998). In addition to germ cells, there are three different somatic cell lineages present in the indifferent gonad: 1) the supporting cell lineage will give rise to Sertoli cells in the testis and follicle cells in the ovary, 2) the steroidogenic cell lineage produces sex steroids required for the development of secondary sexual characteristics of the male embryo: in males, these will form Leydig cells and in females, the theca cells, and 3) the connective cell lineage will lead to the development of testicular cords and ovarian stroma (Swain and Lovell-Badge. 1999). Although clear differences between male and female fetuses become apparent at week seven (E12 in mice) (Soder. 2007, Yao. 2005), the molecular differentiation has already begun. By the end of the sixth week of gestation (E9.5 in mice) (Swain and Lovell-Badge. 1999, Svechnikov and Soder. 2008), the indifferent gonad consists of different cell lineages with predefined maturational paths dependent on sex.

2.2.2 MALE SEXUAL DIFFERENTIATION

2.2.2.1 Development of male gonad (testis)

The genetic sex is determined by the karyotype of the fertilized egg, XX for the female and XY for the male. Testis development begins at the sixth week of gestation in humans (E10.5 in mice) (Koopman et al. 1991, Hacker et al. 1995, Soder. 2007). The development is initiated by expression of the sex-determining region of Y (SRY), which is a DNA-binding transcription factor (Harley et al. 1992). It has been shown to be responsible for the initiation of testicular Sertoli cell development from the somatic supporting cell lineage of the urogenital ridge (Koopman et al. 1991). In mice, it is expressed between E10.5 and E12.5 (Koopman et al. 1991, Hacker et al. 1995). Right after the induction of Sry (at E11.5), Sox9 is upregulated in the developing male gonad, whereas it is turned off in the developing ovary (Swain et al. 1998, Swain and Lovell-Badge. 1999). Wt1 and anti-Müllerian hormone (Amh) genes are suggested to be downstream targets of Sox9 (Sim et al. 2008). Desert hedgehog (Dhh) and fibroblast growth factor 9 (Fgf9) secreted from the newly formed Sertoli cells lead to proliferation of testicular Leydig cells responsible for androgen production, which is essential for the development of male sexual characteristics (Clark et al. 2000, Colvin et al. 2001). Leydig cells and subsequent androgen production appear at week eight (E11.5 in mice) (Siiteri and Wilson. 1974, Kaufman and Bard. 1999, Svechnikov and Soder. 2008). At E12.5, developing germ cells enter mitotic arrest, where they remain until birth (Wilhelm and Koopman. 2006).

2.2.2.2 Development of other male primary sexual characteristics

Androgen action during fetal life is essential for normal male development. It is notable that although sexual development in humans and mice is similar, the developmental time windows differ as reviewed below. As soon as testicular Leydig cells have appeared at week eight of gestation (E11.5 in mice), androgen production starts, and male internal and external genitalia start to develop. During weeks eight and nine (E11-11.5 in mice) (Kaufman and Bard. 1999, Sariola et al. 2003), androgen production stabilizes the Wolffian ducts, the precursors of male internal genitalia. The precursors of female internal genitalia, the Müllerian ducts, also form in males, but they early regress as a result of Amh action. By week 12 in humans, Wolffian ducts have differentiated to male internal genitalia, *i.e.* seminal vesicles, vas deferens, epididymis and the prostate (Reyes et al. 1973, Jirasek. 1977). In mice, the differentiation of internal genitalia continues until birth (Kaufman and Bard. 1999, Cederroth et al. 2007a). Masculinization of external genitalia starts at week ten in humans (E13.5-E14.5 in mice) (Josso. 2004, Kaufman and Bard. 1999) with lengthening of anogenital distance (AGD), followed by fusion of the labioscrotal folds to form the scrotum, and rapid elongation of the genital tubercle to form the phallus, and the closure of the opposing urethral folds to form tubular urethra and the corpus spongiosum around it (Jirasek. 1977). Similar to internal genitalia, the masculinization of male external genitalia is completed by week 11 in humans, whereas in mice the differentiation continues until birth. Testicular descent also differs between humans and mice. In humans, testicular descent occurs between weeks 27 and 35 (Wyndham. 1943). In mice, testicular descent takes place in two phases: the transabdominal phase dependent on insulin-like 3 (Insl3) expression during E15.5-E17.5, and the postnatal inguinoscrotal phase (Koskimies et al. 2003, Adham and Agoulnik. 2004, Cederroth et al. 2007b).

2.2.3 FEMALE SEXUAL DIFFERENTIATION

2.2.3.1 Development of female gonad (ovary)

Less is known about the molecular mechanisms and hormonal control of female sexual differentiation, but some information is available from studies in mouse models. As in males, the primordial germ cells migrate to the urogenital ridge (Kaufman and Bard. 1999, Sariola et al. 2003). Sox9 is expressed at low levels in the indifferent gonad, but it is turned off in the developing ovary (Swain et al. 1998). Also Dax1 is expressed at low levels in the indifferent gonad (E10.5), but is downregulated in males when testis development proceeds, whereas in female fetuses its expression is maintained until E12.5. Dax1 has been shown to antagonize Sry action (Swain et al. 1998). Ovarian development begins approximately at week seven, which is one week later than testis development in humans (E10.5 in mice) (Kaufman and Bard. 1999, Soder. 2007). Unlike in males, both somatic and germ cell lineages are required for gonadal development in females (Svechnikov and Soder. 2008). In the somatic supporting cell lineage, wingless-related MMTV integration site 4 (Wnt4) and follistatin (Fst) are expressed during E11.5-E15.5, antagonizing the formation of the testis vasculature, and they are also required for the survival of the germ cells (Jeays-Ward et al. 2003, Yao et al. 2004). The supporting cells further differentiate into granulosa cells. Forkhead box L2 (Foxl2) is considered a candidate gene for this process. Theca cells producing androgenic precursors for estrogen biosynthesis develop

from stromal steroidogenic precursor cells outside the follicles, but this takes place only after puberty (Swain and Lovell-Badge. 1999, Svechnikov and Soder. 2008). In the germ cell lineage, expression of folliculogenesis-specific basic helix-loop-helix (Figla, Figα) begins at E13.5. It is required for the production of zona pellucida and the formation of primordial follicles (Soyal et al. 2000). At week nine in humans (E15.5-16.5 in mice) (Nagy et al. 2003, Soder. 2007), the germ cells enter meiosis, which commits them to the ovarian fate and controls the onset of oogenesis (Yao. 2005). Differently from the role of T in male development, studies in KO mice have indicated that estrogen production is not essential for the female differentiation pathway in mammals (Couse et al. 1999b, Dupont et al. 2000, Britt and Findlay. 2003).

2.2.3.2 Development of other female primary sexual characteristics

In the absence of androgens in females, the Wolffian ducts regress. During weeks eight and nine (E15.5 in mice), uterine horns, cervix and oviducts begin to develop from the Müllerian ducts (Kaufman and Bard. 1999, Sariola et al. 2003). The differentiation is completed by week 14 (E17.5 in mice). At the same time, the two upper thirds of the vagina are derived from the mesonephric (Wolffian) component (Kaufman and Bard. 1999) or directly from the Müllerian duct, and only assisted by the Wolffian ducts (Drews. 2007), whereas the lower third is suggested to be derived from the urogenital sinus (Kaufman and Bard. 1999), although there is continuing discussion about the role of the involvement of the urogenital sinus in the development of the vagina (Drews. 2007). Similar to males, human female sex differentiation is mainly completed by the end of the first trimester (week 14), whereas in mice the differentiation continues until birth.

Mammary gland development in humans begins during the first trimester, and after midgestation in mice. The prenatal growth of the mammary gland appears to be independent of sex hormones and genetic sex. The development of the human mammary gland is initiated by the formation of milk streaks by ectodermal thickening during the first weeks of pregnancy (Jolicoeur. 2005). Depending on the species, different amounts of mammary glands develop from the streaks (*e.g.* one pair in humans and five pairs in mice) (Sariola et al. 2003). At the age of 12 weeks (by E13.5-15.5 in mice), each future mammary region discloses a small epithelial structure called a primary bud (Maronpot et al. 1999, Jolicoeur. 2005). The next step of development is the formation, downward growth and branching of solid epithelial cords called secondary epithelial outgrowths to the fat pad under the mesenchyme at the beginning of the second trimester of pregnancy (Sariola et al. 2003, Jolicoeur. 2005). By E16.5-17.5 in mice, the nipple is demarcated by invagination of epithelial cells (Maronpot et al. 1999). Mouse mammary tissue does not fully develop in males, because of the inhibitory effect of T secreted by the fetal testes. Although males may have an extensive branching duct system, alveoli and primary ducts, the exterior openings are not present (Maronpot et al. 1999). In contrast, human males also develop mammary glands that are indistinguishable from female mammary glands until puberty (Jolicoeur. 2005).

2.2.4 DISEASES RELATED TO IMPROPER SEX STEROID ACTION DURING DEVELOPMENT

2.2.4.1 Disorders of sexual development

Abnormal sex steroid concentrations during development can cause developmental abnormalities and diseases in later life. Disorders of sexual development (DSD) are defined as congenital conditions in which the development of chromosomal, gonadal, or anatomical sex is atypical. As reviewed by Nabhan and Lee (2007), these include several intersex conditions, *e.g.* hermaphroditisms. Known chromosomal abnormalities resulting in DSDs include Turner's syndrome (45, X) and Klinefelter's syndrome (47, XXY).

Genetic male (46, XY) DSDs include:

1) Defects in gonadal development which can be caused by Swyer syndrome, mutations in WT1, SOX9 or SF1, gonadal regression, or ovotesticular DSD.
2) Disorders of androgen synthesis or action resulting from defects in androgen biosynthetic enzymes (*e.g.* HSD17Bs, 5α-reductase, steroidogenic acute regulatory protein (StAR), P450 oxidoreductase (POR), HSD3B or 17, 20-lyase), Leydig cell hypoplasia or aplasia, or defects in androgen action (*e.g.* CAIS and PAIS).
3) Other disorders, such as hypospadias and micropenis.

Genetic female (46, XX) DSDs include:

1) Defects in gonadal development that can be caused by ovotesticular DSD, gonadal transformation (*e.g.* SRY translocation), or gonadal dysgenesis.
2) Disorders of androgen excess (*e.g.* congenital adrenal hyperplasia (CAH), aromatase deficiency, maternal luteoma of pregnancy, or exogenous androgen exposure).
3) Other disorders, such as congenital anomalies.

2.2.4.2 Polycystic ovary syndrome

In addition to DSDs, abnormal hormone concentrations during development increase the risk of various diseases later in life. PCOS is a disease conspicuously worsening the reproductive health of affected women. PCOS is the most common endocrinopathy in women, and it has been estimated that 5-10% of women of reproductive age are affected by PCOS (Franks. 2002, Jakubowski. 2005). The etiology of PCOS appears to be heterogenic and despite several trials, no clear genetic cause has been identified, although PCOS is considered to be hereditary (Jakubowski. 2005). Symptoms include anovulatory irregular menstrual cycle, increased number of small (2-9 mm) follicles at the edges of the ovary, and often prominent ovarian stroma associated with increased androgen production (Franks. 2002). Increased androgen activity may present either biochemically and/or clinically in the form of acne and hirsutism. In experimental animals, a PCOS-like state is induced in individuals prenatally exposed to androgens (Bruns et al. 2004, Recabarren et al. 2005). About half of the women with PCOS are overweight and suffer from insulin-resistance (Auersperg et al. 2001, Bruns et al. 2004, Recabarren et al. 2005).

2.2.4.3 Testicular dysgenesis syndrome

Cryptorchdism and testis hypoplasia are a typical features observed in rodent males exposed to estrogens during the fetal period (Gill et al. 1979, Stillman. 1982, Cederroth et al. 2007b). Fetal estrogen exposure is suggested to contribute to the development of testicular dysgenesis syndrome also in humans, including symptoms such as cryptorchidism and hypospadias (Toppari et al. 1996, Sharpe. 2001).

2.3 HYDROXYSTEROID (17β) DEHYDROGENASE TYPE 1

2.3.1 HSD17Bs

Hydroxysteroid (17β) dehydrogenases (HSD17Bs) are a family of enzymes that catalyze the conversion between the low active 17-ketosteroids and the highly active 17β-hydroxysteroids, mainly androgens and estrogens. These reactions are dependent on cofactors nicotinamide adenine dinucleotide (NAD(P)(H)) and take place at position C17 of the steroid scaffold (Lukacik et al. 2006, Mindnich and Adamski. 2009). The position is shown in Figure 5 with estrogens as an example. All except one of the HSD17Bs belong to the short-chain dehydrogenase/reductase (SDR) family. Only HSD17B5 is a member of the aldo-keto reductase (AKR) family (Peltoketo et al. 1999a, Persson et al. 2009). At present, 14 mammalian HSD17Bs have been identified (Lukacik et al. 2006, Day et al. 2008b). The enzymes possess a variable sequence homology, expression pattern, cofactor preference, substrate specificity, and subcellular localization (Lukacik et al. 2006, Wu et al. 2007). From an evolutionary perspective, the family can be divided into two groups: 1) the classical HSD17Bs include types 1, 2 and 3. These enzymes have a strict expression pattern and are steroid-specific, but their evolution has been fast, thus, they are not highly conserved, and 2) the more ancient and conserved group of HSD17Bs includes types 4, 7, 8, 10 and 12. Type 14 has been found only recently and little information is available about it (Mindnich and Adamski. 2009). Overall, the complexity of the whole SDR family refers to the occurrence of one or more ancient oxidoreductases, which adapted towards different substrate molecules by gene duplication and transfer events (Baker. 2001, Wu et al. 2007). Functionally, the family can be divided into 1) steroid-inactivating oxidative enzymes dependent on NAD^+ cofactor (types 2, 4, 6, 8, 10, 11, 14), and 2) reductive steroid-activating enzymes dependent on NADPH as cofactor (types 1, 3, 5, 7 and 12) (Lukacik et al. 2006).

2.3.2 HSD17B1

2.3.2.1 Gene

Two genes code for human HSD17B1 (HSD17B1 I and HSD17B1 II) (Luu-The et al. 1990, Peltoketo et al. 1992). The genes are located in tandem in chromosome 17, region q12-21 (Peltoketo et al. 1988, Luu The et al. 1989, Winqvist et al. 1990). They show 89% sequence homology and a similar promoter area, but HSD17B1 I contains a premature stop codon, and thus, is considered a pseudogene (Luu-The et al. 1990, Peltoketo et al. 1992). The correctly transcribed HSD17B1 II (referred to as HSD17B1 in the present study) contains six exons and five short introns, and produces two transcripts

of approximately 1.3 and 2.3kb in size. The two transcripts differ in their 5' untranslated region in that the 1.3kb mRNA starts at 9-10 nucleotides from the upstream ATG start codon, while the 2.2kb mRNA has at least 814 non-coding nucleotides at the 5' end (Luu The et al. 1989, Luu-The et al. 1990). The 1.3kb mRNA expression is associated with estrogen-producing tissues, such as the placenta, ovaries, breast and endometrium, whereas the longer 2.2kb form is more constitutively expressed and does not correlate with HSD17B1 protein level (Luu The et al. 1989, Luu The et al. 1989, Tremblay et al. 1989, Miettinen et al. 1996, Tremblay et al. 1997).

Upstream from the transcription start site of the 1.3kb mRNA, elements typical of a gene promoter have been found. These include a TATA box (nucleotides from -32 to -26), a GC-rich area (nucleotides from -71 to -42) and an inverted CAAT element (nucleotides from -92 to -89). The GC-rich area of the promoter contains binding sites for transcription factors Sp1 (-52 to-43) and AP2 (-62 to -53) (Luu-The et al. 1990, Peltoketo et al. 1992, Piao et al. 1995, Piao et al. 1997). Mutation of the Sp1 binding site has been shown to decrease the promoter activity *in vitro*, suggesting that Sp1 is essential for the promoter function. Binding of AP2 has been shown to inhibit the binding of Sp1 and 3, indicating that AP2 may be a repressor of the promoter activity (Piao et al. 1997). The upstream area also contains an enhancer (-661 to -392) and a silencer (-392 to -78) (Piao et al. 1995). GATA factors bound to the region in the middle of the silencer (-103 to -98) were shown to repress transcription (Piao et al. 1997). Progestin and glucocorticoid -response elements (PRE and GRE, respectively) have been located to start from sites -191 and -144. The retinoic acid response element (RRE) was located between bases -503 and -487 in the HSD17B1 enhancer (Piao et al. 1995). Also several EREs were found upstream of the transcription start site (starting at -490, -419, -315 and -203) as well as a binding site for cAMP (starting at -803) (Peltoketo et al. 1992). Recently, a minimal SMAD-binding element was mapped to locate within 100bp of the start of transcription (Bak et al. 2009).

2.3.2.2 Protein

Human HSD17B1 is a cytoplasmic protein first found by Langer and Engel in 1958 in human placenta. Since then, it has been extensively studied and is currently considered a promising drug target to decrease estrogen action in estrogen-dependent diseases. Rodents also have Hsd17b1 with one major difference compared to the human enzyme: rodent Hsd17b1 can activate both estrogens and androgens with the same catalytic efficiency (Ghersevich et al. 1994a, Nokelainen et al. 1996), while the human enzyme is considered to be estrogen-specific (Poutanen et al. 1993, Puranen et al. 1997b). The gene of human HSD17B1 codes for a protein of 327 amino acids, having a calculated molecular weight of 34853 Da (Peltoketo et al. 1988, Luu The et al. 1989). The core structure of human HSD17B1 is a seven-stranded, parallel β-sheet (βA-βG) surrounded by six parallel α-helices (αB-αG). The sheets βA-βF form a basic α/β-motif with alternating β-strands and α-helices. They make up a classic βαβ -Rossmann fold associated with the cofactor NAD binding. In addition to partial forming of the Rossmann fold, the sheets βD-βG govern quaternary association and substrate binding (Ghosh et al. 1995).

The first 200 amino residues of human HSD17B1 contain conserved sequences, such as Try-X-X-X-Lys and Gly-X-X-X-Gly-X-Gly (where X is any amino acid) that contribute to the formation of the active site and the Rossmann fold, respectively (Baker. 1994, Jornvall et al. 1995). The residues up to Pro150, that also contain the cofactor binding site, are not necessary for substrate specificity (Ghosh et al. 1995). The Tyr and Lys residues of the Tyr-X-X-X-Lys sequence, and Ser142, are highly conserved in the SDRs and have been suggested to directly contribute to the catalytic reaction (Ensor and Tai. 1991, Obeid and White. 1992, Chen et al. 1993, Baker. 1994, Jornvall et al. 1995, Puranen et al. 1997a). The amino residues most essential for catalytic activity include Ser142, Tyr155 and Lys159 (Ghosh et al. 1995, Azzi et al. 1996, Puranen et al. 1997a), but also His210 together with His213 and His221 are essential for enzyme activity (Puranen et al. 1994). The substrate-binding region contains three α-helices and a helix-turn-helix motif that are not observed in other SDRs. The helices are located at one end of the substrate-binding cleft and restrict access to the active site and affect substrate specificity (Ghosh et al. 1995). Studies in human and rat chimeric enzymes have shown that the region responsible for the substrate specificity locates between the residues 148 and 268 (Puranen et al. 1997b), also supported by structural data (Ghosh et al. 1995). Also the most remarkable sequence diversity between human and rodent enzymes is located in this area (Nokelainen et al. 1996, Puranen et al. 1997b). The substrate-binding cleft is narrow and highly complementary to estrogens (Ghosh et al. 1995, Azzi et al. 1996). The binding of the E2 molecule is orientated by four hydrogen bonds between O3 and His221 and Glu282, and between O17 and Tyr155 and Ser142 (Azzi et al. 1996, Zhorov and Lin. 2000, Lin et al. 2006). HSD17B1 uses both NAD(H) and NADP(H) as cofactor, as well as the reduced forms, but it prefers NADP(H) (Jarabak and Sack. 1969). Figure 5 shows the reaction most potentially catalyzed by HSD17B1.

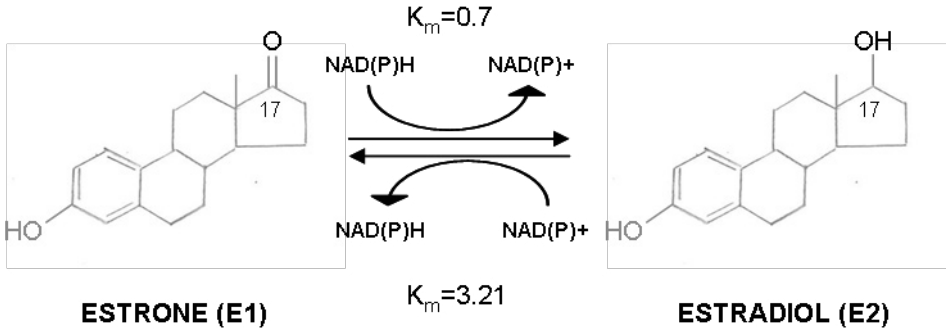


FIGURE 5. The estrogenic reactions catalyzed by human HSD17B1. Human HSD17B1 catalyzes the interconversion between 17-ketosteroid E1 and highly active 17β-hydroxysteroid E2. The reaction takes place in both directions but HSD17B1 has much higher activity towards E1 as substrate, and thus, its major function is to activate estrogens. Both NAD(H) and NADP(H) can serve as cofactor for human HSD17B1, but the enzyme prefers NADP(H).

The functional HSD17B1 protein exists as a homodimer of approximately 68kDa in size (Burns et al. 1972, Nicolas and Harris. 1973, Inano and Tamaoki. 1984, Lin et al. 1992). The structure of human HSD17B1 is shown in Figure 6. The dimerization has been shown to be essential for the enzyme activity, and the residues Leu111, Val113,

Ala170 and Phe172 are essential for the dimer formation (Puranen et al. 1997a). The dimeric interface of the enzyme is formed at the center of a four-helix bundle, comprising two helices (αE-αF) in each monomer. The conserved Tyr and Lys residues of the active site also participate in the formation of the dimer (Ghosh et al. 1995). There have been problems in resolving the structure of the last 38 amino acids of human HSD17B1 (Ghosh et al. 1995). It has been shown that the C-terminus is not needed for the proper folding of the enzyme, but may be involved in its stabilization (Puranen et al. 1997a). Finally, human HSD17B1 contains a consensus sequence for cAMP-dependent phosphorylation and the enzyme is phosphorylated by PKA *in vitro* on Ser134 (Barbieri et al. 1994, Puranen et al. 1997a), but this residue was shown not to affect the enzyme activity.

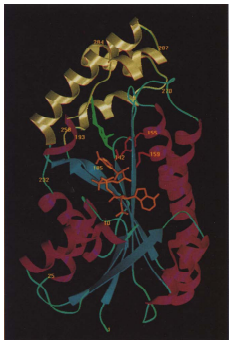


FIGURE 6. Structure of human HSD17B1. A ribbon diagram of human HSD17B1 structure with the substrate-binding domain, comprising helices αG", αG', αH' and αH, highlighted in gold. The modelled substrate, E2, is shown in stick representation. Figure adapted from Ghosh et al. 1995.

2.3.2.3 Physiological role

As indicated by several studies, HSD17B1 efficiently catalyzes the conversion of E1 to E2 *in vitro*. In cultured COS m6 cells, HSD17B1 transfection increased the reductive activity (E1 to E2) approximately 23-fold, while the oxidative activity was not increased (Poutanen et al. 1993). The enzyme also catalyzes the opposite oxidative reaction (E2 to E1), but remarkably less efficiently (Puranen et al. 1994, Miettinen et al. 1996, Puranen et al. 1997b). Although greatly preferring estrogens, HSD17B1 has also been shown to catalyze the conversion of A-dione to T in cultured cells, but this activity was only 20% of that obtained with E1 as substrate (Poutanen et al. 1993, Puranen et al. 1997b). HSD17B1 has been shown to enhance estrogen production also *in vivo* by measuring estrogenic endpoints, such as estrogen-dependent tumor growth and uterus weight (Husen et al. 2006a, Husen et al. 2006b). Because human HSD17B1 is expressed in both estrogen biosynthetic and target tissues, it has been hypothesized that the physiological role of HSD17B1 is to catalyze a reaction essential for ovarian estrogen biosynthesis (Figure 3) and to regulate estrogen concentration tissue-specifically in estrogen target cells at the prereceptor level (Figure 7).

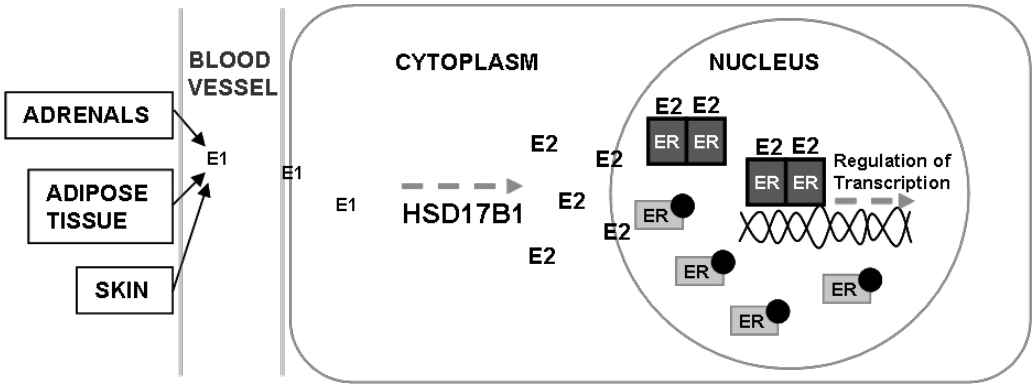


FIGURE 7. Physiological role of human HSD17B1. In estrogen target cell, for example in breast and endometrial epithelial cells, circulating E1 produced by adipose tissue, skin and adrenals diffuses through the plasma membrane and is converted to E2 by cytoplasmic HSD17B1. Within the nucleus, E2 binds to ESR, which binds to the ERE sequence in DNA and regulates transcription of estrogen-dependent genes.

2.3.2.4 Tissue distribution and regulation of expression

The expression of functional human HSD17B1 has been found in the syncytiotrophoplast cells of the placenta (Fournet-Dulguerov et al. 1987, Luu-The et al. 1990, Zhu et al. 2002) and fetal (Vaskivuo et al. 2005) and adult ovarian granulosa cells (Tremblay et al. 1989, Luu-The et al. 1990, Ghersevich et al. 1994c), both being estrogen biosynthetic tissues. Furthermore, HSD17B1 is expressed in estrogen target tissues, including breast (Poutanen et al. 1992, Miettinen et al. 1999), endometrium (Maentausta et al. 1991, Miettinen et al. 1996, Smuc et al. 2006, Dassen et al. 2007, Smuc et al. 2007, Fechner et al. 2007) and bone (Sasano et al. 1997). In males, HSD17B1 is expressed in the prostate (Luu-The et al. 2008). The tissue distribution of the mouse enzyme is somewhat different than in humans. In female mice, Hsd17b1 expression has been found mainly in the ovaries, but also in the adrenals, uterus and brain, but not in placenta. In male mice, Hsd17b1 is expressed in testis and liver (Nokelainen et al. 1996). Table 2 summarizes the expression level of HSD17B1 in different female tissues in humans and mice based on a literature analysis and experimental data obtained in the present study.

Table 2. Comparison of HSD17B1 expression profile between humans and rodents.

TISSUE	HUMAN	MOUSE/RAT	HSD17B1TG MOUSE
Placenta	High	No expression	High
Ovary	High	High	High
Endometrium	Low	No expression	High
Mammary gland	High	No expression	High
Bone	Expressed at unknown level	Expressed at unknown level	High

HSD17B1 expression has been shown to be cAMP-dependent in several studies, but because of conflicting results, cAMP regulation is suggested to be cell type -specific. In cultured human primary granulosa-luteal cells and cultured human primary

cytotrophoblast cells, cAMP analogs decreased HSD17B1 expression (Tremblay et al. 1989), while the expression was increased in cultured diethylstilbestrol (DES)-primed primary rat granulosa cells (Ghersevich et al. 1994b) and human cultured choriocarcinoma cells (Tremblay et al. 1989, Lewintre et al. 1994b). Several hormones regulate HSD17B1 expression. FSH was shown to increase Hsd17b1 expression in a cAMP-mediated manner in cultured DES-primed rat primary granulosa cells (Ghersevich et al. 1994b). Phophol-12-myristate-13-acetate (PMA) attenuated the stimulatory effects of cAMP and FSH in cultured DES-primed rat granulosa cells (Kaminski et al. 1997). DES itself increased Hsd17b1 expression in cultured rat granulosa cells, in the same way as E2, T and DHT, by enhancing the effects of FSH (Ghersevich et al. 1994a, Ghersevich et al. 1994b). Treatment of FSH- or DES- plus FSH-primed rats with human chorionic gonadotrophin (hCG) induced luteinization, resulting in strong downregulation of Hsd17b1 expression (Ghersevich et al. 1994a). Progestins induced HSD17B1 expression in one of two human breast cancer cell lines studied (Poutanen et al. 1990). Progestin level was shown to correlate with HSD17B1 expression in human endometrium (Maentausta et al. 1991, Maentausta et al. 1993), but in another study no correlation was found (Dassen et al. 2007). Retinoic acids have been shown to induce HSD17B1 expression in cultured human choriocarcinoma, cytotrophoblast and breast carcinoma cells (Piao et al. 1995, Zhu et al. 2002). Activin A, a regulator of ovarian E2 biosynthesis, has been shown to induce rodent Hsd17b1 expression in cultured mouse and rat granulosa cells, cultured mouse gonadotroph cells and mouse pituitary (Ghersevich et al. 2000, Bak et al. 2009). Prevention of the binding of GATA transcription factors was shown to increase transcriptional activity of HSD17B1 promoter in human choriocarcinoma cells (Piao et al. 1997).

Several growth factors affect HSD17B1 expression. Similar to cAMP, controversial data on epidermal growth factor (EGF) and transforming growth factor α (TGFα) have been reported: in cultured DES-primed primary rat granulosa cells, Egf and Tgfα decreased Hsd17b1 expression by repressing the effects of FSH (Ghersevich et al. 1994b, Lewintre et al. 1994b, Kaminski et al. 1997), while HSD17B1 expression was increased in two out of three cultured human choriocarcinoma cell lines studied, via the tyrosine kinase activity of the EGF and TGFα receptors (Lewintre et al. 1994b). Basic fibroblast growth factor (bFGF, FGF2) increased HSD17B1 expression in two out of three cultured human choriocarcinoma cell lines studied (Lewintre et al. 1994a), but decreased the expression in cultured DES-primed primary rat granulosa cells (Kaminski et al. 1997). Insulin-like growth factors (IGFs) types 1 and 2 stimulated HSD17B1 expression in cultured human breast cancer cells (Singh and Reed. 1991). Also various cytokines regulate HSD17B1 expression. Tumor necrosis factor α (TNFα) increased HSD17B1 expression in cultured human endometrial glandular epithelial cells (Salama et al. 2009) and cultured human breast cancer cells (Duncan et al. 1994). Interleukin 1β (IL1B) increased HSD17B1 expression in cultured human breast cancer cells (Duncan et al. 1994). For IL6, both a stimulatory effect and no effect have been reported (Adams et al. 1991, Duncan et al. 1994). The regulation of HSD17B1 expression is summarized in Table 3. Interestingly, recent studies have indicated that human HSD17B1 is also regulated by micro RNAs in the endometrium (Pan et al. 2007). The regulatory mechanisms discussed above mainly take place at the expression level. However, HSD17B1 activity may also be regulated

Table 3. Summary of regulation of HSD17B1 expression.

REGULATION OF HSD17B1 EXPRESSION					
REGULATOR	**EFFECT**	**MECHANISM**	**CELL/TISSUE TYPE**	**SPECIES**	**REFERENCE**
cAMP	-	Not specified	Cultured granulosa-luteal cells	Human	(Tremblay et al. 1989)
	+	Not specified	Cultured DES-primed granulosa cells	Rat	(Ghersevich et al. 1994b)
	+	Not specified	Choriocarcinoma cells	Human	(Tremblay et al. 1989, Lewintre et al. 1994b)
	-	Not specified	Cultured cytotrophoblast cells	Human	(Tremblay et al. 1989)
FSH	+	cAMP-mediated	Cultured DES-primed granulosa cells	Rat	(Ghersevich et al. 1994b)
PMA	-	Effects of FSH and cAMP repressed	Cultured DES-primed granulosa cells	Rat	(Kaminski et al. 1997)
Estrogens	+	Effects of FSH enhanced	Cultured DES-primed granulosa cells	Rat	(Ghersevich et al. 1994b)
DES	+	Effects of FSH enhanced	Cultured granulosa cells	Rat	(Ghersevich et al. 1994b, Ghersevich et al. 1994a)
Androgens	+	Effects of FSH enhanced	Cultured DES-primed granulosa cells	Rat	(Ghersevich et al. 1994b)
hCG	-	Induction of luteinizaton	Ovary	Rat	(Ghersevich et al. 1994a)
Progestin	+	Not specified	Cultured breast cancer cells	Human	(Poutanen et al. 1990)
	+	Not specified	Endometrium	Human	(Maentausta et al. 1991, Maentausta et al. 1993)
Retinoic acids	+	Not specified	Cultured choriocarcinoma cells	Human	(Piao et al. 1995, Zhu et al. 2002)
	+	Not specified	Cultured cytotrophoblast cells	Human	(Zhu et al. 2002)
	+	Not specified	Cultured breast cancer cells	Human	(Piao et al. 1995)
Activin A	+	Not specified	Cultured DES-primed granulosa cells	Rat	(Ghersevich et al. 2000)
	+	Alk4, Smad2	Cultured gonadotroph cells	Mouse	(Bak et al. 2009)
	+	Not specified	Pituitary	Mouse	(Bak et al. 2009)
	+	Alk4, Smad2	Cultured granulosa cells	Mouse	(Bak et al. 2009)
GATA	-	Silencer binding	Cultured choriocarcinoma cells	Human	(Piao et al. 1997)
EGF	-	Effects of FSH repressed	Cultured DES-primed granulosa cells	Rat	(Ghersevich et al. 1994b, Kaminski et al. 1997)
	+	Receptor tyrosine kinase activity	Cultured choriocarcinoma cells	Human	(Lewintre et al. 1994b)
TGFα	+	Receptor tyrosine kinase activity	Cultured choriocarcinoma cells	Human	(Lewintre et al. 1994b)
	-	Effects of FSH repressed	Cultured DES-primed granulosa cells	Rat	(Kaminski et al. 1997)
TGFβ	+	Not specified	Cultured DES-primed granulosa cells	Rat	(Kaminski et al. 1997)
bFGF	+	Not specified	Cultured choriocarcinoma cells	Human	(Lewintre et al. 1994a)
	-	Effects of FSH repressed	Cultured DES-primed granulosa cells	Rat	(Kaminski et al. 1997)
IGF1	+	Not specified	Cultured breast cancer cells	Human	(Singh and Reed. 1991)
IGF2	+	Not specified	Cultured breast cancer cells	Human	(Singh and Reed. 1991)
TNFα	+	Not specified	Cultured breast cancer cells	Human	(Duncan et al. 1994)
	+	Not specified	Cultured endometrial glandular cells	Human	(Salama et al. 2009)
Il6	+	Not specified	Cultured breast fibroblast	Human	(Adams et al. 1991)
Il1B	+	Not specified	Cultured breast cancer cells	Human	(Duncan et al. 1994)

at cofactor level, since several abnormal physiological conditions, such as hypoxia, metabolic stress and impairment of glucose uptake may affect intracellular redox states and nicotinamide cofactor gradients (Sherbet et al. 2007).

2.3.2.5 Other HSD17Bs with estrogen reductase activity

In addition to HSD17B1, HSD17B7 and HSD17B12 are also estrogen reductases and based on recent studies there has been discussion that HSD17B12 would actually overtake the proposed function of HSD17B1 in E2 biosynthesis (Luu-The et al. 2006, Day et al. 2008a). However, although HSD17B1 expression in all tissues except the placenta has been shown to be lower than HSD17B7 and HSD17B12 expression, the k_m value of HSD17B1 for E1 is markedly lower than those of HSD17B7 and HSD17B12, indicating that HSD17B1 is a remarkably more efficient enzyme in E2 biosynthesis (Luu-The et al. 2006). Furthermore, recent studies indicate that the major roles of both HSD17B7 and HSD17B12 are in cholesterol biosynthesis and fatty acid metabolism, respectively, *i.e.* elsewhere than in sex steroid -related pathways. HSD17B7 has a role in cholesterol biosynthesis and HSD17B12 is involved in fatty acid metabolism (Moeller and Adamski. 2009). Furthermore, the tissue distributions of both HSD17B7 and HSD17B12 are more abundant than that of HSD17B1. In addition to steroid-related tissues also expressing HSD17B1, both HSD17B7 and HSD17B12 are expressed in a variety of other tissues (Torn et al. 2003, Sakurai et al. 2006). However, in addition to their role in non-steroidal metabolism, these enzymes may also in part contribute to estrogen biosynthesis.

2.4 ROLE OF HSD17B1 IN TISSUE-SPECIFIC REGULATION OF ESTROGEN ACTION

2.4.1 PLACENTA

The placenta is a complex, primarily vascular organ adapted to optimize the exchange of gases, nutrients, and electrolytes between maternal and fetal circulations. In humans, the placenta is also a major endocrine gland capable of producing large amounts of both protein and steroid hormones. It is unique among endocrine glands in that its secretory activity is autonomous and not subject to maternal or fetal signals (Johnson et al. 1998).

2.4.1.1 Human placental steroid biosynthesis

The early human placenta produces steroids, although the role of ovarian hormones in early primate pregnancy is acknowledged. Ovarian corpus luteum secretes P and E2, and their synthesis in the ovary is maintained by hCG secreted by placental trophoblast cells. After seven to eight weeks of gestation, the placenta becomes the dominant source of estrogen and P, and the role of ovaries is diminished. During human pregnancy, large quantities of estrogens are produced, the amount increasing towards the end of pregnancy. In addition to E1 and E2, large amounts of E3 are produced by the placenta, fetal liver and fetal adrenals, covering up to 90% of the total estrogens during pregnancy. In the placenta, E3 is derived from fetal 16OH-DHEA secreted by the fetal liver, and its function is unknown, but it is often used as an indicator of fetal well-being (Simpson

and MacDonald. 1981, Johnson et al. 1998). The human placenta lacks CYP17A1 expression and due to the consequent lack of sex steroid precursor production, placental E1 and E2 are derived from androgenic precursors produced by the fetal and maternal adrenals. Thus, the placenta is indirectly subject to regulation of fetal and maternal ACTH stimulating adrenal androgen biosynthesis (Johnson et al. 1998). The circulating androgenic precursors are further aromatized to estrogens in the placenta (Simpson and MacDonald. 1981, Takeyama et al. 1998). The major endocrine regulator of aromatase expression in the human placenta is hCG (Simpson and MacDonald. 1981). In addition to the lack of steroid precursor production, the ability to synthesize cholesterol in the placenta is limited, and P is produced from circulating maternal cholesterol, its synthesis being stimulated by hCG (Simpson and MacDonald. 1981, Pasqualini. 2005).

2.4.1.2 Role of HSD17B1 in placental steroid biosynthesis

Similar to aromatase, human HSD17B1 is most highly expressed in the syncytiotrophoblasts of human placenta (Fournet-Dulguerov et al. 1987, Poutanen et al. 1995, Takeyama et al. 1998), and consequently, it has been hypothesized that HSD17B1 contributes to placental E2 biosynthesis. Differentiated syncytiotrophoblasts form the most active placental cell type and they produce, *e.g.* cytokines and growth factors, several of which have been shown to regulate HSD17B1 expression (Table 3) (Simpson and MacDonald. 1981, Lewintre et al. 1994b, Lewintre et al. 1994a, Peltoketo et al. 1996). In addition to HSD17B1, another estrogen reductase, HSD17B7, is expressed in human placenta but its significance in estrogen production remains unclear (Torn et al. 2003). Also HSD17B2, which opposes the action of HSD17B1 by converting E2 to E1, is highly expressed in the placenta and is suggested to protect the fetus from a too high estrogen concentration (Takeyama et al. 1998).

2.4.1.3 Rodent placenta

Fetal estrogen supply is different in rats than in humans and mechanisms similar to those in rats are likely to take place in mice. During rat pregnancy, estrogens and P are mainly provided by maternal ovaries, while the placenta has a minor role (Townsend and Ryan. 1970, Matt and MacDonald. 1984). However, in contrast to humans, the rodent placenta expresses Cyp17a1, and thus, can convert P of maternal origin to androgens, which then circulate back to the ovaries to serve as precursors for estrogen biosynthesis (Warshaw et al. 1986, Ben-Zimra et al. 2002). On the other hand, although Hsd17b1 and aromatase are not expressed in the rodent placenta (Akinola et al. 1997), Hsd17b7 and Hsd17b2 are (Mustonen et al. 1997, Nokelainen et al. 2000). Furthermore, both reductive and oxidative activity towards estrogens have been detected in mouse placenta from E9 onwards (Blomquist et al. 1993), suggesting that also the rodent placenta may directly regulate estrogen concentration.

2.4.2 OVARY

2.4.2.1 Folliculogenesis

Folliculogenesis is the basic unit of ovarian activity and has a dual purpose: oocyte maturation and steroid production. Oogonia are the precursors of adult gametes. They

are mitotically dividing, but still premeiotic diploid germ cells representing the pool from which the meiotic oocytes develop and differentiate. The process of meiotic oocyte development from mitotic oogonia is termed oogenesis and it is initiated by premeiotic DNA synthesis at the ninth week of gestation, after which the germ cells are called oocytes. The oocyte is arrested at a final stage of the meiotic prophase, called diplotene, and it is during this stage that the somatic pregranulosa cells enclose the oocyte, forming the primordial follicle (week 12 of gestation), the earliest stage of follicular development (Sathananthan et al. 2006, Soder. 2007, Oktem and Oktay. 2008).

The vast majority of oocytes (~seven million) are lost by atresia during the lifetime of a woman and only about 400-500 oocytes are normally ovulated from puberty to menopause (Sathananthan et al. 2006, Oktem and Oktay. 2008). The recruitment of quiescent primordial follicles into a growing pool of primary follicles starts at fetal age and continues postnatally until the ovarian reserve of primordial follicles is depleted at menopause (Oktem and Oktay. 2008). The formation of primary follicles from the primordial stage is started at week 24 of gestation. By the end of pregnancy, some follicles have already developed to secondary (preantral) follicles (McGee and Hsueh. 2000, Soder. 2007). The progression of primary follicle to secondary follicle is continuously operational until menopause. These transitions from primordial to primary and primary to secondary follicles seem to be gonadotropin-independent, and only antral follicles become responsive to FSH (Oktem and Oktay. 2008). Until puberty, primordial follicles are continuously recruited to mature, but they cannot finalize their maturation and instead turn into atresia. This is called initial recruitment. Cyclic recruitment begins at puberty and is induced by increasing concentrations of FSH during each reproductive cycle (McGee and Hsueh. 2000).

The next step of folliculogenesis is the formation of the antral follicle, which is also the stage from which the cyclic recruitment of follicles begins at puberty (McGee and Hsueh. 2000, Oktem and Oktay. 2008). By this stage, the granulosa cell epithelium surrounding the oocyte has become multilayered and the stromal cells surrounding the follicle differentiate into two layers called theca interna and theca externa (Sathananthan et al. 2006). From the group of growing antral follicles, the one growing best is selected to be the dominant follicle, while the others turn into atresia. Within two weeks, the dominant antral follicle develops into the preovulatory Graafian follicle (McGee and Hsueh. 2000). The larger the follicle grows, the higher the E2 secretion becomes, until it finally triggers positive feedback inducing an LH surge resulting in ovulation within 5-15 hours (Garcia et al. 1981). Ovulation is a process characterized by the rupture of the ovarian surface epithelium and extrusion of the oocyte. Granulosa cells of the remaining follicle become luteinized and the whole structure is transformed into the corpus luteum, synthesizing E2 and P to sustain the possible pregnancy (Oktem and Oktay. 2008). Without fertilization, the corpus luteum regresses to the corpus albicans, and the resulting drop in E2 and P levels induces an increase in the pituitary FSH production, thus starting a new cycle (le Nestour et al. 1993). The ovarian function is thought to be similar in humans and mice, with a few exceptions: firstly, in mice, the menstrual cycle is called estrous cycle and it lasts for only four to seven days, compared to 28 days in humans (Staley and Scharfman. 2005). Secondly, in rodent ovaries, several follicles develop simultaneously instead of the one dominant follicle selected in humans (Nagy et al. 1999).

2.4.2.2 Role of HSD17B1 in ovarian estrogen biosynthesis

The ovary is a steroid-biosynthetic organ producing androgens, estrogens and progestins. The dominant follicle and corpus luteum are the principle sites of estrogen production during the follicular and luteal phases of the cycle, respectively (Shoham and Schachter. 1996). Two different cell populations are involved in ovarian follicular E2 biosynthesis. During the follicular phase, the developing oocyte is surrounded by two layers of cells: the outer layer is composed of steroidogenic theca cells, which synthesize androgens *de novo* from cholesterol. Their function is stimulated by LH. The inner layer is composed of granulosa cells, which express aromatase and HSD17B1, and thus, convert androgens and E1 to E2, with FSH being the major regulator of this process. This is called the "two cells, two gonadotropins" theory (Liu and Hsueh. 1986, Richards. 1994, Peltoketo et al. 1999b).

HSD17B1 expression in the fetal human ovary appears between weeks 16-19 of pregnancy and localizes to granulosa cells. The expression of ESRs in the granulosa cells appears between weeks 20-23 (Vaskivuo et al. 2005), but aromatase expression in fetal human ovaries is very low (Vaskivuo et al. 2002). In the adult human ovary, the expression HSD17B1 is cyclic. Both HSD17B1 and aromatase expressions are related to the differentiation stage of the follicles and correlate with E2 concentration. HSD17B1 is expressed in the granulosa cells of developing follicles; in primary follicles with a single layer of cuboidal-shaped granulosa cells, in preantral follicles with multiple layers of granulosa cells, and in large antral follicles (Sawetawan et al. 1994). Aromatase expression is restricted to antral follicles (Suzuki et al. 1993). In the human corpus luteum, HSD17B1 is expressed in luteinizing granulosa cells (Sawetawan et al. 1994). HSD17B1 expression increases from day two of the luteal phase, reaches its maximum between days eight and nine, and then starts to decrease (Vaskivuo et al. 2002). Aromatase expression in the corpus luteum has a similar pattern (Suzuki et al. 1994). In the rodent corpus luteum, HSD17B1 is sharply downregulated (Ghersevich et al. 1994d).

The capacity of follicles to produce E2 is first apparent in the late preantral stage. Although aromatase and HSD17B1 activities are present in small antral follicles, estrogen production at this stage of development is limited by an inability to produce androgen substrates for aromatization of E2 (Shoham and Schachter. 1996, Drummond and Findlay. 1999). Various factors enable the "antral-to-preovulatory" transition of follicles instead of turning the follicles into apoptosis and atresia. These include FSH, LH, GH, IGF1, EGF, TGFα, FGF2 and IL1B (McGee and Hsueh. 2000). Interestingly, most of these factors also upregulate HSD17B1 expression (Singh and Reed. 1991, Ghersevich et al. 1994a, Ghersevich et al. 1994b, Lewintre et al. 1994b, Lewintre et al. 1994a, Duncan et al. 1994), suggesting that HSD17B1-mediated E2 production has a central role in the survival of follicles.

2.4.2.3 Role of estrogen in the ovaries and disturbances of estrogen action

Esr1 is expressed in the ovary of adult mice only in the theca and not in granulosa cells, whereas Esr2 is localized in the granulosa cells (Schomberg et al. 1999). In the human ovary, ESR1 is expressed in theca interna cells, interstitial gland cells and ovarian surface epithelium. ESR2 is expressed in granulosa cells, interstitial gland

cells and surface epithelium (Pelletier and El-Alfy. 2000). αERKO mice have normal folliculogenesis from the primordial to the antral stage, but thereafter, the mice fail to ovulate, resulting in atresia or hemorrhagic cysts (Schomberg et al. 1999), and a similar phenotype was observed in aromatase KO (ArKO) mice (Britt et al. 2000). However, the arrest of folliculogenesis in αERKO mice could be reversed by gonadotrophins, indicating that Esr1 is not directly necessary for folliculogenesis, but instead acts via the HPGA (Couse et al. 1999a, Rosenfeld et al. 2000). Lack of Esr1 resulted in decreased fertility and inefficient ovulation in mice (Krege et al. 1998). The ovarian phenotype of Esr1 and Esr2 KO (αβERKO) mice was different from that in the single receptor KO mice: the ovaries exhibited follicle transdifferentiation into structures resembling testicular seminiferous tubules, indicating that both Esrs are required for the maintenance of germ and somatic cell lines in the ovary (Couse et al. 1999b). Thus, estrogen is clearly an important and obligatory regulator of folliculogenesis, especially in the postantral stage (Drummond and Findlay. 1999). However, also exposure to exogenous estrogens during early life has been shown to disturb oocyte development. Shortly, neonatal and prepubertal estrogen exposure has been shown to suppress folliculogenesis in mice (Rosa-E-Silva et al. 2003, Susiarjo et al. 2007, Kim et al. 2009). Thus, both lack of estrogens and exposure to exogenous estrogens disturb ovarian function and lead to arrest of folliculogenesis. Esr1 is expressed in all components of the HPGA in mice, whereas Esr2 expression is restricted to granulosa cells (Couse et al. 1999a). Gonadotropins are fundamental for regulating follicle status and development from the antral stage onwards. As shown in Figure 4, estrogens further regulate gonadotropin secretion by both negative and positive feedback. Neonatal or prepubertal treatment of rodents with estrogens reprograms the HPGA, resulting in insufficient phasic secretion of gonadotropins, and thus disturbed menstrual cycle (Rosa-E-Silva et al. 2003, Kato et al. 2003, Nakamura et al. 2008).

Epithelial ovarian cancer is highly lethal among the common gynecologic malignancies (Jemal et al. 2008). The vast majority of ovarian cancers (80-90%) are derived from the ovarian surface epithelium, and epidemiological evidence strongly suggests that steroid hormones, especially estrogens and progestins, are implicated in ovarian carcinogenesis (Ho. 2003). Overweight has been shown to increase the mortality of ovarian cancer, possibly as a consequence of increased conversion of androgens to estrogens in the adipose tissue (Rodriguez et al. 2002). Furthermore, it has been shown that E1 and E2 are equally potent in stimulating the growth of ovarian surface epithelial cells (Syed et al. 2001). This may be important, because E1 is the major circulating postmenopausal estrogen. However, HSD17B1 is not expressed in the surface epithelium of the normal ovary (Sasano et al. 1996), indicating that some other HSD17B may be responsible for this conversion. There is no information available about HSD17B1 expression in the postmenopausal ovary. However, HSD17B1 expression has been shown to positively correlate with the malignancy of human ovarian surface epithelial tumors (Sasano et al. 1996). HSD17B1 expression was also detected in ovarian epithelial cancers in two other studies (Blomquist et al. 2002, Chura et al. 2009). In addition to estrogens, also gonadotrophins and androgens have been suggested to have a stimulatory role in the growth of benign and malignant ovarian surface epithelial cells (Syed et al. 2001).

2.4.3 ENDOMETRIUM

2.4.3.1 Menstrual cycle

The endometrium is a plastic tissue, which responds to ovarian hormones. In each menstrual cycle, if no embryo implantation occurs, the functional layer of the endometrium is shed and within two weeks the complete functional layer is restored. The events underlying this phenomenon are highly complex and include the repair of the endometrium surface, proliferation, angiogenesis, vasculogenesis, cell differentiation and extracellular matrix remodeling. Once the functional layer has successfully been rebuilt, the actions of P change the estrogen-primed endometrium into a receptive state (Groothuis et al. 2007). The menstrual cycle is divided into proliferative and secretory phases. The cycle begins with the proliferative phase, which usually lasts for 14 days, but may also fluctuate between ten and 20 days under physiological conditions. The proliferative phase is further subdivided into early, middle and late proliferative phases. The secretory phase strictly lasts for 14 days under normal ovarian function (Dallenbach-Hellweg and Poulsen. 1985). The proliferative phase is initiated with menstruation approximately during cycle days one to five. At the beginning of the menstrual cycle, the ovaries produce low amounts of E2 and P (Nienstedt et al. 1987). After menstruation, the endometrium regenerates, the epithelium becomes proliferative, and the endometrial glands increase in length and become tortuous. Increasing E2 concentration towards the end of the proliferative phase triggers ovulation via positive feedback to the hypothalamus and pituitary gland, inducing the LH surge (Garcia et al. 1981). The ovulation further shifts the endometrium to the secretory phase, which is regulated by E2 and P secreted by the corpus luteum. During the secretory phase, endometrial glands become increasingly tortuous and secretory, and enlarge their surfaces at the endometrial lumen (Dallenbach-Hellweg and Poulsen. 1985). Epithelial proliferation ceases, and finally, if pregnancy is not on, a new cycle begins with menstruation. The human menstrual cycle lasts for approximately three and a half decades after which the ovaries cease at menopause, and extragonadal steroid biosynthesis becomes dominant (Nienstedt et al. 1987). Similar to humans, the endometrium of rodents undergoes cyclical changes of growth and degeneration and the menstrual cycle is called the estrous cycle. The mouse estrous cycle only lasts for four to seven days. It is subdivided into the proestrus, estrus, diestrus and metestrus, and is not completed with menstrual bleeding (Staley and Scharfman. 2005). Proestrus corresponds to the human proliferative phase, estrus to the human periovulatory phase and diestrus to the human secretory phase. The mouse secretory phase is terminated with the regression of the ovarian corpus luteum and resorption of the endometrium, which corresponds to human menstruation (Groothuis et al. 2007).

2.4.3.2 Role of HSD17B1 in endometrial estrogen biosynthesis

Although the endometrium is greatly dependent on ovarian steroid production, several estrogen-metabolizing enzymes are expressed in the endometrium, indicating a role for local estrogen metabolism in the endometrium. However, the reports about human HSD17B1 expression in the uterus are inconsistent. Northern blot analyses have revealed that the major transcript of HSD17B1 in the human endometrium is the longer, poorly translated 2.3kb transcript (Martel et al. 1992, Miettinen et al. 1996). However, also the 1.3kb transcript, which has been shown to correlate with HSD17B1 activity

in cell culture studies, has been observed (Miettinen et al. 1996, Zeitoun et al. 1998). Furthermore, an additional 1kb transcript has been detected, together with the 2.3kb transcript in the myometrium (Kasai et al. 2004). With RT-PCR, HSD17B1 mRNA has been detected in the human endometrium (Zeitoun et al. 1998, Kasai et al. 2004, Smuc et al. 2006, Fechner et al. 2007, Salama et al. 2009, Smuc and Rizner. 2009). With immunohistochemistry, HSD17B1 protein has been detected in the luminal epithelium (Maentausta et al. 1991, Dassen et al. 2007, Fechner et al. 2007) and stroma (Dassen et al. 2007, Fechner et al. 2007) of the human endometrium. HSD17B1 activity has been detected in the endometrium even during the proliferative phase, suggested to have very low HSD17B1 expression (Delvoux et al. 2007), and significant reductive activity was also detected by another recent study (Delvoux et al. 2009). Inconsistently, according to several studies, HSD17B1 mRNA (Casey et al. 1994, Utsunomiya et al. 2001, Dassen et al. 2007), protein (Marovitz et al. 1980, Utsunomiya et al. 2001) and activity (Utsunomiya et al. 2001) have not been detected.

The human endometrium also reportedly expresses several other estrogenic HSD17Bs, including types 2, 4, 5, 7, 8 and 12. Therefore, the availability of E2 at tissue or cellular level is likely to be tightly regulated by the relative activities of various enzymes with opposing functions (activation or inactivation of estrogens) (Miettinen et al. 1996, Torn et al. 2003, Ito et al. 2006, Smuc et al. 2006, Luu-The et al. 2006, Smuc and Rizner. 2009). HSD17B2 in particular opposes the activity of HSD17B1, while the role of the other HSD17Bs in regulating local estrogen metabolism remains poorly understood. In addition to estrogenic HSD17Bs, androgenic HSD17Bs can promote E2 production via the aromatase pathway (Smuc and Rizner. 2009). The only androgen-producing HSD17B expressed in the endometrium is HSD17B5, also known as AKR1C3 (Rizner et al. 2006, Smuc and Rizner. 2009). In addition to HSD17Bs, several other estrogen-metabolizing enzymes are expressed in the endometrium. These include aromatase, which converts androgens to estrogens (Smuc et al. 2006, Smuc and Rizner. 2009), steroid sulfatase (STS) converting E1-sulfate to E1 and the sulfotransferase family

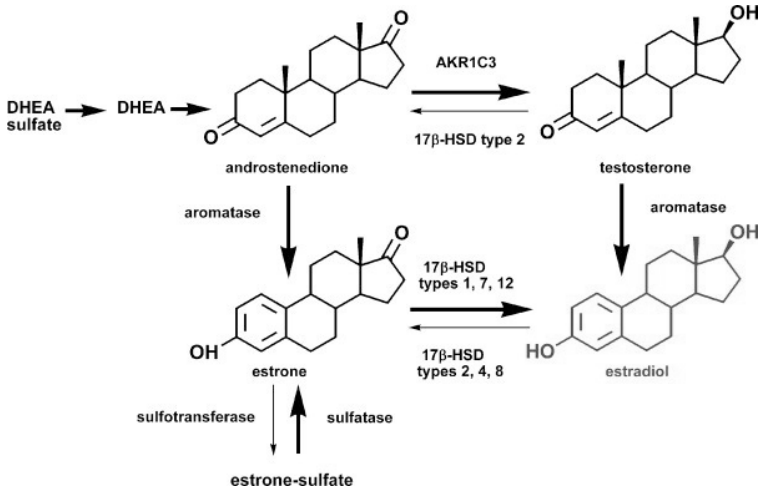


FIGURE 8. Local production of E2 in the endometrium. E2 formation in the human endometrium, showing the aromatase pathway from DHEA-sulfate and DHEA via A-dione and T, and the sulfatase pathway from E1-sulfate. Figure adapted from Smuc and Rizner. 2009.

1E, estrogen-preferring, member 1 (SULT1E1, EST) converting E1 and E2 to their sulfated conjugates (Tanaka et al. 2003, Utsunomiya et al. 2004, Leon et al. 2008, Smuc and Rizner. 2009). However, it has been suggested that the aromatase pathway is not important in the normal endometrium (Watanabe et al. 1995, Leon et al. 2008). Figure 8 summarizes the possible routes of E2 production in the human endometrium.

2.4.3.3 Role of estrogen in the endometrium and its disorders

The endometrium is an estrogen-responsive tissue and ESRs are expressed in the normal and cancerous endometrium. ESR1 is expressed in the endometrial epithelium during proliferative and early secretory phases but is not expressed or the expression is decreased during middle and late secretory phases. In the stroma, ESR1 is also expressed during the midsecretory phase but has disappeared during the late secretory phase (Maentausta et al. 1991, Mertens et al. 2001). The expression of ESR2 is similar for ESR1, but has lower intensity (Mylonas et al. 2007). Estrogens stimulate the proliferation of endometrial cells. The effects of estrogens have been studied in endometrial cell lines during different phases of the cell cycle. Estrogens increase cell proliferation by increasing the expression of cyclins D1 and A, and by decreasing the expression of tumor suppressor genes, including p53, p21 and p27 (Groothuis et al. 2007, Watanabe et al. 2007). Estrogens also stimulate the production of various growth factors, including IGF1 and EGF, and increase the expression of several proto-oncogenes, such as v-myc myelocytomatosis viral oncogene homolog (avian) (MYC), v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), JUN and Harvey rat sarcoma viral oncogene homolog (HRAS) (Ignar-Trowbridge et al. 1993, Fujimoto et al. 1994, Murphy. 1994, Morishita et al. 1995, Salmi and Rutanen. 1996, Naciff et al. 2009). Progestins antagonize estrogen-mediated cell proliferation in the endometrium (Wheeler et al. 2007, Graham and Clarke. 1997) and are, therefore, widely included in HRT to decrease the risk of endometrial hyperplasia and cancer (Beresford et al. 1997, Feeley and Wells. 2001). The endocrinology of the mouse endometrium closely resembles that of humans. As in humans, endometrial hyperplasia and carcinoma can be induced by continuous estrogen exposure (Gunin et al. 2005). In line with this, αERKO mice are resistant and the uterus remains hypoplastic (Couse and Korach. 2001), whereas P receptor KO (PRKO) mice respond to combined estrogen and progestin stimulation by developing abnormally enlarged uteri and endometrial hyperplasia, indicative of unopposed estrogen stimulation (Lydon et al. 1995).

Proliferative uterine disorders, such as endometriosis, endometrial hyperplasia and cancer, are very prevalent during the reproductive years. For example, endometriosis affects up to 10% of women and is a major cause of pelvic pain and infertility (Eskenazi and Warner. 1997, Dassen et al. 2007). Endometrial carcinoma is the most common invasive cancer of the female genital tract and ranks as the fourth most common malignancy in women in Western Europe and in the United States (Smuc et al. 2006, Horn et al. 2007, Kurman and TeLinde. 2002). It is acknowledged that estrogens play a role in proliferative endometrial disorders, but the exact mechanism of estrogen production remains unclear. The correlation between serum E2 levels and the risk of endometrial cancer is controversial (Vermeulen-Meiners et al. 1986, Berstein et al. 2003, Potischman et al. 1996), and increased intratissular E2 concentrations are considered a more definite risk factor (Vermeulen-Meiners et al. 1986, Berstein et al. 2003). Accordingly, reporting

of the expression of estrogen-metabolizing enzymes in endometrial cancer and other endometrial diseases is contentious.

2.4.3.3.1 Endometrial carcinoma

Endometrial carcinomas are subdivided into types I and II. Type II comprises from the high-grade papillary serous and clear cell carcinomas and is usually not preceded by a history of unopposed estrogen exposure (Doll et al. 2008). Estrogen-dependent endometrial carcinoma (endometrioid endometrial carcinoma type I, hereafter referred to as endometrial carcinoma) generally arises from atypical endometrial hyperplasia, whereas hyperplasia without atypia is less likely to become malignant (Horn et al. 2007, Lax. 2007). Patients with typical hyperplasia are usually managed conservatively with progestins, but hysterectomy remains the treatment of choice for atypical hyperplasia and endometrial carcinoma (Kurman and TeLinde. 2002, Lax. 2007). Obesity and anovulation, often associated with PCOS, are major risk factors for endometrial hyperplasia during the reproductive years as is unopposed estrogen replacement therapy in postmenopausal women (Kelsey et al. 1982, Gallup and Stock. 1984, Sherman. 2000, Schneider. 2002). The expression of ESRs in endometrial cancer is controversially reported. Changes in the ESR1/ESR2 ratio have been reported in endometrial cancer (Utsunomiya et al. 2000, Saegusa and Okayasu. 2000), whereas other studies suggest that the expressions of both receptors are decreased in endometrial cancers (Smuc and Rizner. 2009).

HSD17B1 has been detected in endometrial carcinomas by RT-PCR (Smuc et al. 2006), immunohistochemistry (Maentausta et al. 1992), time-resolved immunofluorometric assay (Maentausta et al. 1991) and radioimmunoassay (Maentausta et al. 1990). HSD17B1 activity specified by selective inhibitor treatment has been observed in endometrial cancer cell lines (Fournier and Poirier. 2009). However, other research groups consistently detect neither HSD17B1 expression nor activity in normal and diseased human endometrium (Utsunomiya et al. 2001, Ito et al. 2001, Utsunomiya et al. 2003). According to previous studies, HSD17B1 expression is decreased in endometrial hyperplasia, cancer and cancer cell lines (Maentausta et al. 1990, Maentausta et al. 1991, Smuc et al. 2006, Smuc and Rizner. 2009), but interestingly, one study showed increased HSD17B1 expression in serous endometrial carcinomas (Smuc and Rizner. 2009). In addition to HSD17B1, other estrogenic HSD17Bs are expressed in cancerous endometrium. These include estrogen-activating reductases HSD17B7, HSD17B12 and HSD17B5, and estrogen-inactivating oxidases HSD17B2, HSD17B4 and HSD17B8 (Smuc and Rizner. 2009). Similar to HSD17B1, HSD17B7 was downregulated in endometrial carcinomas, whereas HSD17B12 was expressed at the same level as in normal endometrium (Smuc et al. 2006, Smuc and Rizner. 2009). The downregulation of estrogen reductases may serve as a compensatory mechanism to decrease estrogen exposure of the endometrium. The expression of oxidative HSD17B2 has been shown to be both increased and decreased in cancerous endometrium. In one study, HSD17B2 immunoreactivity and enzymatic activity were found to be decreased in endometrial hyperplasia and carcinoma as compared with normal endometrium (Utsunomiya et al. 2001), whereas in another study, HSD17B2 mRNA was reported to be upregulated (Smuc et al. 2006). Decreased HSD17B2 activity could enhance the accumulation of estrogens in the endometrium. Two other endometrial oxidases, HSD17B4 and HSD17B8, are expressed in cancerous

endometrium, but their expression level was not altered between normal and cancerous endometrium (Smuc and Rizner. 2009). HSD17B5, which mainly activates androgens, has consistently been shown to be upregulated in the cancerous endometrium, and could provide androgenic substrates for aromatase (Ito et al. 2006, Smuc and Rizner. 2009). High aromatase activity has been shown in benign and malignant endometrial lesions (Jongen et al. 2005). Similarly, high aromatase expression was detected in cancerous endometrium as compared with normal endometrium in another study (Watanabe et al. 1995). However, in two other studies, no significant changes in aromatase expression were observed between normal and cancerous endometrium (Smuc et al. 2006, Smuc and Rizner. 2009), but aromatase was, however, upregulated in 50% of samples (Smuc and Rizner. 2009). The upregulation of aromatase would enhance local estrogen production from androgenic precursors. However, also the sulfatase pathway can affect local estrogen concentration. According to one study, the expression of estrogen-activating STS was increased and estrogen-inactivating EST decreased in endometrial carcinomas (Utsunomiya et al. 2004). In another study, STS expression was unaltered, whereas EST expression was decreased (Smuc and Rizner. 2009). In a third study, both STS and EST expressions were decreased (Smuc et al. 2006).

In conclusion, decreased HSD17B2 activity, increased HSD17B5 activity associated with increased aromatase activity, and decreased EST activity could all result in an accumulation of E2, whereas the downregulation of estrogen reductases HSD17B1 and HSD17B7 could be explained as a compensatory mechanism to decrease local E2 accumulation. However, the data are highly controversial and uniform conclusions on the role of each pathway cannot be drawn. This kind of variation in the results between different groups could result for example from samples sets composed of different disease subtypes, pre-/postmenopausal samples and different menstrual cycle phases, non-standardized sample preparation procedures, or simply from the high heterogeneity of endometrial carcinomas.

2.4.3.3.2 Endometrium of PCOS patients

In two recent studies, altered estrogen metabolism has been detected in the normal and hyperplastic endometrium of PCOS patients. PCOS patients have an increased risk of developing endometrial hyperplasia (Gadducci et al. 2005). This could be explained by the obesity often associated with PCOS and the consequent increase in aromatase activity of adipose origin, or alternatively, unopposed estrogen stimulation resulting from anovulation which is also a characteristic symptom of PCOS. However, it has been shown (Leon et al. 2008) that there is increased STS and decreased EST activity, and interestingly, increased HSD17B1 expression in the endometrium of PCOS patients as compared with the secretory endometrium of healthy control women. This indicates enhanced local estrogen accumulation in the PCOS endometrium that could also explain the higher risk of PCOS patients to develop endometrial hyperplasia. However, another study by the same group (Bacallao et al. 2008) compared the prolifative endometria of healthy women to PCOS endometria and showed decreased STS, increased EST and decreased HSD17B1 expression within the PCOS endometria that would limit E2 accumulation. The conflict between the two studies was explained by the different phase of the menstrual cycle of the control samples. They also showed an increased HSD17B1/

HSD17B2 ratio and decreased HSD17B2 activity in the PCOS endometrium as compared with the control endometrium that would in turn favor E2 accumulation.

2.4.3.3.3 Endometriosis

Endometriosis is an estrogen-dependent disease characterized by the presence of endometrium-like tissue outside the uterus (Dassen et al. 2007, Fechner et al. 2007). Similar to endometrial carcinoma, local estrogen biosynthesis may enhance local estrogen concentration, and thus, stimulate the proliferation of endometriotic lesions: this hypothesis has been widely studied. In deep endometriotic lesions, HSD17B1 and aromatase both favoring estrogen production, have been shown to be upregulated and the oxidases HSD17B2 and HSD17B4 downregulated (Dassen et al. 2007). In another study of extraovarian endometriotic lesions, HSD17B1 expression was unaltered, but HSD17B2 expression was downregulated (Zeitoun et al. 1998). In ovarian endometriosis, HSD17B7, HSD17B12 and STS expressions, in addition to HSD17B1 and aromatase, were shown to be upregulated, whereas HSD17B2 and HSD17B8 expressions were downregulated (Smuc et al. 2007, Smuc et al. 2008). Collectively, the data indicate that the expression pattern of estrogen-metabolizing enzymes in endometriosis favors local estrogen accumulation.

2.4.4 BREAST

The mammary gland is the organ that, in mammals, produces milk for the sustenance of the young. In humans, one mammary gland is located in each breast. These exocrine glands are enlarged and modified sweat glands and give mammals their name. The mammary glands of most mammalian species, including humans, are not fully developed and functional at birth. Instead there is a small, primitive ductal structure extending a small distance from the nipple. At puberty, under the influence of ovarian and pituitary hormones, the gland undergoes the first phase of allometric growth (Anderson and Clarke. 2004). The duct system grows and branches under the influence of estrogens. Surrounding stromal and fat tissues also proliferate (Johnson et al. 1998). In mice, this is initiated at about 21 days of age and is characterized by enlargement of the duct termini to form terminal end buds (TEBs). TEBs are the major sites of proliferation through which ductal elongation and ramification into the mammary fat pad are achieved. In the human mammary gland, the site of active epithelial cell proliferation is also a TEB-like structure. Alveolar budding at the ends of terminal ductules eventually gives rise to the terminal ductal lobular units (TLDUs), which are the functional units of the mammary gland, situated at the end of the terminal ducts. The entire ductal system is lined by a continuous layer of luminal epithelial cells which, in turn, are surrounded by delimiting fibroblasts and embedded in a specialized intralobular stroma (Anderson and Clarke. 2004). P in combination with E2 promotes the growth and branching of the lobuloalveolar tissue, but for these steroids to be effective, prolactin, growth hormone and cortisol must also be present (Johnson et al. 1998). In humans, once menstruation is established, there is a cyclical increase in the proliferation associated with the luteal phase. The second phase of allometric growth of the mammary gland occurs during pregnancy (Anderson and Clarke. 2004).

2.4.4.1 Role of estrogen in the breast and its disorders

The clinical and epidemiological evidence of an obligate role for E2 in mammary gland development and tumor formation is considerable. The clinical management of girls with E2 deficiency from, for example, gonadal dysgenesis or gonadotrophin insufficiency demonstrates that E2 is strictly necessary, although not sufficient, to induce pubertal breast development (Anderson and Clarke. 2004). Both ESR1 and ESR2 are expressed in the human mammary gland. Normal, non-lactating human breast tissue contains a small but distinct population of ESR1-positive luminal epithelial cells comprising approximately 7% of the whole epithelial cell content (Petersen et al. 1987, Clarke et al. 1997, Shaw et al. 2002, Taylor et al. 2009). ESR2 expression is more abundant, locating to epithelial cells, myoepithelial cells, lymphocytes, fibroblasts and other stromal cells (Shaw et al. 2002, Palmieri et al. 2004). Similar to humans, Esr1 in mice is mainly expressed in the luminal epithelial cells, but is also present in the dense fibrous stroma around the nipple area and the primary mammary ducts, and is also expressed heterogeneously in the adipocytes of the mammary fat pad (Shyamala et al. 2002). Also Esr2 is expressed in the lobules and ducts of the mouse mammary gland (Wang et al. 2008). Studies in KO mice have indicated that Esr1 is essential for the postnatal development of the mammary gland. αERKO and αβERKO mice only develop a rudimentary ductal structure limited to the nipple region and lacking TEBs and alveolar development, whereas βERKO mice have normal mammary glands (Bocchinfuso and Korach. 1997, Hewitt and Korach. 2003). Molecular mediators of estrogen action in the mammary gland are not well characterized, but may include cyclin D1, IGF, EGF, TGFα and TGFβ (Bocchinfuso and Korach. 1997, Anderson and Clarke. 2004, Lamote et al. 2004). As reviewed by Anderson and Clarke (2004), the role of estrogen in breast cancer has been indicated by various studies. Decreasing the exposure of the mammary gland to cyclically altering concentrations of E2 and P, for example, by early menopause, substantially lowers the risk of breast cancer, whereas early menarche, late menopause and late first pregnancy increase the risk of breast cancer. The role of estrogens is also supported by the fact that in men, the occurrence of breast cancer is 1% of that seen in women, and that the users of oral contraceptives and HRT have elevated breast cancer risks, whereas the use of antiestrogen tamoxifen in the high-risk population decreases breast cancer risk. The ESR pathway is the key to survival and progression in a significant proportion of breast cancers, and several studies have shown ESR expression also in breast cancer, with some studies reporting changes in ESR1/ESR2 ratio and others not (Clarke et al. 1997, Shaw et al. 2002, Lofgren et al. 2006, Badve and Nakshatri. 2009). It has been shown that in the normal mammary gland, the ESR1-positive cells do not proliferate, whereas in cancerous tissue the proliferating cells contain ESR1 (Clarke et al. 1997).

2.4.4.2 Role of HSD17B1 in local estrogen biosynthesis in the breast

The breast has good vascular support and ovarian and pituitary hormones are supplied via the circulation. During reproductive years, the ovaries are the main source of estrogens. Additionally, the breast is capable of local estrogen biosynthesis from the circulating precursors, and the role of local estrogen biosynthesis is especially emphasized after menopause (Nagasaki et al. 2009). Intratumoral metabolism and synthesis of estrogens as a result of the interactions of various enzymes are considered to play very

important roles in the pathogenesis and development of hormone-dependent breast carcinoma (Nagasaki et al. 2009, Jansson. 2009). Breast tumors tend to have higher E2 concentration than normal breast tissue and circulating plasma (Labrie et al. 2000, Jansson. 2009). Similar to the endometrium, several enzymes participating in local steroid biosynthesis are expressed in the breast and breast cancer, including HSD17B1, HSD17B2, HSD17B5, HSD17B7, HSD17B12 and HSD17B14 (Nagasaki et al. 2009, Jansson. 2009). Various factors, including IGF1, IGF2, IL6, IL1 and TNFα that stimulate breast cancer proliferation also upregulate HSD17B1 expression in breast cancer cells (Nagasaki et al. 2009). High HSD17B1 expression has been associated with poor prognosis of breast cancer (Gunnarsson et al. 2001, Gunnarsson et al. 2003, Oduwole et al. 2004, Gunnarsson et al. 2005, Gunnarsson et al. 2008). HSD17B2 expression is decreased in breast cancer according to several studies (Nagasaki et al. 2009), and high a HSD17B1/HSD17B2 ratio is associated with decreased survival in ESR1-positive breast cancer patients (Gunnarsson et al. 2001, Gunnarsson et al. 2005). Like with HSD17B1, increased HSD17B5 expression has been associated with poor prognosis and significantly higher relapse risk (Oduwole et al. 2004, Jansson et al. 2006). The recently found HSD17B14, which converts E2 to E1, has been associated with higher recurrence-free survival of breast cancer patients (Jansson et al. 2006). Aromatase and STS expressions are significantly higher in cancerous than in normal breast (Suzuki et al. 2008, Sasano et al. 2009). Together, these studies suggest that local estrogen biosynthesis plays a central role in the development and prognosis of breast carcinoma.

2.4.5 BONE

2.4.5.1 Bone function

Bone is a dynamic tissue that is remodeled throughout life. The purpose of bone remodeling is to repair microdamage, adapt the skeleton to mechanical loading and maintain calcium and phosphorus homeostasis. Bone remodeling is the result of a coupled action of osteoclasts (bone-resorbing cells) and osteoblasts (bone-forming cells). The bone remodeling cycle starts with the degradation of old bone by osteoclasts, which takes from two to four weeks. Osteoblasts then synthesize extracellular bone matrix and coordinate its mineralization. The bone-forming process takes from four to six weeks. After the formation and mineralization of new bone, the remodeling unit enters a resting phase (Pietschmann et al. 2008).

2.4.5.2 Role of estrogen in normal and diseased bone

Bone remodeling is affected by several factors. For example, multiple hormones modulate the rate of bone remodeling. Sex steroids play an important role in the regulation of skeletal growth and maintenance of the adult skeleton. Among these, estrogens are of particular interest. Both human and rodent osteoblasts (Eriksen et al. 1988, Masuyama et al. 1992), osteocytes (Hoyland et al. 1997, Gu et al. 2005) and growth plates (Nilsson et al. 1999, Van Der Eerden et al. 2002) express ESR1 and ESR2, indicating estrogen-responsiveness of bone tissue, although their distributions within bone differ and concentrations are lower than in reproductive tissues (Bland. 2000, Riggs et al. 2002). ESR expression, especially in rodent osteoclasts, is controversial (Bland. 2000), but both

human and rodent osteoclasts have been shown to be estrogen-responsive (Hughes et al. 1996, Nakamura et al. 2007, Michael et al. 2007). Common mediators of estrogen action in bone include IGF1 (Ernst et al. 1989), TGFβ1 (Hughes et al. 1996) and FasL (Nakamura et al. 2007). Estrogens conserve bone mass and the balance between bone formation and resorption by suppressing bone turnover (Manolagas. 2000), by decreasing the activity of osteoclasts and by inducing osteoclast apoptosis (Hughes et al. 1996). Moreover, estrogens are suggested to increase osteoblast production, differentiation, proliferation and function (Chow et al. 1992, Majeska et al. 1994).

Osteoporosis is one of the most common conditions associated with aging. It is defined as a skeletal disorder characterized by compromised bone strength, predisposing a person to an increased risk of fractures, which lead to pain, occasional disability and even increased mortality (Pietschmann et al. 2008). Estrogens have an essential role in the maintenance of bone, and estrogen deprivation has been associated with bone loss, development of osteoporosis, and tall stature (Simpson. 2000, Pietschmann et al. 2008, Folkestad et al. 2009). Tall stature, inability to ossify growth plates and severe osteoporosis were reported in a man with homozygous inactivation of ESR1 (Smith et al. 1994) and similar phenotype has been reported in men deficient in aromatase (Simpson. 2000). Osteoporosis is also associated with postmenopausal estrogen deprivation (Pietschmann et al. 2008). Male αERKO and αβERKO mice have decreased bone mineral content (BMC), while the bones of βERKO males and bone mineral density (BMD) in all three genotypes are normal (Vidal et al. 2000). Female αERKO and βERKO mice have increased BMD and BMC, whereas both are significantly decreased in αβERKO mice, indicating that Esr1 and Esr2 may have compensatory roles for each other in rodent bone (Windahl et al. 1999, Lindberg et al. 2001). ArKO female and male mice suffer from bone loss, whereas TG male mice overexpressing human aromatase (Arom+ mice) have increased BMD (Oz et al. 2000, Peng et al. 2004). However, the effects of estrogens on bone length are somewhat different in rodents than in humans. In humans, low plasma levels of estrogens at the beginning of puberty initiate the growth spurt, whereas high estrogen levels induce growth plate fusion at the end of puberty (Cutler. 1997). In contrast, rodents retain open epiphyses after sexual maturation (Kennedy et al. 1999, Weise et al. 2001), increasing the complexity of the use of rodent models. In contrast to humans, deletion of Esr1 and aromatase results in decreased bone length in male and female mice (Vidal et al. 1999, Vidal et al. 2000, Oz et al. 2001). Controversially, also overexpression of human aromatase in Arom+ mice resulted in decreased bone length (Peng et al. 2004). Deletion of Esr2 resulted in longer bones in female mice and unaltered bone length in males (Vidal et al. 2000, Lindberg et al. 2001). Thus, both lack of and increase in estrogen can decrease bone length in mice.

2.4.5.3 Role of local estrogen biosynthesis in bone

The role of aromatase in local estrogen biosynthesis in bone has been widely acknowledged. Deficiency of aromatase results in bone loss in both rodents and humans (Simpson. 2000, Oz et al. 2000, Oz et al. 2001) and the use of aromatase inhibitors in the treatment of breast cancer also induces osteoporosis (Folkestad et al. 2009). Together with aromatase, at least two HSD17Bs, HSD17B1 and HSD17B2, are simultaneously expressed in bone. In rat tibia metaphysis, Hsd17b1, Hsd17b2 and aromatase mRNA expressions have been detected during and after sexual maturation. Hsd17b1 was detected only in osteoblasts,

whereas Hsd17b2 and aromatase were detected also in the osteoclasts (van der Eerden et al. 2004). Additionally, the expression of Hsd17b1, Hsd17b2 and aromatase has been detected in rat tibia growth plate. The expression of Hsd17b1 and Hsd17b2 was weak, but Hsd17b2 expression increased during sexual maturation. Hsd17b1 and aromatase activities were significantly increased during sexual maturation in rat tibia growth plates, but Hsd17b2 activity was not determined in the study (Van Der Eerden et al. 2002). In human tibia, co-expression of HSD17B1 and aromatase has been detected by immunohistochemistry in lining cells, osteoblasts, chondrocytes of articular cartilage and adipocytes adjacent to trabecularie (Sasano et al. 1997). There are as yet no studies on the physiological role of HSD17B1 in bone, but it was recently shown that overexpression of human HSD17B2 results in severe bone loss (Shen et al. 2008). Thus, the data are in line with the hypothesis that extragonadal sex steroid biosynthesis plays a role in both rodent and human bone.

2.5 HSD17B1 AS A DRUG TARGET

2.5.1 INHIBITORS OF LOCAL ESTROGEN BIOSYNTHESIS

Hormone-dependent diseases rank high in mortality in the modern world, and thus, there is an urgent need for new drugs to treat these diseases. The use of antiestrogens in the treatment of estrogen-dependent diseases is limited by their side effects, and inhibition of local estrogen biosynthesis is considered to provide a softer treatment approach. Altered expression of human HSD17B1 has been associated with several estrogen-dependent diseases, including breast cancer, endometriosis, endometrial hyperplasia and cancer, and ovarian epithelial cancer. Because of its putative role in E2 biosynthesis in ovaries and peripheral target tissues, HSD17B1 is considered a promising drug target for estrogen-dependent diseases. In addition to HSD17B1 inhibitors, efforts have been made to develop inhibitors for other estrogenic HSD17Bs (mainly HSD17B7 and HSD17B12) and enzymes of the sulfatase pathway. Additionally, aromatase inhibitors are already on the market and are widely used in the treatment of breast cancer.

HSD17B1 inhibitors are classified as steroidal and non-steroidal. Most of the development has been focused on steroidal inhibitors including: E1- and E2-derived inhibitors with large substitutions at position 16; E1- and E2-derived inhibitors with small substitutions at position 16; 6-, 16- and 17-substituted E1 derivatives; 2-, 3- and 6-substituted derivatives of E1 and E2; C15 derivatives of E1; E-ring heterocyclic derivatives of E1; 17-fluoro-substituted estrogens; 2-substituted estra-1,2,5(10)-trien-17-ones; 2-substituted D-homo-estra-1,2,5(10)-trienes and tibolone and its metabolites (Brozic et al. 2008, Messinger et al. 2008). However, steroidal inhibitors may have residual hormonal activity, and thus, also non-steroidal inhibitors have been under investigation. These include phytoestrogens, gossypol and related compounds, and thiophenepyrimidinone derivatives (Brozic et al. 2008, Bey et al. 2008, Brozic et al. 2009, Al-Soud et al. 2009). Several preclinical studies indicate that HSD17B1 inhibitors are indeed capable of modulating estrogen responses *in vitro* and *in vivo*. It has been demonstrated (Husen et al. 2006b) that breast cancer cells transfected with human HSD17B1 formed E1-induced tumors in nude mice. Moreover, treatment with a HSD17B1 inhibitor decreased E1-stimulated growth of

the xenografts in the nude mice. Similarly, it has been shown (Day et al. 2008a) that HSD17B1 expression correlated with E2 production in breast cancer cells, and again, treatment with a HSD17B1 inhibitor decreased the growth of E1-stimulated breast cancer xenografts in nude mice. HSD17B1 inhibitors have also been shown to decrease E1-induced proliferation of breast cancer cells *in vitro* (Laplante et al. 2007).

In addition to HSD17B1, several other enzymes also participate in the regulation of local estrogen biosynthesis. Special interest has been shown in aromatase, STS, HSD17B7 and HSD17B12. Aromatase inhibitors are already in clinical use mostly in the treatment of breast cancer (Attar and Bulun. 2006, Perez. 2007), but are also being tested for endometriosis (Attar and Bulun. 2006) and bone growth disorders (Dunkel. 2009). However, resistance of breast cancer to the inhibitors has been universally gained (Urruticoechea. 2007) and the use of aromatase inhibitors induces bone loss (Perez. 2007, Pant and Shapiro. 2008, Folkestad et al. 2009, Hadji. 2009). STS inhibitors are also suggested to be effective against breast carcinoma and are expected to be in therapeutic use along with aromatase inhibitors (Nagasaki et al. 2009, Sasano et al. 2009). In addition to HSD17B1, also HSD17B7 and HSD17B12 are suggested to contribute to E2 biosynthesis, but the enzymes have major functions other than sex steroid -related pathways (Moeller and Adamski. 2009). Recent studies on breast, uterine and cervical cancer cells have indicated that even the combination of all the three enzyme inhibitors (HSD17B1, HSD17B7 and HSD17B12) could not completely block the conversion of E1 to E2, suggesting that yet another enzyme is contributing to this reaction. Nevertheless, the results suggest that combining the different inhibitors of local estrogen biosynthesis may be a useful approach to obtain a complete block of local E2 production *in vivo* (Fournier and Poirier. 2009, Laplante et al. 2009).

3 AIMS OF THE STUDY

HSD17B1 inhibitors are new drugs that will enter clinical trials in the near future. The inhibitors are designed to decrease local estrogen concentration in estrogen-dependent diseases, including breast cancer and endometriosis. Based on studies *in vitro*, the role human HSD17B1 plays in estrogen biosynthesis is widely acknowledged, whereas the weak activity towards androgens as substrate has been ignored. Nevertheless, HSD17B1 is considered a promising drug target, and so it is of importance to understand the full physiological role of the enzyme. The aim of the present study was to increase our understanding of the physiological role of human HSD17B1 and to map novel disease indications for HSD17B1 inhibitors. The approach chosen was to characterize the phenotype of transgenic FVB/N mice overexpressing human HSD17B1 (HSD17B1TG mice).

Specific aims of the present study were:

I) To characterize the substrate specificity and physiological response of human HSD17B1 using non-clinical models and human samples

   i. Generating TG mice expressing human HSD17B1.

   ii. Determining the substrate specificity of human HSD17B1 *in vivo*.

II) To find the potential clinical outcomes of overexpression of human HSD17B1 in TG female mice, and to discover novel disease indications for HSD17B1 inhibitors by

   i. Studying the role of human HSD17B1 in androgen-dependent diseases by characterizing the androgen-dependent disease phenotype of HSD17B1TG mice.

   ii. Studying the roles of human HSD17B1 in estrogen-dependent diseases by charactering the estrogen-dependent disease phenotype of HSD17B1TG mice.

III) On the basis of the literature and the data of the present study, to evaluate the clinical relevance of our findings in corresponding human diseases.

4 MATERIALS AND METHODS

4.1 TRANSGENIC MICE (I-V)

4.1.1 HSD17B1TG MICE (I-II, IV-V) AND MMTV-HSD17B1TG MICE (III)

A cDNA fragment of human HSD17B1, encoding for the full-length enzyme was cloned under the chicken β-actin promoter (kindly provided by Jun-ichi Miyazaki, Osaka University, Osaka, Japan) (HSD17B1TG construct) or under mouse mammary tumor virus (MMTV) promoter (MMTV-HSD17B1TG construct). Reductive HSD17B activity for the transgene constructs was verified in cultured cells as previously described (Miettinen et al. 1996), with minor modifications. In brief, HEK-293 cells were transfected with the transgene construct using the lipofectamine method (Invitrogen, Carlbad, CA). Transfected cells (4×10^5 cells/well) were applied in 12-well plates in DMEM/F12 with 10% fetal calf serum, including penicillin and streptomycin. After overnight culture, the medium was aspirated from the wells, and 2ml of serum-free medium containing 200nM [^{3}H]E1 (2×10^5cpm/ml; PerkinElmer, Waltham, MA) was applied to the cells. The cells were then incubated at 37°C for 1, 2, 4, 8 and 24h in the cell culture conditions. After the incubation, E1 and E2 were separated with a high performance liquid chromatography (HPLC) system (Waters™ 2695, Waters Corporation, Milford, MA), and the radioactivities were measured with an online β-counter.

For microinjection the HSD17B1 transgene constructs were cleaved from the plasmid backbone by *XmnI/HindIII* digestion, purified by a Quick-Pick Electroelution Capsule Kit (Qiagen, Valencia, CA) and Elutip DEAE columns (Schleicher & Schüell, Dassel, Germany), and diluted to the final concentration of 2ng/μml. TG founder mice were generated in the genetic background of the FVB/N strain, which was used in all the experiments of the present study, by microinjecting the DNA into pronuclei of fertilized oocytes using standard pronucleus injection techniques (Hogan et al. 1986). Integration of the transgene was verified by Southern blot analysis and PCR screening of DNA isolated from tail or ear biopsies by the salting-out method (Miller et al. 1988). The PCR reaction consisted of a denaturation cycle (5min at 97ºC, 1min at 56ºC, 30s at 72ºC), 31 cycles of amplification (1min at 95ºC, 1min at 56ºC, 30s at 72ºC), and a termination cycle (10min at 72ºC). The following primer pairs were used for genotyping and RT-PCR analyses: HSD1Fw2 5'-CTTCAGATCCATCCCAGAGC-3' - HSD1Ex32 5´-GCCCAGGCCTGCGTTACAC-3´ and HSD1Fw562 5'-ACACCTTCCACCGCTTCTAC-3' - HSD1Rev562 5'-GAACGTCGCCGAACACTT-3'. The founder mice were mated with wild type (WT) FVB/N mice to create several HSD17B1TG and MMTV-HSD17B1TG mouse lines. HSD17B1TG females were infertile, and therefore, HSD17B1TG males were bred with WT females to maintain mouse lines. Heterozygous offsprings were used for analyses. MMTV-HSD17B1TG mice were similarly maintained. The strongest expression lines (013 and 023) were used for HSD17B1TG and MMTV-HSD17B1TG mice, unless otherwise stated.

4.1.2 BI-TG MICE

The bi-TG (HSD17B1TGxHSD17B2TG) mice were generated by crossbreeding HSD17B1TG males (strongest expression line 013) with HSD17B2TG females (line

141). The generation and maintenance of HSD17B2TG mouse lines was performed similarly with HSD17B1TG and MMTV-HSD17B1TG mice and is described elsewhere (Zhongyi et al. 2007). Bi-transgene genotyping was performed by PCR with HSD17B1- and HSD17B2-specific primers simultaneously (Zhongyi et al. 2007).

4.1.3 ANIMAL EXPERIMENTATION (I-V)

Mice were housed under controlled environmental conditions (12h light/12h darkness, at 21±1˚C) at the Central Animal Laboratory of the University of Turku. Soy-free SDS RM3 (Special Diet Service; Witham Essex, UK) and tap water were available *ad libitum*. To obtain tissue samples, adult mice were terminally anesthetized with a 600-1000µl 2.5% tribromoethanol (Avertin; Sigma-Aldrich, St. Louis, MO or Alfa Aesar, Karlsruhe, Germany) (Weiss and Zimmermann. 1999) injection *i.p.*, and blood was withdrawn from the heart followed by euthanasia by cervical dislocation. Tissues were weighed and frozen at -80˚C or processed for histology as described in Section 4.12. Phenotypic analysis of sex steroid -responsive tissues was performed. The molecular profiles of the different proliferative lesions observed were not characterized in the present study. Animal experiments were approved by the Finnish Animal Ethics Committee: the institutional policies on animal experimentation fully meet the requirements as defined in the NIH Guide on animal experimentation.

4.2 HORMONE DETERMINATIONS (I-II)

Blood samples were incubated at +4˚C for 15-24h. Serum was separated by centrifugation and stored at -20˚C until hormone measurements. Organic extracts were prepared by diethyl ether extraction from serum or tissue samples for T and E2 determinations. E2 concentration was measured by IFMA, using the human E2 Delfia kit (PerkinElmer) adapted for mouse samples (Rulli et al. 2002). Serum and tissue T levels were measured by RIA (Huhtaniemi et al. 1985). Concentrations of LH and FSH were measured using time-resolved immunofluorometric assays as previously described (Haavisto et al. 1993, van Casteren et al. 2000). Intra-tissular hormone concentrations were normalized to protein concentration. Protein concentrations from tissue homogenates were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

4.3 RT-PCR (I, III, V)

4.3.1 QUANTITATIVE RT-PCR (I)

For quantitative transgene expression analysis, total RNA was isolated by the RNeasy Mini Kit (Qiagen) from hearts of mice from all HSD17B1TG mouse lines generated. RNA samples were then DNAse-treated (DNase I Amplification Grade Kit, Invitrogen, Life Technologies, Paisley, UK), and used for quantitative RT-PCR reactions. The reaction conditions are described in Table 4. The data were normalized by relating HSD17B1 expression to a mouse housekeeping gene ribosomal protein L19 (Rpl19) analyzed in a similar way to HSD17B1, except for the use of an annealing temperature of 54.5ºC. Primer sequences used to detect Rpl19 were as follows: Rpl19Fw 5'-CTGAAGGTCAAAGGGAATGTG-3'

and Rpl19Rev 5'-GGACAGAGTCTTGATGATCTC-3'. Primer sequences used to detect transgenic human HSD17B1 were the same as used for genotyping and are described in section 4.1.1. QuantiTect SYBR Green RT-PCR Kit (Qiagen) was used for quantitative RT-PCR of HSD17B1 and Rpl19 following the manufacturer's instructions. For quantitative mouse Hsd17b2 expression analysis, total RNA was isolated using the NucleoSpin RNA II (Macherey-Nagel, Düren, Germany) RNA isolation kit. Quantitative RT-PCR was performed utilizing the DyNAmo™ HS qRT-PCR kit for 2-step SYBR® Green qRT-PCR (Finnzymes Oy, Espoo, Finland). Primer sequences used to detect mouse endogenous Hsd17b2 expression were as follows: mHSD17B2Fw 5'-GAGCGTCTTTCAGTGCTCCAG-3' and mHSD17B2Rev 5'-CCTTGGACTTTCTAAGTAGAGGCA-3'. The data were normalized by relating HSD17B2 expression to Rpl19. The reaction conditions are presented in Table 4.

4.3.2 SEMI-QUANTITATIVE RT-PCR (I, III, V)

To confirm ubiquitous expression of the transgene in HSD17B1TG and MMTV-HSD17B1TG mice, semi-quantitative RT-PCR was carried out from various tissues. Total RNA for RT-PCR analysis was isolated from frozen tissues utilizing single-step phenol chloroform extraction (Chomczynski and Sacchi. 1987). For bones, total RNA was extracted from tibias with the guanidinium isothiocyanate method (Chirgwin et al. 1979). Briefly, frozen samples were pulverized into fine powder prior to homogenization in 4M guanidinium isothiocyanate solution. Total RNA was isolated by sedimentation through 5.7M cesium chloride density gradient by ultracentrifugation. RNA samples were then DNase-treated as described above, and semi-quantitative RT-PCR analysis was performed according to the manufacturer's instructions (Promega, Madison, WI). The reaction conditions are presented in Table 4.

Table 4. Reaction conditions for quantitative and semi-quantitative RT-PCR.

GENE ANALYZED	HSD17B1 (I)	HSD17B1 (I, III, V)	Hsd17b2 (I)
RT-PCR TYPE	Quantitative	Semi-quantitative	Quantitative
KIT	QuantiTect SYBR Green RT-PCR Kit	Promega	DyNAmo™ HS qRT-PCR kit for 2-step SYBR® Green qRT-PCR
REVERSE TRANSCRIPTION	Performed simultaneously with PCR	According to manufacturer's instructions (1µg RNA template)	According to Manufacturer's instructions (0.5µg RNA template)
PCR			
Reaction volume (µl)	25	25	20
Template amount	50ng RNA	1µl 1:10 diluted cDNA	1µl 1:10 diluted cDNA
Primers (10 µM)	HSD1FW562-HSD1Rev562	HSD1Fw562-HSD1Rev562	mHSD17B2Fw-mHSD17B2Rev
1. Reverse transcription	50˚C, 30min		
2. Initial Denaturation	95˚C, 15min	97˚C, 5min	95˚C, 15min
3. Denaturation	95˚C, 15s	95˚C, 1min	94˚C, 10s
4. Annealing	55.8˚C, 30s	55.8˚C, 1min, 30s	60˚C, 30s
5. Extension	72˚C, 30s	72˚C, 1min, 30s	72˚C, 30s
6. Small product denaturation	80˚C, 15s	-	78˚C, 1s
7. Final extension	72˚C, 10min	72˚C, 10min	72˚C, 10min
Cycles (Steps 3-6)	35	35	40

4.4 ACTIVITY MEASUREMENTS *IN VITRO* (I)

HSD17B1 activity *in vitro* was determined in the heart tissue of the various HSD17B1TG mouse lines and WT mice using the method described by Tseng and Gurpide (1974), with minor modifications. In brief, tissues were homogenized in 10mM KH_2PO_4 pH7.5, 1mM EDTA, 0.02% NaN_3, and protein concentrations of the homogenates were determined (BioRad protein assay, Hercules, CA). Different amounts of protein (10-800µg) were mixed with [^{3}H]E1 (PerkinElmer; about 500000cpm) and unlabeled E1 (Sigma-Aldrich) to a final concentration of 37µmol/l. The reaction was started by adding 50µl of NADPH (Sigma-Aldrich), to a final concentration of 1.4mmol/l, and the tubes were incubated at +37˚C for 3, 6, 9 and 12min. The reactions were stopped by freezing the reaction tubes in a dry-ice ethanol bath. Thereafter, the steroids were extracted twice with 2ml of diethyl ether (Merck, Whitehouse Station, NJ), evaporated under nitrogen flow and dissolved in 150µl of acetonitrile-water (48%:52%, vol:vol). The amount of E1 converted to E2 was analyzed by separating the [^{3}H]E1 and [^{3}H]E2 using HPLC (Waters), connected to an online β-counter.

4.5 ACTIVITY MEASUREMENTS *IN VIVO* (I, III, IV)

4.5.1 DETERMINATION OF SUBSTRATE SPECIFICITY AND INHIBITION OF HSD17B1 ACTIVITY IN HSD17B1TG AND MMTV-HSD17B1TG MICE (I, III)

HSD17B1 activity in HSD17B1TG and MMTV-HSD17B1TG mice was measured *in vivo* in anesthetized (400-1000µl 2.5% tribromoethanol *i.p.*) 2-3-month-old mice (n=5-6). WT mice were used as controls. [^{3}H]E1 and [^{3}H]A-dione and corresponding unlabeled substrates (PerkinElmer) in a final concentration of 35µg/kg were used as substrate. The substrate concentration, administration route and timing were optimized by various experiments. The substrate was dissolved in 25% ethanol in water, and an *i.v.* injection of 2.5µl/g was given via the tail vein. Blood was withdrawn from the heart of anesthetized mice 2min after the substrate injection, and the mice were euthanized by cervical dislocation. Thereafter, the steroids were extracted twice with 2ml of diethyl ether (Merck), evaporated under nitrogen flow and dissolved in 150µl of acetonitrile-water (48%:52%, vol:vol). The amount of E1 converted to E2 and A-dione converted to T was analyzed by separating the [^{3}H]E1/[^{3}H]E2 and [^{3}H]A-dione/[^{3}H]T using HPLC (Waters), connected to an online β-counter. The efficiency of a HSD17B1 inhibitor (Messinger et al. 2008; compound 50) (hereafter referred to as Inhibitor 1) to inhibit human HSD17B1 activity was tested in MMTV-HSD17B1TG mice *in vivo*. A 25mg/kg dose of the compound dissolved in 10% DMA, 40% polyethylene glycol 400 (PEG-400; VWR International, West Chester, PA) in water was applied *i.p.* 60 and 10min prior to the substrate delivery. The efficiency of HSD17B1-inhibition was determined by analyzing the E1 to E2 conversion in MMTV-HSD17B1TG mice with and without (vehicle only) the inhibitor treatment.

4.5.2 DETERMINATION OF E2 ACCUMULATION IN HSD17B1TG TISSUES (IV)

Radioactive [^{3}H]E1 combined with non-radioactive E1 (Perkin Elmer; Sigma-Aldrich) dissolved in ethanol:saline (20%:80%, vol:vol) was slowly injected *i.v.* (61µg/kg, 2.5µl/g,

555000cpm/µl, ~1.6Mbq/mouse) into adult HSD17B1TG female mice. The mice were terminally anesthetized with 600-1000µl 2.5% tribromoethanol (Sigma-Aldrich; Alfa Aesar) injection *i.p.,* and 15min after the substrate injection, blood was withdrawn from the heart, followed by euthanasia by cervical dislocation. Tissues were dissected, immediately frozen in liquid nitrogen and stored at -80˚C. Thereafter, the tissues were homogenized by Ultra-Turrax in 500µl ice-cold 50mM Tris-HCl buffer (pH7.4). Steroids were then extracted by isopropylether extraction by adding 2ml isopropylether to 500µl homogenate or 150µl serum. After mixing, samples were centrifuged at 700rpm for 10min at room temperature, and extraction was repeated for the organic phase. The organic phase was evaporated under nitrogen flow, dissolved in acetonitrile-water (48%:52%, vol:vol) and centrifuged at 700rpm for 10min at room temperature. Finally, 50µl of samples were applied for HPLC (Waters) connected with an online β-counter.

4.6 UTERUS WEIGHT TEST (IV)

Mice were ear marked at the age of 12 days (d), genotyped and divided into experimental groups. Between postnatal days 15-19, WT and HSD17B1TG female mice were given either placebo (corn oil; Sigma-Aldrich), 1µg/kg/d E1 (Sigma-Aldrich) or 50µg/kg/d E2 (Sigma-Aldrich) in a 50µl volume *i.p* once a day. At the age of 20d, mice were terminally anesthetized with 250-500µl of 2.5% tribromoethanol (Sigma-Aldrich; Alfa Aesar); following by euthanasia by cervical dislocation, the uterus was dissected and uterus weight was recorded.

4.7 PRENATAL FLUTAMIDE TREATMENT (I, II)

Pregnant WT female mice, bred with HSD17B1TG males, were treated with an antiandrogen flutamide (Sigma-Aldrich) from pregnancy day 13.5 until the day of parturition (n=3-6 pregnant females). Dosing was performed once a day by injecting 50mg/kg flutamide in 100µl volume *s.c.* PEG-400 was used as a vehicle. Female pups were divided in groups based on the genotype (WT and TG) and the agent given to the mother (flutamide or vehicle). The pups were sacrificed at the age of 4 months, external morphology was analyzed, and samples were dissected for further analysis.

4.8 OVARY TRANSPLANTATIONS (I)

Prepubertal HSD17B1TG females (or prenatally flutamide-treated prepubertal HSD17B1TG females) were anesthetized with 100-300µl 2.5% tribromoethanol (Sigma-Aldrich; Alfa Aesar) (n=7) given *i.p.* Anesthetic analgesia was achieved by giving 0.2-0.5mg/kg buprenorphine (Temgesic, Schering-Plough, Kenilworth, NJ) preoperatively. HSD17B1TG ovaries on each side were replaced with a half of the ovary of a prepubertal WT mouse. As a control, WT ovaries were similarly transplanted to another WT female (n=10). Postoperative analgesia was performed similarly to preoperative analgesia for three postoperative days. Ovary donors were euthanized by cervical dislocation. Females with transplanted ovaries were sacrificed as described in Section 4.1.3, and analyzed at the age of four months.

4.9 SUPEROVULATION TREATMENT (IV)

To induce ovulation, adult HSD17B1TG females were treated with 5IU of pregnant mare serum gonadotropin (PMSG; Sigma-Aldrich) provided *i.p.* in 100µl in phosphate-buffered saline (PBS). After 49h, mice were injected with 5IU of hCG (Schering-Plough) *i.p.* in 100µl PBS. The length of the mouse estrous cycle typically varies from four to seven days (Staley and Scharfman. 2005) and two superovulation treatments were performed within a period of eight days to mimic the normal estrous cycle. On day nine after starting the treatment, mice were euthanized, and ovarian and uterine samples were dissected for histological analyses.

4.10 PHENOTYPE RESCUING INHIBITOR TREATMENTS (IV-V)

The effect of two novel HSD17B1 inhibitors was tested: 10mg/kg/d of Inhibitor 2 (Messinger et al. 2008, compound 49; Husen et al. 2006a) was given in 50% DMSO/50% propanediol (vol:vol) (Merck) to WT and HSD17B1TG mice for six weeks by subcutaneous minipumps (Alzet #2004, Cupertino, CA) with the constant drug-release of 6µl/d. Alternatively, placebo was given. Untreated WT mice were used as control. Minipumps were inserted as follows: at the age of four months, mice were anesthetized with 450-800µl 2.5% tribromoethanol (Sigma-Aldrich; Alfa Aesar) given *i.p.* and 0.15mg/kg buprenorphine (Schering-Plough). Minipumps were inserted subcutaneously under the loose skin on the back of mice. Postoperative analgesia was obtained by injecting 0.1mg/kg buprenorphine (Schering-Plough) *s.c.* daily for three postoperative days. Additional analgesia was provided when needed with 0.1mg/kg buprenorphine (Schering-Plough) or 5mg/kg carprofen (Rimadyl; Pfizer, New York, NY) *s.c.* After three weeks dosing, minipumps were substituted with new ones as described above. To dissect samples, mice were euthanized at the end of the dosing period and samples were dissected for histological analyses. For Inhibitor 3 (Messinger et al. 2008; compound 21), subcutaneous pellets (Innovative research of America, Sarasota, FL) containing 20mg of the drug (22 mg/kg/d, n=9) releasing 0.33mg/d or placebo (n=7) were used. Anesthesia was performed as described above concerning the minipumps and two pellets were inserted under the loose skin on the back of the mice. Additional analgesia was provided when needed with 0.1mg/kg buprenorphine (Schering-Plough) or 5mg/kg carprofen *s.c.* For sample dissection, mice were euthanized at the end of the dosing period and samples were dissected for histological analyses.

4.11 PROGESTIN TREATMENT (IV)

Medroxyprogesterone acetate (MPA) was provided for two weeks by a subcutaneous pellet releasing the hormone 16mg/kg/d (Innovative Research of America). Adult HSD17B1TG mice were anesthetized with 450-800µl 2.5% tribromoethanol (Sigma-Aldrich; Alfa Aesar), *i.p.* and 0.15mg/kg buprenorphine (Schering-Plough) preoperatively. The pellets were inserted subcutaneously under the loose skin on the back of the mice. Postoperative analgesia was obtained by injecting 0.1mg/kg buprenorphine (Schering-Plough) *s.c.* daily for three postoperative days. Two weeks later, mice were euthanized, and samples were dissected for histological analyses.

4.12 MOUSE HISTOLOGY (I-II, IV)

Tissues were fixed in 4% paraformaldehyde at room temperature for 15-20h. After fixation, tissues were dehydrated and paraffin-embedded. Sections, cut to 5µm thickness were stained with hematoxylin-eosin (HE) for microscopic analysis.

4.13 IMMUNOHISTOCHEMISTRY (I-II)

4.13.1 FETAL MICE (I-II)

An anesthetized pregnant dam was euthanized. Embryos were dissected and decapitated. Whole embryos were fixed in 4% paraformaldehyde at RT for one week, after which fetal ovaries and internal genitalia were dissected. Immunohistochemistry for smooth muscle actin (Sma) and Ar was performed on internal genitalia recovered from WT and TG animals at the age of E17.5, using standard avidin peroxidase protocols (Welsh et al. 2007) and using a Bond-X automated immunostaining machine (Vision Biosystems, Newcastle, UK). Antibodies used for immunohistochemistry, their dilutions and sources are listed in Table 5. Sections were deparaffinized in xylene, rehydrated in graded ethanols and washed in water. For some antibodies (detailed in Table 5), high temperature antigen retrieval was performed using 0.01M citrate buffer, pH6.0. Sections were pressure-cooked for 5min at full pressure, left to stand for 20min and then cooled under running water. Endogenous peroxidase activity was blocked by washing sections in 3% H_2O_2 in methanol for 30min at room temperature.

All washes between antibody or reagent incubations comprised two 5-min washes at room temperature in Tris-buffered saline (TBS; 0.05M Tris-HCl, pH7.4, 0.85% NaCl). Non-specific binding sites were blocked by incubating sections in normal goat serum (NGS; Autogen Bioclear UK Ltd, Wiltshire, UK) diluted 1:4 in TBS containing 5% bovine serum albumin (BSA; Sigma-Aldrich). Sections were incubated overnight at 4°C with primary antibodies diluted in NGS/TBS/BSA as shown in Table 5. Sections were incubated with the appropriate secondary antibody, either biotinylated goat anti-rabbit or biotinylated goat anti-mouse, diluted 1:500 in NGS/TBS/BSA for 30min at room temperature prior to incubation for 30min with avidin-biotin conjugated with peroxidase diluted in 0.05M Tris-HCl, pH7.4 according to the manufacturers instructions (ABC-HRP; DAKO, Ely, UK). Antibody localization was determined using 3,3'-diaminobenzidine (liquid DAB^+; DAKO) until staining was optimally detected in control sections; the reaction was stopped by immersing the sections in distilled water. All sections were then lightly counterstained in Harris's hematoxylin, dehydrated in graded ethanols, cleared in xylene and mounted using Pertex (Cell Path, Hemel Hempstead, UK). Cellular sites of expression of Ar and Sma were determined and compared to the well established normal pattern of expression for each protein to observe any deviation from the normal pattern. Active Ar is a nuclear protein and nuclear localization was used to determine specific positive staining. Sma is a cytoplasmic protein, thus, cytoplasmic staining was used to determine specific positive staining. To ensure the reproducibility of results, the sections from WT and TG animals with appropriate positive and negative controls were processed in parallel on at least two occasions.

Table 5. Antibodies: source, dilution, retrieval and species.

ANTIBODY	SOURCE	DILUTION	RETRIEVAL	SPECIES RAISED IN
Ar	Santa Cruz Biotechnology Inc	1:200	Citrate	Rabbit
Sma	Sigma-Aldrich	1:4000	None	Mouse

4.13.2 HUMAN ENDOMETRIUM

Tissue microarrays (TMAs) of human endometrial cases were constructed using core biopsies from sections from a hysterectomy or endometrial biopsy specimen of cases treated at the Imperial College NHS Trust. The use of the tissues was approved by the Local Ethical Committee. The tissues were fixed in 4% formalin for at least 1h at room temperature depending on sample size. TMA multiblocks were constructed by identifying areas of interest from HE-stained sections. Paraffin blocks containing the samples of interest were retrieved and three punch cores (1mm diameter, 2mm depth) were taken from each specimen using the manual tissue microarrayer (Beecher Instruments, Sun Prairie, WI), and these cores were punched into the recipient block. As a positive control for HSD17B1, human placenta was included in every TMA. Sections were then cut at 4-5μm thickness for immunohistochemistry. Immunohistochemistry was performed with an Autosteiner machine (Lab Vision, Fremont, CA). Sections were deparaffinized in xylene, rehydrated in graded ethanols and washed with water. Antigen retrieval was done with 10mM Tris-EDTA, pH9, at 850W for 3min followed by 100W for 15min. The sections were left to stand for 20min at room temperature and washed with distilled water. To block unspecific binding, the sections were then incubated in 3% BSA/0.05M Tris-HCl/0.05% Tween, pH7.4, at room temperature. Between antibody or reagent incubations, sections were washed with 0.05M Tris-HCl/0.05% Tween, pH7.4 for 3x2min at room temperature. Thereafter, the sections were incubated with primary antibody (mouse anti-human HSD17B1; Abnova, Taipei, Taiwan) diluted 1:100 in 3% BSA/0.05M Tris-HCl/0.05% Tween, pH7.4, for 1h at room temperature. Endogenous peroxidase activity was blocked by 3% H_2O_2 in 0.05M Tris-HCl/0.05% Tween, pH7.4 for 10min at room temperature. Secondary antibody (goat anti-mouse, Dako Envision+, DAKO) was applied for 30min at room temperature according to the manufacturer's instructions. Antibody localization was determined using 3,3'-diaminobenzidine (liquid DAB+; DAKO) until staining was optimally detected in control sections. The reaction was stopped with distilled water. Finally, sections were stained with Mayer's hematoxylin for 1min at room temperature and the reaction was again stopped with distilled water. In the end, sections were rehydrated with graded ethanols, cleared in xylene and mounted using Pertex® (HistoLab, Gothenburg, Sweden). HSD17B1 is a cytoplasmic protein and, thus, cytoplasmic epithelial staining was used to determine specific positive staining, with staining intensity being scored from 0 to 4 (0= no staining, 1= weak staining, 2= moderate staining, 3= strong staining and 4= very strong staining).

4.14 PERIPHERAL QUANTITATIVE COMPUTATIVE TOMOGRAPHY (V)

Tibias and femurs were stored in 40% ethanol at +4˚C for peripheral quantitative computative tomography (pQCT) analyses. After removing soft tissues, bones were inserted in a plastic tube (8mm diameter) and scanned with pQCT equipment (XCT540; Stratec Medizintechnik, Pforzheim, Germany). For tibias, the reference line was placed at the proximal end of the tibia. Two to three cross-sections at 0.25mm intervals were analyzed approximately 1.7mm from the reference line, referred to as the metaphysis area. Measurements were also carried out from the diaphysis (midshaft) of the tibias. For femurs, two sections were measured starting 1.8mm from the distal end and one section 6mm from the distal end. Special Software version 5.40 (Stratec Medizintechnik) was used to analyze the images of each section, with a voxel size of 0.7mm. Standardized analysis (peel mode 2, cort mode 1, contour mode 1, threshold 0.25mg/cm^3 for trabecular bone and 0.71mg/cm^3 for cortical bone) was applied. Bone strength was also estimated by calculating the strength strain index (SSI) and polar moment of inertia (PMI) from the pQCT measurements, and bone length was measured.

4.15 STATISTICS (I-V)

Statistical analyses were carried out using the following tests: Student's t-test or Mann-Whitney test when applicable to compare two groups and One Way Analysis of Variance or Kruskal-Wallis One Way Analysis of Variance of Ranks when applicable to analyze many groups. Significance was set as $p<0.05$, and mean values ± standard error of the mean (SEM) are presented.

5 RESULTS

5.1 GENERATION OF HSD17B1TG MICE (I)

In order to study the physiological role of human HSD17B1 *in vivo*, TG mice universally overexpressing human HSD17B1 were generated using standard pronucleus injection techniques (HSD17B1TG mice). The transgene construct is presented in Figure 9. Five HSD17B1TG mouse lines were generated and universal transgene expression was confirmed to correlate with enzyme activity and penetration of the phenotype (I: Figure 1, Table 2). The mouse line with the strongest expression (line 013) was chosen for further studies. The transgene expression levels in other TG mouse lines compared to line 013 were 46%, 10.6%, 14.5% and 0%, for lines 050, 012, 020 and 016, respectively. The conversions of E1 to E2 *in vitro* as compared with line 013 were 54.8%, 6.7%, 5.0% and 0%, for lines 050, 012, 020 and 016, respectively. Concisely, HSD17B1TG female mice suffered from several estrogen-dependent disorders, but surprisingly, also androgen-dependent disorders.

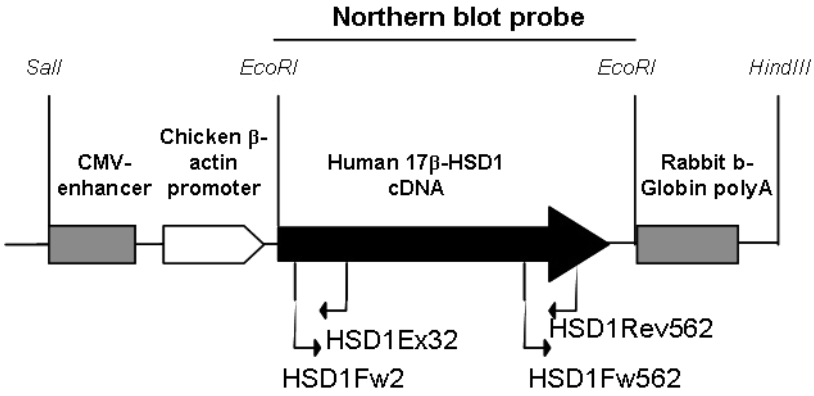


FIGURE 9. Trangene construct. Schematic presentation of HSD17B1 transgene construct. Human HSD17B1 cDNA was inserted between CMV-enhanced chicken β-actin promoter and rabbit β-globin poly-A tail to form the transgene construct.

5.2 HORMONAL STATUS OF HSD17B1TG MICE (I)

As presented in Table 6, the concentrations of intra-tissular T and E2 were measured from fetuses on the last day of gestation (E17.5), as well as from adult mice at the ages of two and four months (I: Table 3). Both E2 and T concentrations were significantly higher in HSD17B1TG than in WT female fetuses, whereas the placental T concentration was normal (I: Table 3). The T concentration in HSD17B1TG female fetuses was approximately at the same level as in WT males as indicated in Table 6. The result that placental T was not increased indicates that elevated T was not of maternal source, and is in line with the fact that HSD17B1TG mice were born from a WT dam. Despite this, although differences in sex steroid concentrations were detected before birth, serum E2 and T concentrations were not significantly different in adult WT and HSD17B1TG females. Moreover, LH and FSH concentrations were within normal range (I: Table 3).

Table 6. Prenatal intra-tissular E2 and T concentrations in male and female fetuses at E17.5.

	MALE			FEMALE		
	WT	TG	p	WT	TG	p
E2 (pg/ml)	29.7±11.1 (n=7)	70.0±11.7 (n=4)	NS	14.2±4.6 (n=4)	47.8±7.7 (n=5)	*
T (pg/ml)	108.7±14.5 (n=6)	191.1±14.7 (n=4)	**	34.0±5.3 (n=7)	107.2±21.5 (n=10)	**

*p<0.05, **p<0.01, ***p<0.001, NS=not significant

5.3 SUBSTRATE SPECIFICITY OF HUMAN HSD17B1 *IN VIVO* (I, III-IV)

Based on studies *in vitro*, human HSD17B1 is considered estrogen-specific, with only very weak androgenic activity (Poutanen et al. 1993, Puranen et al. 1997b), a finding which has been ignored. Because fetal androgen concentration was significantly increased in HSD17B1TG females, substrate specificity of human HSD17B1 expressed in the TG mice was determined *in vivo*. Enzyme activities using E1, E2 and A-dione as substrate were determined in line 013 and in WT mice. With a substrate concentration of 35µg/kg, both the estrogen and androgen reductase activities were significantly increased in HSD17B1TG mice as compared with WT mice (Figure 10). Furthermore, using estrogens as substrates, both the reductive (E1 to E2) and oxidative (E2 to E1) activities were significantly increased. However, similarly to that shown previously in cultured cells, the conversion of E1 to E2 was markedly higher than the opposite reaction in HSD17B1TG mice *in vivo* (Figure 10A). The results suggest that the main activity of human HSD17B1 *in vivo* is to convert E1 to E2, but the enzyme also catalyzes the reverse reaction, although markedly less efficiently. Furthermore, the data suggest that human HSD17B1 has significant androgen reductase activity *in vivo*.

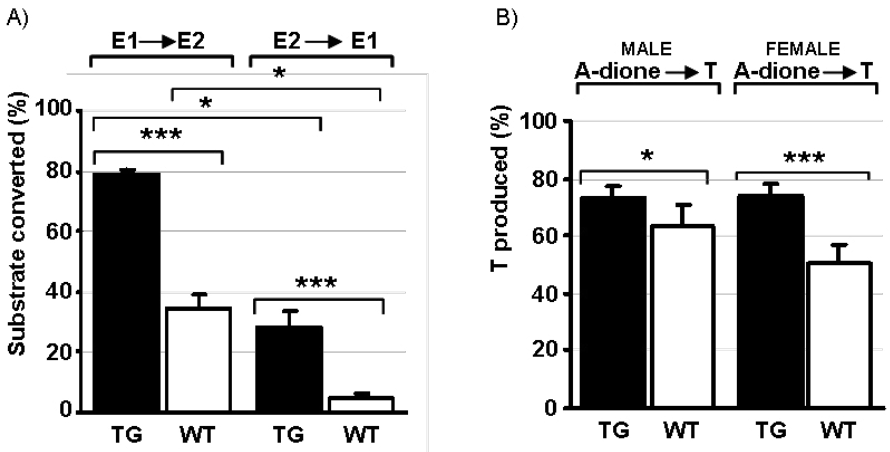
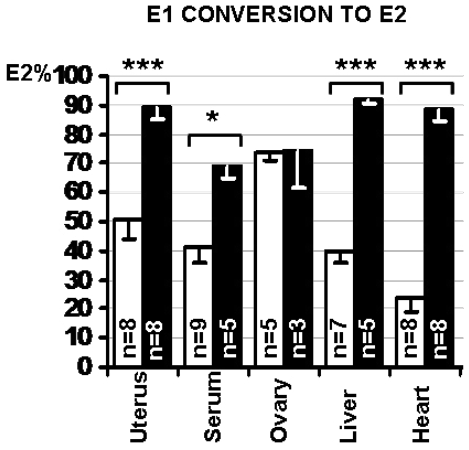


FIGURE 10. Substrate specificity of human HSD17B1. The substrate specificity of human HSD17B1 was determined *in vivo* in HSD17B1TG mice. A) Reductive (E1 to E2) and oxidative (E2 to E1) activities were measured in TG and WT males. Reductive activity was significantly higher than oxidative activity in both TG and WT mice, and in TG mice the reductive activity was significantly higher than the oxidative conversion, indicating that the major activity of human HSD17B1 *in vivo* is the activation of estrogens. B) The reductive androgenic activity (A-dione to T) was also determined from males and females and, surprisingly, was shown to be significantly higher in the HSD17B1TG mice, indicating that human HSD17B1 has significant androgen reductase activity *in vivo*.

The inevitable role of human HSD17B1 in estrogen metabolism was further studied in detail. To test whether E2 accumulates in tissues according to the hypothesis, we analyzed the reductive HSD17B capacity, *i.e.* conversion of E1 substrate to E2, in the uterus and other tissues of WT and HSD17B1TG female mice *in vivo*. Compared to WT animals, the E2/E1 ratio in HSD17B1TG mice was significantly higher in all tissues examined, except in the ovaries (Figure 11A), which have high expression of mouse endogenous Hsd17b1. To further examine the modulation of estrogen responses in the uterus by HSD17B1 we performed a uterus weight test, which is a classical bioassay for estrogen action in mice *in vivo*. Immature (age of 15d) HSD17B1TG females and WT littermates were treated for five days with vehicle (placebo), 1µg/kg/d of E1, or 50µg/kg/d of E2. Uterine weight increased in both E1-treated WT and HSD17B1TG animals, but the relative increase in uterus weight was four times higher in HSD17B1TG animals (Figure 11B). As expected, E2 also markedly stimulated uterine growth, and the magnitude of this response was identical in WT and HSD17B1TG mice. Notably, uterine weight was modestly higher in placebo-treated HSD17B1TG mice, possibly reflecting more efficient conversion of endogenous E1 to E2 (IV: Figure 2).

A)



B)

UTERUS GROWTH IN E1 TREATED MICE

	Uterus weight (mg)		
	Pla	E1	Growth (mg)
TG	11.1	19.5***	8.4
WT	9.8	11.8	2

***p<0.001 between E1 treated TG and WT uterus weight containing secretory products

FIGURE 11. Accumulation of E2 in TG tissues expressing human HSD17B1. A) Conversion on E1 to E2 was studied in various HSD17B1TG female tissues *in vivo*. The mice were injected with [^{3}H]E1 *i.v.* and samples were collected 15min after the injection. With substrate concentration of 61µg/kg, the conversion of E1 to E2 was markedly higher in HSD17B1TG than in WT tissues, indicating that E2 accumulates in HSD17B1TG tissues. In WT and HSD17B1TG ovaries, the conversion was equal, probably because of the high endogenous mouse HSD17B1 expression in the ovaries. B) The growth of HSD17B1TG uterus induced with E1 was four times greater than in WT mice, indicating that HSD17B1 expression enhances the response to E1 in the uterus.

MMTV-HSD17B1TG mice were generated similarly to HSD17B1TG mice, except that MMTV promoter was used instead of chicken β-actin promoter. These mice appeared to be more suitable for measuring the degree of inhibition of HSD17B1 activity by HSD17B1 inhibitors. Therefore, MMTV-HSD17B1TG mice were used to test the effect of a HSD17B1 inhibitor in the inhibition of estrogenic HSD17B1 activity *in vivo*. Male and female mice were treated with a single *i.p.* injection of Inhibitor 1 (25mg/kg) prior

to the activity measurement *in vivo*. As a result, a significant decrease in the conversion of E1 to E2 was obtained in both male and female mice. In males, the inhibition was on average 75%, while in females it was only 33.1%. The effect negatively correlated with TG expression, which was shown to be higher in MMTV-HSD17B1TG female mice than in male mice (III: Figure 3). The results indicate that HSD17B1 activity can indeed be inhibited by specific HSD17B1 inhibitors *in vivo*.

5.4 ANDROGEN-DEPENDENT DISORDERS IN HSD17B1TG FEMALES (I-II)

5.4.1 FEMALE DSD (I-II)

In line with increased fetal T concentration, HSD17B1TG females suffered from female DSD (feminine pseudohermaphroditism), which is a known sign of fetal androgen exposure. As a result, both external and internal genitalia of HSD17B1TG females were masculinized: AGD was increased, nipple development was suppressed, there was lack of vaginal opening, and the vagina was combined with the urethra in the lowest third area (I: Figure 3). Furthermore, enlarged remnants of Wolffian ducts in the mesovarium (II: Figure 1) and enlarged Skene paraurethral gland (or the female prostate) (II: Figure 2) were observed. The androgen-dependency of the phenotypes was confirmed by a rescuing experiment using prenatal treatment with the antiandrogen flutamide (I: Figure 3 and Table 4, II: Figures 1-2). Because masculinized phenotypes were observed, and HSD17B2 could possibly exert an antiestrogenic effect, we analyzed the putative alteration in the endogenous mouse Hsd17b2 expression in HSD17B1TG mice in the two tissues with the highest level of Hsd17b2 expression in mice, *i.e.* the liver and intestine. However, no significant induction of Hsd17b2 expression was observed in these tissues (I: Table 1).

5.4.2 OVARIAN BENIGN SEROUS CYSTADENOMAS (I)

Although differences in sex steroid concentrations detected before birth were normalized by adulthood, HSD17B1TG females developed benign ovarian serous cystadenomas by the age of four months. These were characterized by hyperplasia of ovarian surface epithelium, and cysts surrounding the ovaries (I: Figure 4A, E, F). The changes were absent at the age of two months. The mice were followed until the age of 12 months, but no overt ovarian cancer development was observed. Because there were no significant alterations in adult serum hormone concentrations, we analyzed the dependence of ovarian benign serous cystadenomas on prenatal masculinization. Interestingly, the formation of the lesions was efficiently prevented by prenatal flutamide treatment (I: Figure 4C), and development of ovarian surface epithelial hyperplasia was also suppressed in WT ovaries prepubertally transplanted to HSD17B1TG females (I: Figure 4B). The effect of WT ovary transplantation on ovarian cysts was not evaluated because the method itself induced a cystic appearance of the ovaries. The lesions were also observed in the weaker expression line 050, while lines 012, 020, and 016 were not analyzed.

5.5 ESTROGEN-DEPENDENT DISORDERS IN HSD17B1TG MICE AND THEIR TREATMENT WITH HSD17B1 INHIBITORS (IV-V)

5.5.1 ENDOMETRIAL HYPERPLASIA (IV)

5.5.1.1 Characterization of endometrial phenotype of HSD17B1TG females

Endometrial hyperplasia was invariably observed in HSD17B1TG females from the age of two months onwards. The mice also presented with anovulation, as characterized by inefficient luteinization of ovarian follicles (IV: Figure 1). The prevalence of endometrial hyperplasia and anovulation was 100% in HSD17B1TG females at the age of four months, and both typical and atypical hyperplasias were found (Table 7). While approximately 25% of animals developed complex atypical hyperplasia, progression to endometrial cancers was not observed by the age of 12 months. At one month of age, before the onset of puberty, the endometrium was normal on histology. Although our analyses were carried out predominantly in a mouse line that strongly expresses human HSD17B1 (line 013), endometrial hyperplasia and anovulation were also observed in low-expressing HSD17B1TG lines (data not shown).

Table 7. Prevalence of endometrial hyperplasia.

	Age (months)	NORMAL	SIMPLE TYPICAL	COMPLEX TYPICAL	SIMPLE ATYPICAL	COMPLEX ATYPICAL
TG	1 (n=5)	100%	0%	0%	0%	0%
	4 (n=8)	0%	25%	50%	0%	25%
WT	1 (n=4)	100%	0%	0%	0%	0%
	4 (n=6)	100%	0%	0%	0%	0%

5.5.1.2 Effect of various rescuing treatments on endometrial hyperplasia

We reasoned that as in humans, endometrial hyperplasia in HSD17B1TG mice could be a consequence of persistent anovulation abolishing cyclic P secretion and resulting in unopposed estrogen action in target tissues like the uterus. To test this, animals were treated with exogenous gonadotropins to induce ovulation and consequent luteinization of ovarian follicles. Two PMSG/hCG-injections (5IU) were given at a four-day interval to mimic the normal estrous cycle in mice. The treatment induced ovulation and this was sufficient to reverse endometrial hyperplasia in HSD17B1TG mice (IV: Figure 4A-B), indicating that the endometrial phenotype was indeed a consequence of ovarian dysfunction. In addition, a treatment with 16mg/kg of progestin (MPA) administrated for two weeks with a subcutaneous pellet reversed the endometrial hyperplasia but, as expected, did not induce ovulation (IV: Figure 4C-D). To test whether HSD17B1 could serve as a drug target for the treatment of endometrial hyperplasia, HSD17B1TG females were treated with 10mg/kg of Inhibitor 2, administrated via subcutaneous minipumps for six weeks. Similarly to MPA, the inhibitor completely reversed the hyperplastic morphology of the glandular endometrial compartment in the presence of the anovulatory ovary (IV: Figure 5A-C). The luminal epithelial cells, however, remained proliferative in

50% of animals treated with this inhibitor (IV: Figure 5B) and this was often associated with focal endometrial inflammation, characterized by the accumulation of eosinophil granulocytes and other leucocytes (data not shown). The effects of different rescuing treatments are summarized in Table 8.

Table 8. Endometrial hyperplasia after different treatments in HSD17B1TG mice.

Treatment	Normal	Proliferative	SIMPLE Typical	COMPLEX Typical	SIMPLE Atypical	COMPLEX Atypical
PMSG/hCG (n=4)	100%	0%	0%	0%	0%	0%
MPA (n=5)	100%	0%	0%	0%	0%	0%
Inhibitor 2 (n=6)	50%	50%	0%	0%	0%	0%
Placebo (n=7)	0%	0%	14.3%	42.9%	14.3%	28.6%

PMSG/hCG= pregnant mare serum gonadotropin, hCG= human chorionic gonadotropin, MPA= medroxyprogesterone acetate

5.5.1.3 Expression of HSD17B1 in normal and malignant human endometrium

While our analysis of HSD17B1TG mice suggests that HSD17B1 is a promising drug target for estrogen-dependent uterine disorders, the expression of this enzyme in the cycling human endometrium and estrogen-dependent endometrial diseases is controversial, and mRNA expression analysis is further complicated, because two transcripts of HSD17B1 are expressed. Thus, we used immunohistochemistry to examine the relative expression levels of HSD17B1 protein in normal, hyperplastic and malignant human endometrial tissue sections. As shown in Figure 12, HSD17B1 immunoreactivity was apparent in normal and abnormal endometrium and localized predominantly to the epithelial cell compartment. Expression levels were comparable between the proliferative and secretory endometrium, but significantly lower in atypical endometrial hyperplasia and carcinoma when compared to the normal cycling endometrium. This expression profile fits well with the observations in HSD17B1TG female mice, suggesting a major contribution of HSD17B1 in the transition of the proliferating endometrium to hyperplasia but not in the subsequent transformation to neoplasia.

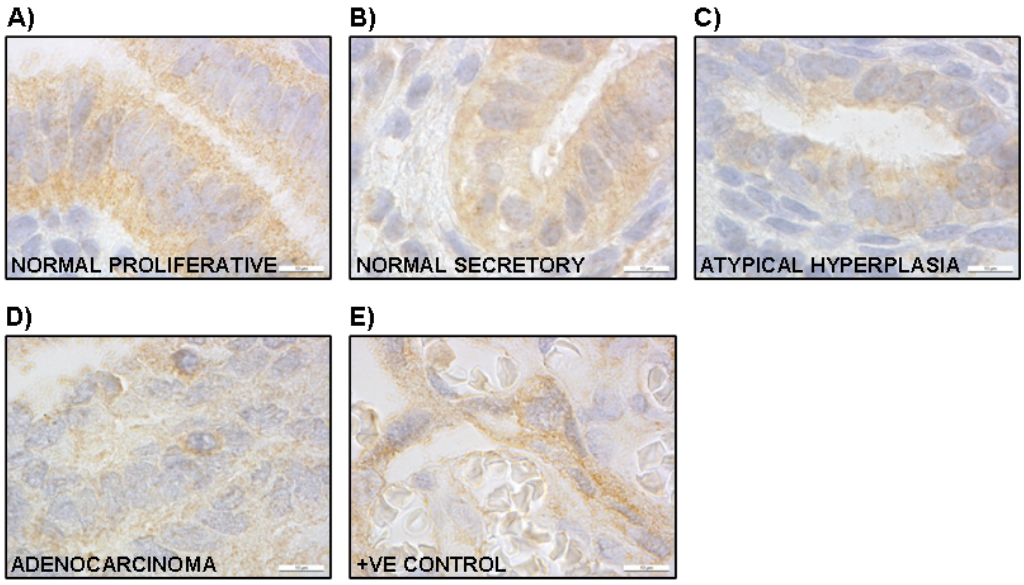


FIGURE 12. HSD17B1 is expressed in the human endometrium. Because HSD17B1 expression has been controversially reported in the literature, we studied the expression by immunohistochemistry. We found that HSD17B1 expression was detected in normal human proliferative (A) and secretory (B) endometrium. HSD17B1 expression was found, but was decreased, in atypical endometrial hyperplasia (C) and endometrioid adenocarcinoma (D), which may represent a compensatory role to decrease endometrial estrogen concentration locally. Human placenta was used as positive control (E).

5.5.2 BONE QUALITY AND LENGTH (V)

5.5.2.1 Characterization of bone phenotype in HSD17B1TG females

Bone phenotype of HSD17B1TG females was characterized at the ages of four and nine months and results were compared to WT mice. Proximal metaphysis and midshaft areas of tibia and distal metaphysis and midshaft areas of femur were scanned by pQCT. The observed phenotype was stronger in the femur than the tibia and in midshaft as compared with the metaphysis. Interestingly, significant changes were only observed in cortical bone, whereas trabecular bone was normal, except for the increase in trabecular BMD of the metaphysis area at the age of four months. Total BMD in the femur metaphysis of HSD17B1TG females was significantly increased at the ages of four and nine months as compared with WT mice. Trabecular BMD of metaphysis was significantly increased in HSD17B1TG females at the age of four months, but not at the age of nine months. In contrast, cortical BMD of the metaphysis was significantly increased in HSD17B1TG females at the age of nine months as compared with WT, but less so at the age of four months. In the femur midshaft area, total and cortical BMDs were significantly increased at the ages of four and nine months in HSD17B1TG females compared to WT, whereas no changes were observed in trabecular bone. Cortical thickness, PMI and SSI were significantly increased in the femur midshaft area at the ages of four and nine months, whereas in the metaphysis, significant increases were only observed at the age of nine

months (V: Table 1). The phenotype observed in tibias of HSD17B1TG females was similar to that of femurs, but weaker (data not shown). However, although the tibia length of HSD17B1TG females was significantly decreased at the age of four months, the differences normalized by the age of nine months. Femur lengths were normal (V: Table 2). Figure 13 summarizes the effects of HSD17B1 in bone.

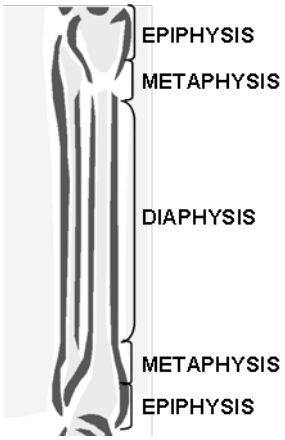


FIGURE 13. Bones sites affected by human HSD17B1 expression. Tibia is presented as an example. Overexpression of human HSD17B1 in TG mice had effects on all areas of tibia, i.e. epiphysis, metaphysis and diaphysis. The major effect in metaphysis and diaphysis was an increase in cortical bone, whereas epiphyses participate in the regulation of bone length, which was decreased in HSD17B1TG females. In the femur, HSD17B1 only affected the metaphysis and diaphysis areas.

5.5.2.2 Effect of HSD17B1 inhibitors on bone

The effect of two novel HSD17B1 inhibitors on bone was studied. Adult HSD17B1TG and WT females were treated with two different HSD17B1 inhibitors, Inhibitor 2 (10mg/kg/d) and Inhibitor 3 (22mg/kg/d) administrated via subcutaneous minipumps and pellets, respectively, for six weeks. Surprisingly, the treatments had no effect on either femoral or tibial bone pQCT parameters analyzed (data not shown), but interestingly, treatment of HSD17B1TG mice with HSD17B1 Inhibitor 3 significantly increased the length of tibia compared to placebo-treated HSD17B1TG females (Table 9). The same tendency was observed with Inhibitor 2, but statistical significance was not reached. The inhibitor treatments did not affect femur length.

Table 9. Effect of HSD17B1 inhibitor on bone length.

	TG+PLA2	TG+INH2	*p*	TG+PLA3	TG+INH3	*p*
Tibia	18.1 (±0.12)	18.5 (±0.20)	NS	18.2 (±0.07)	18.5 (±0.10)	*
Femur	15.5 (±0.10)	15.6 (±0.20)	NS	14.7 (±0.05)	14.8 (±0.10)	NS

*p<0.05 between placebo- and inhibitor-treated TG, ***p<0.001 between WT and placebo-treated TG, n=6-9, PLA=placebo, INH=inhibitor, NS=not significant

5.5.2.3 Effect of HSD17B2 overexpression on HSD17B1TG bone

The effect of the opposite enzyme for HSD17B1, *i.e.* HSD17B2, was studied by crossbreeding HSD17B1TG and HSD17B2TG mice to generate bi-TG mice. Similarly to HSD17B1TG mice, HSD17B2TG mice universally overexpressed human HSD17B2. In the bi-TG mice, bone quality and length were dramatically decreased (data not shown), indicating that HSD17B2 may have a more pronounced role in bone than HSD17B1 or that, in bone, these enzymes affect by different mechanisms.

ANOVULATION (I-V)

HSD17B1TG females suffered from anovulatory ovaries appearing as absent corpora lutea, known to correlate with anovulation. To understand this, the ovarian phenotype was analyzed after the various rescuing experiments performed, *i.e.* prenatal flutamide treatment, ovary transplantations, superovulation treatment and treatments with Inhibitors 2 and 3. However, of these, only superovulation treatment rescued ovulation and induced the formation of corpora lutea, indicating that the ovarian phenotype was dependent on LH. However, as the circulating LH level was normal in HSD17B1TG females (I: Table 3), the data suggest aberration in the LH surge rather than in the basal LH level. Extragonadal failure is also suggested by the fact that WT ovaries prepubertally transplanted into HSD17B1TG females turned anovulatory, while WT ovaries transplanted to WT mice had normal corpus luteum formation. The results indicate that extragonadal exposure of HSD17B1TG females to estrogens reprograms the HPGA feedback system, resulting in lack of LH surge and anovulation as summarized in Table 10 below.

Table 10. Origin of anovulatory phenotype in HSD17B1TG females.

TREATMENT	TIMING	MECHANISM	EFFECT
Flutamide	E13.5-birth	Overcomes the effect of prenatal masculinization	No effect
Transplantation of WT ovaries to untreated HSD17B1TG mice	< 30d	Excludes gonadal disorder	No effect
Inhibitors 2 and 3	4-5.5 months	Excludes the effect of estrogen exposure during adulthood	No effect
Superovulation	4 months	Creation of artificial LH surge	**Ovulation induction**

6 DISCUSSION

6.1 SUBSTRATE SPECIFICITY OF HUMAN HSD17B1 *IN VIVO*

The properties of human HSD17B1 have been extensively studied *in vitro*. Based on these studies, human HSD17B1 is considered highly estrogen-specific with markedly higher K_m values for androgenic substrates (Poutanen et al. 1993, Miettinen et al. 1996, Peltoketo et al. 1999b). Structural studies have also indicated that the narrow substrate-binding tunnel is highly complementary to estrogens (Azzi et al. 1996, Lin et al. 2006). Furthermore, the enzyme catalyzes both oxidative and reductive estrogenic HSD17B activity with a proper cofactor added *in vitro*. In cultured cells, human HSD17B1 has been shown to catalyze predominantly the reductive reaction, and the expression of human HSD17B1 decreased the oxidative activity (converting E2 to E1) while the reductive activity converting E1 to E2 was markedly increased (Poutanen et al. 1993, Miettinen et al. 1996, Puranen et al. 1997b, Day et al. 2008a). Recent studies have further provided evidence for reductive estrogenic activity of human HSD17B1 *in vivo*. Xenografting HSD17B1-transfected MCF7 cells into immunodeficient nude mice resulted in enhanced estrogen-dependent tumor growth in the presence of E1, while no such effect was detected in the absence of HSD17B1 (Husen et al. 2006b).

Several human HSD17Bs are capable of converting E1 to E2, including HSD17B1 (Poutanen et al. 1993, Husen et al. 2006b), HSD17B7 (Torn et al. 2003) and HSD17B12 (Luu-The et al. 2006), but the role of each of these in extragonadal E2 formation remains unclear. Although HSD17B1 expression in various peripheral tissues is low, its catalytic efficacy is markedly higher than that of HSD17B7 or HSD17B12 (Luu-The et al. 2006), suggesting a central role for HSD17B1 in peripheral E2 formation. In the present study, we further analyzed the role of human HSD17B1 in estrogen metabolism *in vivo* using HSD17B1TG mice. The observation that a small dose of E1 suffices to markedly increase the uterine weight of immature HSD17B1TG mice further demonstrates the ability of HSD17B1 to enhance estrogen action in target tissues. Increased local E2 production in response to E1 was apparent in all TG tissues with the exception of the ovaries, which in mice express high levels of endogenous Hsd17b1 (Nokelainen et al. 1996). Moreover, overexpression of human HSD17B1 in TG mice under weak MMTV promoter resulted in significantly increased reductive estrogenic activity *in vivo*, while it was significantly decreased by treatments with HSD17B1 inhibitors. Our data suggest that HSD17B1 plays a major role in determining the gradient between the E2 concentrations in serum and peripheral tissues, as has been reported for postmenopausal breast cancer (Pasqualini et al. 1996), a tissue with abundant HSD17B1 expression (Miyoshi et al. 2001, Shibuya et al. 2008).

There is a marked difference in the steroid specificity between the human and rodent HSD17B1 enzymes. Human HSD17B1 is considered estrogen-specific, while the catalytic efficacy for the rodent enzymes *in vitro* is similar for both androgens and estrogens. HSD17B1 is composed of 327 and 344 amino acids in humans and rodents, respectively (Peltoketo et al. 1988, Nokelainen et al. 1996). The identity between the rat and human amino acid sequences is 63%, the highest identity (81%) being located at the region composed of the 200 N-terminal amino acids (Puranen et al. 1997b). The

cofactor-binding region comprises the first ~100 amino acids (Ghosh et al. 1995), while the next 50 amino acids are involved in the dimerization (Puranen et al. 1997b). Studies with chimeric enzymes composed of rat and human sequences have shown that the region between the residues 148–266 determines most of the steroid specificity in the HSD17B1 enzyme, with a consistently decreasing E1/A-dione substrate ratio, along with more amino acid residues identical to the rat enzyme. Especially $Asn^{152}His$, $Asp^{153}Glu$ and $Pro^{187}Ala$ variations were found to be closely related to steroid specificity in this region (Puranen et al. 1997b). However, the difference in the steroid-binding regions (residues 148-171) between human and rat enzymes is only two amino acids (Puranen et al. 1997b). Furthermore, as shown by a recent structural study, the pseudosymmetry of C19 steroids can lead to alternative substrate binding, resulting in the multispecificity of human HSD17B1. As reviewed by Lin et al. (2006), the enzyme can also catalyze the formation of A-diol from DHEA, and 3β-diol from DHT. However, the significance of these activities *in vivo* remains unclear. Interestingly, despite the high k_m value of human HSD17B1 towards A-dione as a substrate (Puranen et al. 1997b, Lin et al. 2006), the activity obtained was 20% of that observed with E1 in cultured COS 6m cells (Poutanen et al. 1993). This suggests that the human enzyme possesses considerable androgenic activity with the preference for estrogenic substrates. Data from the present study further support the theory that human HSD17B1 is not fully estrogen-specific but has significant androgenic activity *in vivo*. Significantly increased conversion of A-dione to T was observed in both female and male HSD17B1TG mice. Furthermore, significantly increased T concentration was observed in fetal HSD17B1TG females, resulting in various androgen-dependent disorders.

6.2 ROLE OF HUMAN HSD17B1 IN ANDROGEN-DEPENDENT FEMALE DISORDERS

6.2.1 FETAL ANDROGEN ACCUMULATION

Various animal experiments (Stinnakre. 1975, Wolf et al. 2002, Wolf et al. 2004, Welsh et al. 2008) have shown that androgen excess during fetal life disturbs normal fetal development, resulting in masculinization of the female reproductive tract. This is also suggested by clinical observations in human patients with CAH, in which female fetuses have increased adrenal T production, resulting in masculinization of the external genitalia (Merke and Bornstein. 2005). AR is expressed in both female and male reproductive tissues during development, and consequently, also the female reproductive tract is capable of responding to androgens (Bentvelsen et al. 1995). Along with the significant androgenic activity and increased fetal T concentration, HSD17B1TG females presented with well-known androgen-dependent phenotypic changes including female DSD indicated by increased AGD, suppressed nipple development, lack of vaginal opening, combination of vagina with urethra, enlarged Skene paraurethral gland, and enlarged Wolffian duct remnants in the mesovarium. T concentration in HSD17B1TG females preceding birth was in the same range as that of WT males, which is naturally sufficient to fully retain the Wolffian ducts. However, the Wolffian duct remnants in the mesovarium, which were enlarged in HSD17B1TG females, are also typical for WT mice and women (Kaufman and Bard. 1999, Kurman and TeLinde. 2002), and thus, the classical T-induced rescuing

of Wolffian ducts did not take place in HSD17B1TG females. It is suggested, however, that different structures in the female reproductive tract may differ in their sensitivity to androgen exposure: for example, the female external genitalia are highly sensitive to the effects of T, whereas rescue and differentiation of the female Wolffian duct appears to depend on higher levels of T (Swanson and Werff ten Bosch. 1965, Ogawa and Nozawa. 1969, Stinnakre. 1975, Wolf et al. 2002). In the present study, T levels in HSD17B1TG female fetuses were only measured at E17.5, which is the last day of pregnancy in FVB/N mice. Therefore, it is possible that the androgen concentration has been lower at earlier time points, sufficient to induce the lengthening of AGD, but not to rescue Wolffian ducts.

The female DSD in HSD17B1TG females was effectively treated by prenatal antiandrogen administration, confirming the dependence of the phenotype on androgens. In humans, masculinization of external genitalia begins with lengthening of AGD at gestational week ten, followed by the fusion of the labioscrotal folds and closure of the rims of the urethral groove (Josso. 2004). In FVB/N mice, this occurs at E13.5-E14.5, after which the genitalia continue to develop until the time of birth (Kaufman and Bard. 1999). Based on this information, flutamide administration was timed from E13.5 until birth, and this efficiently rescued the increase in AGD. It is acknowledged that as a response to androgens the vagina fails to completely descend to the perineum, causing a common urogenital canal or sinus with incomplete separation of the vagina and urethra, as observed in HSD17B1TG females (MacLaughlin and Donahoe. 2004). Nipple development has also been shown to be androgen-dependent. Male rodents normally lack nipples, but for example in rats, the nipple development can be induced by undermasculinizing males with prenatal flutamide treatment (Miyata et al. 2002, Foster and Harris. 2005). Consequently, prenatal flutamide exposure retained nipple development and rescued vaginal morphology in masculinized TG females. Interestingly, the urethra of HSD17B1TG females was surrounded by an enlarged gland identified as the Skene paraurethtral gland, also referred to as the female prostate, at the site of fusion of the vagina and urethra. The female prostate has been reported to be present in several species, including humans and rodents, and similar to the male prostate, it has been shown to respond to androgens (Santos et al. 2006). Like the other androgen-dependent malformations in HSD17B1TG females, flutamide treatment during fetal life suppressed the gland development. The concentration of T in HSD17B1TG females was not increased in adulthood, suggesting that the fetal exposure to androgens is enough to stimulate the growth of the female prostate during adulthood.

These data unequivocally show that overexpression of human HSD17B1 leads to increased androgen exposure during embryonic development. However, the source of androgen could not be determined in the present study. The fact that placental T was not increased indicates that elevated T was not of maternal origin, which would have been unlikely, because HSD17B1TG mice were born from a WT dam. Mouse adrenals express Cyp17a1 during fetal development, whereas adult mouse adrenals lack both Cyp17a1 expression and sex steroid biosynthesis (Keeney et al. 1995), and we hypothesized that the adrenals were the source of the androgen excess during the fetal development of HSD17B1TG females. However, no increase in T production was identified in fetal HSD17B1TG adrenals, as determined in the present study. Interestingly, several studies have indicated that prenatal exposure to androgens results in PCOS and

metabolic syndrome in experimental animals (Bruns et al. 2004, Recabarren et al. 2005, Padmanabhan et al. 2006). This is also clinically manifested in human patients with CAH having an increased risk of developing PCOS (McKenna and Cunningham. 1995, Xita and Tsatsoulis. 2006). HSD17B1 has been shown to be expressed in fetal human ovaries (Vaskivuo et al. 2005), and thus, is likely to contribute to the maintenance of the normal sex steroid balance during development. Therefore, aberrant HSD17B1 expression could lead to hormonal imbalances and masculinization-induced disorders, such as PCOS, in females.

6.2.2 OVARIAN CARCINOGENESIS

Epithelial ovarian tumors are among the most variable tumors of any organ, in that they may express properties related to fallopian tube epithelium (serous tumors), endometrium (endometrioid tumors), endocervix or colonic epithelium (mucinous tumors), or the urogenital tract (clear cell tumors) (Auersperg et al. 2002). The vast majority of ovarian cancers (80-90%) are derived from the ovarian surface epithelium. Furthermore, serous adenocarcinomas comprise approximately 80% of all epithelial ovarian cancers (Auersperg et al. 2001). Similarly to most epithelial tumors, ovarian epithelial tumors follow the adenoma-carcinoma sequence scheme, where normal epithelial cells undergo genetic alterations of increased complexity towards a state that is phenotypically consistent with the tumor cell. High-grade ovarian serous epithelial tumors are different from all other types of ovarian tumors, including low-grade serous tumors in such a way that they progress rapidly, and no precursor lesion has been indentified (Levanon et al. 2008). Histopathologically detectable early malignant changes occur more frequently in surface epithelium-lined clefts and inclusion cysts than on the ovarian surface that faces the pelvic cavity (Auersperg et al. 2001, Levanon et al. 2008). With neoplastic progression, the ovarian surface epithelial cells become increasingly committed to complex epithelial phenotypes which include the formation of glandular structures and papillas (Auersperg et al. 2002). A dysfunctional ovary is also a risk factor for ovarian epithelial carcinogenesis (Vanderhyden. 2005).

Between the ages of two and four months, HSD17B1TG females developed benign ovarian serous cystadenomas. These are common, slowly-proliferative benign lesions that can be precursors of ovarian serous borderline tumors, which can, in turn, progress to low-grade serous carcinomas (Cheng et al. 2004). The development of the benign serous cystadenomas in HSD17B1TG females was prevented by treating the mice prenatally with flutamide, or by transplanting WT ovaries to HSD17B1TG females. The data thus provide evidence for a connection between fetal androgen production, dysregulation of HSD17B1 expression in the ovaries and the development of benign serous cystadenomas. More specifically, the data indicate that increased T concentration is required locally in the ovaries during fetal life to induce the development of ovarian benign serous cystadenomas later in life in mice, and one way to induce masculinization is the overexpression of HSD17B1 during fetal life. Developmentally, the ovarian surface epithelium is derived from the embryonic coelomic epithelium in the presumptive gonadal area, which also gives rise to the Müllerian ducts (Auersperg et al. 2001, Auersperg et al. 2002). Starting at about the tenth week of gestation and continuing until the fifth month of human gestation, the human fetal ovarian surface epithelium changes from a flat-to-cuboidal simple epithelium with a fragmentary basement

membrane to a multistratified, papillary epithelium on a well-defined basement membrane, but it reverts to a monolayer by term. It has been postulated that the growth signals for the fetal ovarian surface epithelium include intragonadal steroid hormones (Auersperg et al. 2001). In mice, the surface epithelium encloses the ovary by E15.5 (Kaufman and Bard. 1999). Interestingly, androgen exposure during the second half of pregnancy in our mouse model seems to program adult HSD17B1TG ovaries to develop benign serous cystadenomas. However, further cancer progression was not observed, suggesting that other factors in addition to human HSD17B1 are required for malignant transformation of the ovarian surface epithelium.

It has been hypothesized that the pathogenesis of ovarian cancer could be related to the role of androgens in stimulating epithelial cell proliferation (Risch. 1998), but a clear connection has not been demonstrated (Olsen et al. 2008). In experimental animals, a dysfunctional ovary, which is a definite risk factor for ovarian surface epithelial invagination, inclusion cysts, and further cancer develoment, can be caused by prenatal androgen exposure (Vanderhyden. 2005, Padmanabhan et al. 2006). Furthermore, benign serous cystadenomas have been induced in guineapigs with T (Silva et al. 1997). Fetal masculinization has not been directly linked to ovarian surface epithelial pathologies in humans, but ovarian surface epithelium-lined surface invaginations and epithelial inclusion cysts, which are considered as a starting site for ovarian carcinogenesis, are more frequent in PCOS patients than in healthy individuals (Auersperg et al. 2001). In a recent study, it was found that ovarian serous borderline tumors were positively associated with a history of PCOS, thus, with a history of (fetal) hyperandrogenism (Olsen et al. 2008). HSD17B1 expression has been linked to ovarian epithelial cancers and was shown to positively correlate with an increasing malignancy of ovarian surface epithelial tumors (Sasano et al. 1996, Blomquist et al. 2002, Chura et al. 2009). Thus, both estrogens and androgens have been associated with ovarian epithelial carcinogenesis (Syed et al. 2001, Ho. 2003). Therefore, HSD17B1 may promote ovarian carcinogenesis via increasing estrogen concentration, but as suggested by the present study, also via enhanced androgen production. The present study also provides further evidence for the putative role of androgens in ovarian carcinogenesis.

6.3 ROLE OF HUMAN HSD17B1 IN ESTROGEN-DEPENDENT DISEASES AND AS DRUG TARGET

6.3.1 ENDOMETRIAL HYPERPLASIA AS A DISEASE INDICATION FOR HSD17B1 INHIBITORS

Endometrial hyperplasia is a precursor of endometrial carcinoma and is highly dependent on the estrogen/progestin balance. Thus, both increased estrogen and decreased progestin concentrations can induce endometrial hyperplasia and cancer. This type of hormonal imbalance could be caused, for example, during estrogen replacement therapy or tamoxifen treatments, both of which increase the risk of endometrial carcinoma (Kelsey et al. 1982, Sherman. 2000, Schneider. 2002). Most endometrial carcinomas occur postmenopausally, but obese women with increased estrogen production in the adipose tissue, and PCOS patients with enhanced steroid biosynthesis and anovulatory menstrual cycles, have an

increased risk of developing endometrial carcinoma during reproductive years (Gallup and Stock. 1984). The correlation between serum E2 levels and the risk of endometrial cancer is controversial (Vermeulen-Meiners et al. 1986, Potischman et al. 1996, Berstein et al. 2003) and increased intratissular E2 concentrations are considered a more definite risk factor (Vermeulen-Meiners et al. 1986, Berstein et al. 2003). Progestins antagonize estrogen-mediated cell proliferation in the endometrium (Graham and Clarke. 1997, Wheeler et al. 2007) and are, therefore, widely included in HRT to decrease the risk of endometrial hyperplasia and cancer (Beresford et al. 1997, Feeley and Wells. 2001).

We showed that overexpression of human HSD17B1 in mice enhances estrogen action in the uterus and, in combination with persistent anovulation, causes endometrial hyperplasia, ranging from simple to complex hyperplasia with atypia. However, endometrial carcinomas were not observed in HSD17B1TG mice, indicating that other mechanisms, such as phosphatase and tensin homolog (PTEN) inactivation, loss of forkhead box O subclass transcription factor 1 (FOXO1), and hyperactivity of the PI3K pathway, are important pathways to endometrial carcinogenesis (Goto et al. 2008). Endometrial hyperplasia in HSD17B1TG mice closely resembled human disease and was efficiently reversed upon normalization of the estrogen/progestin ratio in response to either ovulation induction or exogenous progestins. Treatment with Inhibitor 2 also restored endometrial glandular morphology but incompletely blocked proliferation of luminal epithelial cells. The compound also often caused an endometrial inflammatory response for reasons as yet unclear. However, ovulation was not induced by the treatment. One explanation for this could be the failure in the programming of the HPGA, known to be induced by abnormal concentrations of estrogen and androgens during fetal development and after birth (Rosa-E-Silva et al. 2003, Kato et al. 2003, Robinson. 2006, Nakamura et al. 2008), and the consequent lack of LH surge, which would normally induce ovulation. Furthermore, it was shown in a recent study that fetal androgen excess disturbs E2 negative feedback, whereas E2 positive feedback is reprogrammed by estrogen excess during fetal life or early in life (Veiga-Lopez et al. 2009). This is in line with our data showing that the anovulation in HSD17B1TG females was caused by a lack of LH surge, which was not rescued by prenatal antiandrogen treatment and thus is likely to be an estrogen-dependent disorder.

Expression of HSD17B1 in the human endometrium and endometrial cancer is contentious, with some studies reporting expression at mRNA or protein levels and others not (Marovitz et al. 1980, Maentausta et al. 1990, Maentausta et al. 1991, Maentausta et al. 1992, Martel et al. 1992, Casey et al. 1994, Miettinen et al. 1996, Zeitoun et al. 1998, Utsunomiya et al. 2001, Utsunomiya et al. 2003Kasai et al. 2004, Smuc et al. 2006, Dassen et al. 2007, Fechner et al. 2007). In addition to HSD17B1, various other estrogen-metabolizing enzymes including HSD17B2, HSD17B5, HSD17B7 and HSD17B12, aromatase, STS and EST, are differently expressed in the endometrium under different pathological conditions, such as endometrial cancer (Smuc et al. 2006, Rizner et al. 2006), endometriosis (Dassen et al. 2007, Delvoux et al. 2009), and PCOS (Leon et al. 2008, Bacallao et al. 2008) and the various disease subtypes. In our studies, HSD17B1 was detectable by immunohistochemistry in the normal endometrium, atypical endometrial hyperplasia and endometrioid adenocarcinoma, which is in line with previous studies (Smuc et al. 2006). HSD17B1 predominantly, but not exclusively, localized to endometrial epithelial cells. Expression levels were lower in endometrial hyperplasia and cancer as compared with the normal endometrium, which

possibly points towards a compensatory mechanism aimed at limiting local estrogen action. However, HSD17B1 is very effective enzyme and is likely to contribute to endometrial estrogen production even when expressed at a low level. Our data and a literature analysis suggest that HSD17B1 is not the main enzyme in the estrogen production of the normal and malignant endometrium, but nevertheless, the enzyme is expressed in both tissues. Furthermore, studies indicate that oxidative activity is higher in the endometrium than reductive activity, thus, estrogen inactivation is higher than synthesis (Delvoux et al. 2007, Delvoux et al. 2009). However, significant reductive activity has been detected in the human endometrium (Delvoux et al. 2007, Delvoux et al. 2009) and inhibition of the reductive activity would further decrease the estrogen synthesis/inactivation ratio, thus decreasing local estrogen concentration and consequently estrogen-induced proliferation. Recent studies in endometrial and cervical cancer cell lines indicate that reductive activity can be inhibited by using the inhibitors of HSD17B1, HSD17B5, HSD17B7 and HSD17B12, indicating that all four enzymes contribute to estrogen synthesis in the cancer cell lines. However, full inhibition was not obtained even when using a combination of the inhibitors, indicating that there are still other HSD17B(s) participating in endometrial estrogen synthesis (Fournier and Poirier. 2009). Thus, the combination of the activities of these enzymes ultimately determines the hormonal status of the endometrium and the drug combination to be used. Collectively, the data suggest that HSD17B1 inhibition is one of several possible approaches to reduce endometrial estrogen production.

6.3.2 LOCAL ESTROGEN BIOSYNTHESIS AND ITS PREVENTION IN BONE

Estrogens have an important role in the maintenance of bone, and estrogen deprivation is associated with bone loss, osteoporosis, and tall stature (Simpson. 2000, Pietschmann et al. 2008, Folkestad et al. 2009). The loss of aromatase activity or ESR1 results in bone loss in mice and humans (Smith et al. 1994, Windahl et al. 1999, Simpson. 2000, Vidal et al. 2000, Oz et al. 2000, Lindberg et al. 2001, Peng et al. 2004). Furthermore, the presence of estrogen-metabolizing enzymes in human and rodent bone suggests a role for local regulation of estrogen concentration in bone (Sasano et al. 1997, Van Der Eerden et al. 2002, van der Eerden et al. 2004). *In vivo* and/or clinical studies on the role of aromatase (Oz et al. 2000, Oz et al. 2001, Peng et al. 2004, Dunkel. 2009, Folkestad et al. 2009) and HSD17B2 (Shen et al. 2008, Bagi et al. 2008) in bone are available, but the role of HSD17B1 has not been previously studied. Thus, in the present study, we demonstrated that overexpression of human HSD17B1 in female TG mice resulted in increased cortical BMD and BMC. Similarly, universal overexpression of aromatase in Arom+ mice and local overexpression of aromatase in bone in Coll-1α1-Arom mice has been shown to result in increased BMD, but only in male mice, and the authors hypothesized that due to a lack of androgenic substrates, aromatase has a less pronounced role in female than male bone (Peng et al. 2004, Sjögren et al. 2009).

Interestingly, long term treatments of HSD17B1TG females with two different human HSD17B1 -specific inhibitors did not affect pQCT parameters, and thus, for example BMD and BMC were unaltered after the inhibitor treatments in HSD17B1TG females. The effect of Inhibitor 2 on WT female bone was also tested, and no differences were found compared to untreated WT (data not shown). Aromatase inhibitors are in clinical use in the treatment of breast cancer and endometriosis, but several studies have indicated that their use results

in bone loss, and resistance to these drugs is universally obtained (Urruticoechea. 2007, Perez. 2007, Pant and Shapiro. 2008, Folkestad et al. 2009, Hadji. 2009). The role of human HSD17B1 in breast cancer and endometriosis has been acknowledged, and it is considered a promising drug target (Gunnarsson et al. 2001, Gunnarsson et al. 2003, Oduwole et al. 2004, Gunnarsson et al. 2005, Dassen et al. 2007, Smuc et al. 2007, Gunnarsson et al. 2008, Day et al. 2008b, Day et al. 2008a, Smuc et al. 2008, Delvoux et al. 2009, Nagasaki et al. 2009). Thus, HSD17B1 inhibitors are likely to compete with aromatase inhibitors in the treatment of breast cancer and endometriosis in the future, and it would be a big advantage if HSD17B1 inhibitors had no effect on BMD and BMC, as is suggested by our data. Further *in vivo* /clinical studies are needed to confirm our results.

In humans, aromatase deficiency in men is associated with tall stature despite normal T concentration, indicating a role for aromatase in the regulation of bone length (Simpson. 2000), and aromatase inhibitors have been successfully clinically tested in the treatment of premature growth plate fusion in humans (Dunkel. 2009). The role of estrogens in the regulation of bone length in mice is different from in humans, as suggested by studies in genetically modified mice, indicating that both exposure to and lack of estrogens can result in bone shortening (Vidal et al. 1999, Vidal et al. 2000, Oz et al. 2001, Peng et al. 2004). Interestingly, the tibias of HSD17B1TG females were significantly shorter than in WT mice. Moreover, despite the fact that HSD17B1 inhibitors did not affect pQCT parameters, treatment with Inhibitor 3 significantly increased bone length in HSD17B1TG females, and the same tendency was obtained with Inhibitor 2. No effect on the length of WT bone was observed (data not shown). The data suggest that HSD17B1-mediated local estrogen action regulates bone length even under ovarian E2 production in female mice, and the effect can be reversed by HSD17B1 inhibitor treatment, while the effect on BMD and BMC was not reversible. There are as yet no studies about the effect of inactivating HSD17B1 mutations on human or mouse bone, and thus, the relevance of HSD17B1 in the regulation of bone length in humans remains to be studied further. However, our data suggest that diseases related to improper growth are a promising new indication for HSD17B1 inhibitors.

Human HSD17B1 and HSD17B2 are simultaneously expressed in several tissues, for example, in the normal endometrium and breast, and the enzymes have opposite activities in cultured cells (Miettinen et al. 1996). It has been hypothesized that the relative activity of these enzymes could locally regulate the amount of E2 in target cells, but the hypothesis has not been previously tested in bone. In a recent study, overexpression of human HSD17B2 in TG mice has been shown to dramatically decrease the amount, strength and length of bone (Shen et al. 2008). In line with this, treatment of cynomolgus monkeys with HSD17B2 inhibitors maintained bone formation and bone strength after ovariectomy (Bagi et al. 2008). On the other hand, recent studies have also revealed that human HSD17B2 is involved in sex steroid -independent pathways with only moderate modulation of sex steroids (Zhongyi et al. 2007, Rantakari et al. 2008). In the present study, we showed that simultaneous overexpression of both human HSD17B1 and HSD17B2 under the same promoter in bi-TG mice did not rescue either phenotype, but instead resulted in a bone phenotype resembling that observed in HSD17B2TG mice (Shen et al. 2008). The data suggest that HSD17B1 and HSD17B2 act along different pathways in bone, or alternatively, that the effect of HSD17B2 on bone estrogen metabolism is much greater that that of HSD17B1.

7 SUMMARY AND CONCLUSIONS

As has been acknowledged for a long time, human HSD17B1 is an estrogen-specific enzyme that participates in estrogen biosynthesis. It is also expressed in some extragonadal estrogen target tissues, and increased expression of the enzyme in breast cancer and endometriosis leads to accumulation of E2 in the diseased tissues. Some studies have indicated that the enzyme may also have androgenic activity, but these have been ignored. Although the function of human HSD17B1 *in vivo* is only superficially known, HSD17B1 is considered a promising drug target and, therefore, it is essential to understand the full physiological role of the enzyme. The present study aimed at increasing our understanding of the physiological role of human HSD17B1 using non-clinical TG mouse models and human samples, and to find novel disease indications for HSD17B1 inhibitors. The conclusions are as follows:

I) Human HSD17B1 is not an estrogen-specific enzyme. It has significant reductive activity towards androgens *in vivo*. Universal overexpression of human HSD17B1 in TG mice resulted in enhanced androgen production during fetal life, which further led to several androgen-dependent disorders in HSD17B1TG mice. These included female DSD (increased AGD, suppressed nipple development, lack of vaginal opening, combination of vagina with urethra, enlarged Skene paraurethral gland and enlarged Wolffian duct remnants in the mesovarium) and ovarian benign serous cyctadenomas during adult life.

II) Fetal androgen exposure has been linked to PCOS and metabolic syndrome during adulthood in experimental animals and humans, but the genes involved in PCOS are largely unknown. A putative mechanism to accumulate androgens during fetal life by HSD17B1 overexpression was shown in the present study.

III) Benign serous cystadenomas found in HSD17B1TG females are considered to be precursors of low-grade ovarian serous tumors. The results, together with the literature analysis, suggest that HSD17B1 has a role in ovarian epithelial carcinogenesis, and especially in the development of serous tumors. Estrogens have been shown to promote ovarian epithelial carcinogenesis, but the role of androgens is considered controversial. However, androgens have been positively shown to associate with serous epithelial tumors in PCOS patients, who often have a history of (fetal) hyperandrogenism. The present study provides evidence for the putative role of androgens in ovarian carcinogenesis, and directly links HSD17B1-induced prenatal androgen exposure to ovarian epithelial carcinogenesis.

IV) Our data and literature analysis suggest that HSD17B1 is not the main enzyme in the estrogen production of the normal and malignant human endometrium, but nevertheless, the enzyme is expressed in both tissues, and its overexpression resulted in endometrial hyperplasia in mice. Furthermore, treatment with a HSD17B1 inhibitor reversed the endometrial hyperplasia in HSD17B1TG mice. Collectively, the data and literature analysis suggest that HSD17B1 inhibition is one of several possible approaches to decrease endometrial estrogen production in endometrial hyperplasia and cancer.

V) HSD17B1 expression has been found in the bones of humans and rats. The data of the present study suggest that human HSD17B1 is likely to have an important role in the regulation of bone formation, strength and length during the female reproductive years. Bone density in HSD17B1TG females was highly affected in femurs, but, in lesser amounts, also in tibias. Especially the tibia growth plate, but not other regions of bone, was susceptible to respond to HSD17B1 inhibition, while the inhibitors did not affect bone density. Thus, HSD17B1 inhibitors could be safer than aromatase inhibitors in regard to bone in the treatment of breast cancer and endometriosis. Furthermore, diseases related to improper growth are a promising new indication for HSD17B1 inhibitors.

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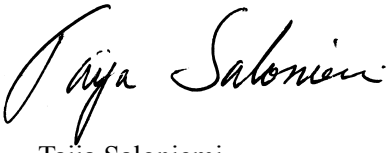

Taija Saloniemi

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