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HYDROXYSTEROID (17BETA) DEHYDROGENASE 1 EXPRESSION ENHANCES ESTROGEN ACTION AND SERVES AS A POTENTIAL DRUG TARGET FOR REDUCING ESTROGEN SIGNALING IN THE UTERUS AND MAMMARY GLANDS

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ABSTRACT

Päivi Järvensivu

Hydroxysteroid (17beta) dehydrogenase 1 expression enhances estrogen action and serves as a potential drug target for reducing estrogen signaling in the uterus and mammary glands

University of Turku, Faculty of Medicine, Institute of Biomedicine, Physiology, Drug Research Doctoral Programme (DRDP)
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Hydroxysteroid (17beta) dehydrogenase type 1 (HSD17B1) is a steroid-metabolizing enzyme with a preface for converting the low active estrogen estrone (E1) to highly active estradiol (E2). Accordingly, HSD17B1 is expressed particularly in E2-producing tissues such as the human placenta and ovaries. Moreover, HSD17B1 is expressed in peripheral estrogen target tissues such as the breast and uterus, where it controls local intratissue E2 concentration. Exposure to elevated concentrations of estrogens is associated with increased risk of several diseases, including breast cancer, which is the most common cancer in women worldwide. To investigate the significance of HSD17B1 in steroid production *in vivo* and in estrogen-dependent diseases, transgenic mice universally expressing human HSD17B1 enzyme (HSD17B1TG mice) were used. These mice showed increased peripheral conversion of E1 to E2 in a variety of tissues, including the uterus and mammary glands. Female HSD17B1TG mice developed endometrial hyperplasia, a precursor to endometrial carcinoma. The hyperplasia was reversed by ovulation induction, while initially the HSD17B1TG females failed to ovulate. Treatment with an HSD17B1 inhibitor also partly reversed the hyperplastic morphology. The HSD17B1TG females developed mammary cancer at older age. Mammary gland epithelial restricted HSD17B1 expression formed mammary lesions with a disrupted myoepithelial cell layer and inflammatory cell infiltration that was reduced by blocking estrogen receptor signaling with an antiestrogen, ICI 182, 780. The HSD17B1TG mice were also successfully used as a preclinical model for screening the efficacy of HSD17B1 inhibitors *in vivo* by crossing HSD17B1TG mice with estrogen reporter mice (ERELuc mice). In these bi-TG mice, both an immature uterus growth response and estrogen receptor activity were used as readouts, and both were reduced by HSD17B1 inhibitor treatment. These studies elucidate the potential of HSD17B1 enzyme to enhance the action of E1 in peripheral tissues, thus, indicating that inhibition of HSD17B1 is a plausible approach to treating estrogen-dependent diseases.

Keywords: HSD17B1, estrogen, endometrial hyperplasia, mammary cancer

TIIVISTELMÄ

Päivi Järvensivu

Hydroksisteroidi (17beta) dehydrogenaasi 1 ilmentyminen lisää estrogeeniaktivaatiota ja toimii potentiaalisena lääkekohteena vähentämällä estrogeenisignalointia kohdussa ja maitorauhasissa

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Fysiologia, Lääketutkimuksen tohtoriohjelma (DRDP)
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Hydroksisteroidi (17beta) dehydrogenaasi tyyppi 1 (HSD17B1) on steroideja metaboloiva entsyymi, joka pääasiassa muuttaa vähemmän aktiivista estrogeeniä, estronia (E1), biologisesti aktiivisemmaksi estradioliksi (E2). Ihmisillä HSD17B1 ilmentyy erityisesti estrogeenejä tuottavissa kudoksissa kuten ihmisen istukassa ja munasarjoissa, joissa se osallistuu estradiolin tuotantoon. Lisäksi HSD17B1 ilmentyy perifeerisissä estrogeenin kohdekudoksissa, kuten rinnassa ja kohdussa, joissa se säätelee paikallista kudoksen sisäistä E2-konsentraatiota. Estrogeenien kohonnut altistus on liitetty useiden sairauksien kohonneeseen riskiin kuten rintasyöpään, joka on naisten yleisin syöpä maailmanlaajuisesti. HSD17B1-entsyymin merkityksen tutkimiseksi käytettiin muuntogeenisiä hiiriä, jotka tuottavat ihmisen HSD17B1-entsyymiä (HSD17B1TG-hiiret). Näillä hiirillä on lisääntynyt perifeerinen E1:n muuntuminen E2:ksi useissa kudoksissa, kuten kohdussa ja maitorauhasessa. HSD17B1TG-naarashiirille kehittyi kohdun limakalvon liikakasvua, joka on kohtusyövän esiaste. Lisäksi HSD17B1TG-hiiret eivät ovuloineet ja indusoimalla ovulaatio myös kohdun limakalvon liikakasvu korjautui. HSD17B1-inhibiittorihoito lievensi kohdun limakalvon liikakasvua. HSD17B1TG-naaraille kehittyi myös vanhemmalla iällä maitorauhassyöpiä. Maitorauhaseen rajattu HSD17B1:n ilmentyminen aiheutti maitorauhasleesioita, joissa epiteelisolujen proliferaatio oli lisääntynyt, tiehyiden myoepiteelikerros oli hajonnut ja niihin oli kertynyt inflammatorisia soluja. ICI 182,780, antiestrogeenihoito, vähensi leesioiden määrää. HSD17B1-inhibiittoreiden testaamiseksi *in vivo* kehitettiin uudenlainen hiirimalli, jossa HSD17B1TG-hiiret risteytettiin estrogeeni-reseptorin aktivoitumista raportoivaan hiireen (ERELuc-hiiret). Näissä kaksoissiirtogeenisissä hiirissä esipubertaalisen kohdun kasvua ja lusiferaasiaktiivisuutta käytettiin vasteena ja HSD17B1-inhibiittorihoitolla nämä vasteet vähenivät. Nämä tutkimukset vahvistavat, että HSD17B1-entsyymin ylituotto lisää E1:n vaikutusta perifeerisissä kudoksissa, joten HSD17B1:n esto voisi olla uudenlainen lähestymistapa kohtu- ja rintasyövän hoidossa.

Avainsanat: HSD17B1, estrogeeni, kohdun limakalvon liikakasvu, rintasyöpä

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ABBREVIATIONS

A-dione	androstenedione
cAMP	cyclic adenosinemonophosphate
AP-1	activator protein 1
DAB	3,3´-diaminobenzidine
DCIS	ductal carcinoma in situ
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
E1	estrone
E2	estradiol
E3	estriol
EGF	epidermal growth factor
ERE	estrogen responsive element
ESR	estrogen receptor
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
HE	hematoxylin-eosin
HSD17B	hydroxysteroid (17beta) dehydrogenase
HSD3B	hydroxysteroid (3beta) dehydrogenase
HPLC	high performance liquid chromatography
IDC	intraductal carcinoma
IGF	insulin-like growth factor
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
miR	microRNA
MMTV	mouse mammary tumor virus
NAD	nicotinamide adenine dinucleotide
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen

PMSG	pregnant mare serum gonadotrophin
PTEN	phosphate and tensin homolog
s.c.	subcutaneous
SDR	short-chain dehydrogenase/reductase
SEM	standard error of the mean
SF1	steroidogenic factor 1
SP-1	trans-acting transcription factor 1
STS	steroid sulfatase
TLDU	terminal ductal lobular unit
TEB	terminal end bud
TGFalpha	tumor necrosis factor alpha
TG	transgenic
WT	wild type

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to using Roman numerals (I-III) in the text.

I. Saloniemi T, **Järvensivu P**, Koskimies P, Jokela H, Lamminen T, Ghaem-Maghami S, Dina R, Damdimopoulou P, Mäkelä S, Perheentupa A, Kujari H, Brosens J, Poutanen M. Novel hydroxysteroid (17beta) dehydrogenase 1 inhibitors reverse estrogen induced endometrial hyperplasia in transgenic mice.
Am J Pathol. 2010 Mar;176(3):1443-51.

II. **Järvensivu P**, Saloniemi-Heinonen T, Awosanya M, Koskimies P, Saarinen N, Poutanen M. HSD17B1 expression enhances estrogen signaling stimulated by the low active estrone, evidenced by an estrogen responsive element-driven re-porter gene *in vivo*.
Chem Biol Interact. 2015 Jun 5;234:126- 34.

III. **Järvensivu P***, Heinosalo T*, Hakkarainen J, Kronqvist P, Saarinen N, Poutanen M. HSD17B1 expression induces inflammation-aided rupture of mammary gland epithelium.
Revised manuscript. *Equal contribution

1 INTRODUCTION

Breast cancer is the most common cancer and corpus uterine cancer is the fourth common cancer in women according to Finnish Cancer Registry (http://stats.cancerregistry.fi/stats/eng/veng0021i0.html). Breast cancer is the leading cause of cancer-related death among females worldwide. The highest breast cancer incidence is in high-income countries that reflects the use of breast cancer screening as well as higher prevalence of breast cancer risk factors (Torre et al., 2016). Particularly, the incidence of estrogen receptor -positive breast cancer is rising that suggest an increase in hormonal risk factors (Dall and Britt, 2017). However, the breast cancer mortality rates have been decreasing in many countries, as a consequence of early detection and improved treatment (Torre et al., 2016). The most common gynaecological cancer in developed countries is endometrial cancer and the incidence is rising at the moment (Siegel et al., 2016). The majority of risk factors that increase the prevalence of endometrial cancer are related to exposure of excess estrogens (Colombo et al., 2016). Several studies have shown the association of estrogens with the pathogenesis and growth of breast and endometrial cancers in women (Bulun and Simpson, 2008; Chuffa et al., 2017; Dall and Britt, 2017). Estrogens also play a role in the etiology of various other diseases in women, including endometriosis, which affects up to 10% of premenopausal women, causing severe pain and infertility (Bulun, 2009; Giudice, 2010).

The classical endocrine organs, such as the ovary, synthesize estradiol (E2) and distribute the estrogenic E2 to all tissues throughout the body by blood circulation. In the mechanism of intracrinology each tissue is able to synthesize *i.e.* estrogens locally where they are acting without biologically significant release into the circulation. These tissue-specific enzymes control the amount of active sex steroids in peripheral tissues to meet the specific need of each tissue, without affecting to other tissues (Labrie, 2015). Intratissue metabolism and synthesis of estrogens, regulated by the activities of various enzymes, are considered to play an important role in the pathogenesis and development of hormone-dependent diseases such as uterine and breast cancers (He et al., 2016; Hilborn et al., 2017a; Simpson et al., 2000).

The objective of this study was to enhance the general understanding of the role of HSD17B1 enzyme, particularly in uterine and breast carcinogenesis. The approach chosen was to generate and apply transgenic mice expressing human HSD17B1 and to validate the model for HSD17B1 inhibitor efficacy testing. The study provides preclinical evidence of a putative use of HSD17B1 inhibitors as a treatment for estrogen-dependent diseases.

2 REVIEW OF LITERATURE

2.1 Estrogens

Estrogens are female sex hormones with various physiological functions in the human body. They are responsible for the development and regulation of the female reproductive system and secondary sex characteristics. The most potent endogenously produced estrogen is 17beta-estradiol (E2). In addition, there are weaker endogenous estrogens estrone (E1) and estriol (E3) (Coelingh Bennink, 2004; Lipovka and Konhilas, 2016). E2 is the predominant estrogen during reproductive years in women and is primarily secreted by ovaries. After the suppression of ovarian function in menopause, the main physiological estrogen is E1 which is synthesized in peripheral tissues from dehydroepiandrosterone (DHEA) produced by the adrenals. E3 is specially produced by the placenta during pregnancy (Coelingh Bennink, 2004). Estrogen biosynthesis in the ovary is regulated by the cyclic release of gonadotrophins from the anterior pituitary in premenopausal women. During pregnancy, E2 is involved in uterine growth, placental development, parturition, and the development of the mammary glands (Coelingh Bennink, 2004; Gruber et al., 2002). E2 affects cell proliferation, immunity, inflammatory response, cardiovascular factors, bone integrity, cognition and behavior (Castoria et al., 2010).

These physiological effects of estrogen are mainly mediated by classical estrogen receptors alpha (ESR1) and beta (ESR2) (Lipovka and Konhilas, 2016; Pearce and Jordan, 2004). The ESRs are members of the nuclear receptor superfamily of transcription factors. In addition, this family comprise thyroid hormone receptor, vitamin D receptor, retinoic acid receptors, aryl hydrocarbon receptor, orphan receptors and other steroid receptors such as the glucocorticoid receptor, androgen receptor, progesterone receptor and mineralocorticoid receptor (Hernández-Ochoa et al., 2009; Pearce and Jordan, 2004; Yen, 2015). The ESRs are steroid hormone receptors that modulate the transcription of target genes when a ligand is bound. The activity of these transcription factors is regulated by ligand binding but also by post-translational modifications, including phosphorylation and, interaction with coregulators (Pearce and Jordan, 2004).

In the classical model of the estrogen response, estrogens diffuse across the cell membranes and nuclear membranes and bind to ESRs primarily in the nucleus, but also in cytoplasm, which results in a conformational change in the receptor-complex and transforms it into a state that enables dimerization (Lipovka and Konhilas, 2016). Estrogen binds to the ligand-binding domain of the receptor, which dissociates from cytoplasmic chaperones, and an estrogen-receptor-

complex is formed (Gruber et al., 2002). In the nucleus, these receptor complexes bind to specific sequences of DNA called estrogen-response elements (EREs) as homodimers or heterodimers with nuclear-receptor coactivators or repressors. The ligand-ERE-bound receptor complex then employs coregulator molecules, such as FoxA1, leading to modulation of the transcription rates of the responding genes (Hewitt et al., 2016).

The classical transcriptional pathway results in modulation of gene transcription by binding directly to promoters of target genes. In the non-classical pathway, the activated ESR does not bind directly to DNA but instead forms protein-protein interactions with other transcription factors (Hewitt et al., 2016). Moreover, the rapid effects of E2 are mediated through non-ESR signaling. The receptors involved in this type of signaling are the G-protein-coupled estrogen receptor 1 (GPER1, originally GPR30) and possibly certain variants of ESR1 and ESR2 (Lipovka and Konhilas, 2016; Revankar et al., 2005). In the ligand-independent activation of ESRs, ESR1-mediated gene expression is activated via mitogen-activated protein kinase by peptide growth factors in the absence of E2 (Hewitt et al., 2016).

The ESRs are composed of five functional domains, an N-terminal domain (NTD) or A/B domain, the DNA-binding (DBD or C) domain, a hinge (D) region, ligand-binding domain LBD (LBD or E) and a C terminal domain (Hewitt et al., 2016). The main functional domains in classic steroid receptor signaling are the NTD, DBD and LDB (Lipovka and Konhilas, 2016). The DBD domain binds to the EREs and modulate recruitment of coregulators. The transcriptional activity of ESRs are regulated by two activation function (AF) domains, AF-1 and AF-2, located in NTD and LBD respectively (Lipovka and Konhilas, 2016). These domains synergize via the recruitment of coregulator proteins, particularly members of the p160 family (Hewitt et al., 2016; Pearce and Jordan, 2004). E2 can also influence the expression of genes that do not contain EREs in their promoter region, for example, ESRs can interact with other transcription factors such as Sp1 (Lipovka and Konhilas, 2016) and AP-1 (Dahlman-Wright et al., 2012).

ESR1 and ESR2 are encoded by two different genes located in different chromosomes (Lipovka and Konhilas, 2016). The structures of ESR1 and ESR2 are different as ESR1 has both AF-1 and AF-2 functional domains, whereas ESR2 lacks the AF-1 domain (Pearce and Jordan, 2004). Ligand-dependent transcriptional activation is mediated through AF-2, whereas AF-1 regulates ligand-independent activation of ESRs (Heldring et al., 2007). In addition to the full-length 66 kD of ESR1, two other isoforms have been characterized of this receptor; a 46 kD splice variant ESR1-46 and a 36 kD ESR1-36 (Sołtysik and Czekaj, 2015). The ESR1-46 receptor variant only lacks the functional domain AF-

1, and is otherwise completely identical to the full-length ESR1. The function of ESR1-46 is not exactly known, but it may have significance in cancer progression (Chantalat et al., 2016). The other splice variant ESR1-36 lacks both functional domains, AF-1 and A-F2, but have an intact DNA-binding domain and a truncated ligand-binding and a partial dimerization domain. ESR1-36 is reported to be predominantly associated with the plasma membrane and mainly mediates non-genomic estrogen signaling (Yan et al., 2017). It is postulated that ESR1-36 inhibit transcriptional activity of other ESRs and would be a negative regulator of hormone-independent transcription (Sołtysik and Czekaj, 2015). ESR2 has multiple isoforms resulting from alternative splicing of the last coding exon (ESR2-2, ESR2-3, ESR2-4 and ESR2-5) (Moore et al., 1998). Only the ESR2-1 variant is fully functional, whereas the ESR2-2, ESR2-4 and ESR2-5 isoforms modulate receptor activity when bound to ESR2-1 (Leung et al., 2006). The effect of ESR1, ESR2 and their isoforms differ by having distinct tissue distributions and signaling responses (Ramsey et al., 2004). The majority of the ESR2 isoforms promote E2 signaling, but some isoforms can act as inhibitors of ESR1 signaling (Matthews et al., 2006).

2.1.1 Estrogen effects in mammary glands

The maturation of the mammary gland epithelial tree is initiated by the ovarian steroids estrogen and progesterone in puberty. At the onset of puberty, the ducts grow from the nipple to the surrounding fat pad located under the skin. The tips of the ducts enlarge to form specialized cub-shaped structures called terminal end buds (TEBs), which contain highly proliferative cells and are responsible for ductal elongation (Howlin et al., 2006; Mallepell et al., 2006). The TEB is composed of cap cells, which differentiate into myoepithelial cells and body cells; in turn, these cells become ductal cells. Mature ducts and alveoli feature three different layers: the epithelial cell layer, the myoepithelial cell layer and the basement membrane layer, which surrounds the previous two layers (Howlin et al., 2006). Other hormones needed for mammary gland development and normal function are progesterone and prolactin. Progesterone and progesterone receptor in the epithelium are required for ductal side-branching and alveolar growth (Brisken et al., 1998; Lydon et al., 1995). Prolactin signaling in the epithelium is also required for lobuloalveolar development and for functional alveolar cell differentiation, during lactation (Brisken et al., 1999).

Estrogen triggers ductal elongation during puberty, which is mediated by both stromal and epithelial ESRs (Mueller et al., 2002). Gene knockout studies have demonstrated that the presence of ESR1 in mammary epithelium and stroma, but

not of ESR2, is necessary for the postpubertal development of the female mouse mammary glands (Korach et al., 1996; Krege et al., 1998; Mallepell et al., 2006); thus, the mammary glands of ESR1 knockout females have only a primitive ductal rudiment tree that does not develop after puberty (Korach et al., 1996). Deletion of ESR2 in female mice was shown to result in normal mammary gland development (Krege et al., 1998). These findings indicate that ductal elongation and proliferation is dependent of the presence of ESR1 not ESR2 in the mammary epithelium and stroma (Mallepell et al., 2006).

In human breast tissue, both ESR1 and ESR2 are expressed in ducts and alveoli (Huang et al., 2015). The density of ESRs in breast tissue is highest in the follicular phase of the menstrual cycle and declines after ovulation (Söderqvist et al., 1993). ESR1 is mainly detected in the nuclei and to a lesser extent in the cytoplasm of epithelial cells of intralobular acini and interlobular ducts. In addition, certain stromal cells also exhibit ESR1 nuclear staining (Pelletier and El-Alfy, 2000). ESR2 expression is more widespread; in addition to being expressed in epithelial cells, ESR2 is expressed in myoepithelial cells and occasionally in surrounding stromal and endothelial cells and in lymphocytes (Speirs et al., 2002). In postmenopausal women, the expression of ESR1 declines, and expression is detected in less than 10% of normal mammary epithelial cells, while ESR2 is expressed in more than 50% of normal mammary epithelial cells (Cheng et al., 2013).

2.1.2 Estrogen effects in endometrium

The endometrium is the inner mucosal lining of the uterus. It is a target tissue for steroid hormones and is composed of two functional layers: the transient superficial functional layer (stratum functionalis) and the permanent basal layer (stratum basalis) below the functional layer and adjacent to the myometrium. The functional layer consists of the luminal epithelium, the superficial glandular epithelium and stroma and is completely shed and regenerated during the menstrual cycle and after childbirth (Gargett et al., 2008; Hapangama et al., 2015; Jabbour et al., 2006). The basal layer contains the terminal part of the endometrial glands and tightly organized stroma and is not shed during menstrual bleeding or at parturition. After the cessation of ovarian function, the basal layer remains as an atrophic, inactive postmenopausal endometrium (Hapangama et al., 2015).

There are three classical phases of the menstrual cycle: the preovulatory (follicular), postovulatory (secretory) and menstrual phases. The menstrual cycle is controlled by a series of changes in hormone levels; thus, estrogen is responsible for proliferative alterations during the follicular phase of the menstrual cycle and

initiates the formation of a new layer of endometrium (Gargett et al., 2008; Jabbour et al., 2006). Progesterone is responsible for differentiation of the endometrium, making the endometrium receptive to implantation during the secretory phase (Jabbour et al., 2006). Estrogen exerts its effects on the endometrial cells mainly via ESR1; thus, the highest expression of ESR1 occurs in the follicular phase, and the expression of ESR1 declines in the secretory phase (Critchley et al., 2001; Jabbour et al., 2006; Lessey et al., 1988; Snijders et al., 1992). Progesterone is essential for the initiation and maintenance of pregnancy after the estrogen-priming of the endometrium in the follicular phase. Both estrogen and progesterone are critical for successful implantation and the early stages of pregnancy. The hormones initiate gene expression of local growth factors that mediate the effect of steroid hormones in an autocrine/paracrine and even intracrine manner in the endometrium (Critchley and Saunders, 2009; Jabbour et al., 2006). In addition, endometrial estrogen concentrations are actively regulated by local estrogen-metabolizing enzymes within the tissue (Dassen et al., 2007; Delvoux et al., 2009; Huhtinen et al., 2012a), and the tissue concentration does not reflect the corresponding serum levels (Huhtinen et al., 2012b). As an example, in the proliferative phase, the endometrial E2 concentration was observed to be 5–8 times higher than in the serum, whereas in the secretory phase, the tissue E2 concentration was approximately half of that in the serum (Huhtinen et al., 2012b).

The physiological responses of estrogens in the uterus are biphasic, and the responses can be divided into events that occur early, within the first hours after E2 administration (early phase), and subsequent responses that follow 24 h after E2 administration (late phase) (Hewitt et al., 2003). In the early phase, after ESR activation, transcription of early-phase genes such as *c-fos* is initiated, leading to fluid uptake (water imbibition), hyperemia, and infiltration of immune system cells such as macrophages and eosinophils into the uterine tissue (Hewitt et al., 2003; Perez et al., 1996). The late-phase responses are the transcription of the late-phase genes, which affects, for example, lactoferrin production; increase in uterine wet weight; further accumulation of immune system cells and the transition of the epithelial layer into columnar secretory epithelial cells; and subsequent mitosis, which mainly occurs in the epithelial cell layer (Gunin, 2001; Hewitt et al., 2003). ESR1 plays a predominant role in the genomic responses that occur in the rodent uterus; thus, ESR1 is required to mediate the full biochemical and biological uterine response to estrogen (Hewitt et al., 2003; Korach et al., 1996; Winuthayanon et al., 2010).

2.2 Estrogen biosynthesis

In premenopausal women, the principal site for estrogen biosynthesis is the ovary. The major function of the ovary is the production of mature oocytes for fertilization. Oocyte maturation occurs in follicles, which consist of granulosa cells, and the outer layers of thecal cells surrounding the granulosa cells (Hsueh et al., 2015). The primary sources of E2 in the ovary are the granulosa cells of the ovaries and the luteinized derivatives of these cells (Hsueh et al., 2015; Shoham and Schachter, 1996). In the human ovary, follicle development starts before birth; primordial follicles begin to form at 15 weeks of gestation; they transform into primary follicles by 24 weeks of gestation and develop into secondary follicles by 26 weeks of gestation. Antral follicles develop in the third trimester of gestation. At the antral stage, follicle-stimulating hormone (FSH) released by the pituitary gland after puberty induces the progression of few of the follicles into the preovulatory stage. These dominant Graafian follicles are the main source of the ovarian estrogens secreted in women of reproductive age (Hsueh et al., 2015; McGee and Hsueh, 2000). The surviving dominant follicles secrete high levels of estrogens and inhibins, which in turn suppress pituitary FSH release, and the development of the remaining antral follicles is discontinued. Usually, one Graafian follicle is formed each month for mature oocyte release (McGee and Hsueh, 2000). Estrogen production starts in granulosa cells of preovulatory follicles induced by FSH, which up-regulates the expression of aromatase (CYP19A1, cytochrome P450 family 19 subfamily A member 1) and, in turn, converts androgens to estrogens in granulosa cells (Oktem and Urman, 2010; Suzuki et al., 1993). According to the “two-cell” theory of estrogen synthesis, the androgens produced in thecal cells diffuse to the granulosa cells where androgens are aromatized to estrogens (Hillier et al., 1994); however, these two types of cells have the ability to form both androgens and estrogens (Gruber et al., 2002). Estrogen synthesis is directed by FSH, and only small amounts of luteinizing hormone (LH) are needed for follicular estrogen production (Shoham and Schachter, 1996). After ovulation each month, the remaining thecal and granulosa cells undergo transformation, and the Graafian follicle become the corpus luteum (McGee and Hsueh, 2000). In rodents, cyclic recruitment of early antral follicles and selection of dominant follicles are similar to those of humans, with the exception that multiple follicles become dominant during each mouse estrous cycle (McGee and Hsueh, 2000). In mouse, the reproductive cycle is referred to as the estrous cycle, which is commonly divided into four stages: proestrus, estrus, metestrus, and diestrus; the cycle length is 4–5 days (Cora et al., 2015).

The naturally occurring estrogens E1, E2, and E3 are C18 steroids that are synthesized from cholesterol in the ovary in premenopausal women. During the synthesis of estrogens, cholesterol is first metabolized to pregnenolone by

cytochrome P-450 side-chain cleavage enzyme CYP11A1 (cytochrome P450 family 11 subfamily A member 1). Pregnenolone is then converted to progesterone by hydroxysteroid (3beta) dehydrogenase 2 (HSD3B2) or to 17alpha-hydroxy-pregnenolone by CYP17A1 (cytochrome P450 family 17 subfamily A member 1). 17Alpha-hydroxy-pregnenolone is further converted to 17alpha-hydroxy-progesterone by HSD3B2. 17Alpha-hydroxy-progesterone can also be metabolized from CYP17A1-mediated hydroxylation of progesterone. Dehydroepiandrosterone (DHEA) results from CYP17A1-medated conversion of 17alpha-hydroxy-pregenolone. DHEA is metabolized to androstenedione (A-dione) by HSD3B2. In addition, A-dione can result from CYP17A1-mediated oxidation of 17alpha-hydroxy-progesterone. Finally, A-dione is converted to E1 and testosterone and then to E2 by aromatase (CYP19A1). A-dione can be converted to testosterone by HSD17Bs, and E1 is converted to E2 in tissue by HSD17Bs, in particular, HSD17B1 (Baker et al., 2015; Blair, 2010). Estrogen biosynthesis is illustrated in Figure 1.

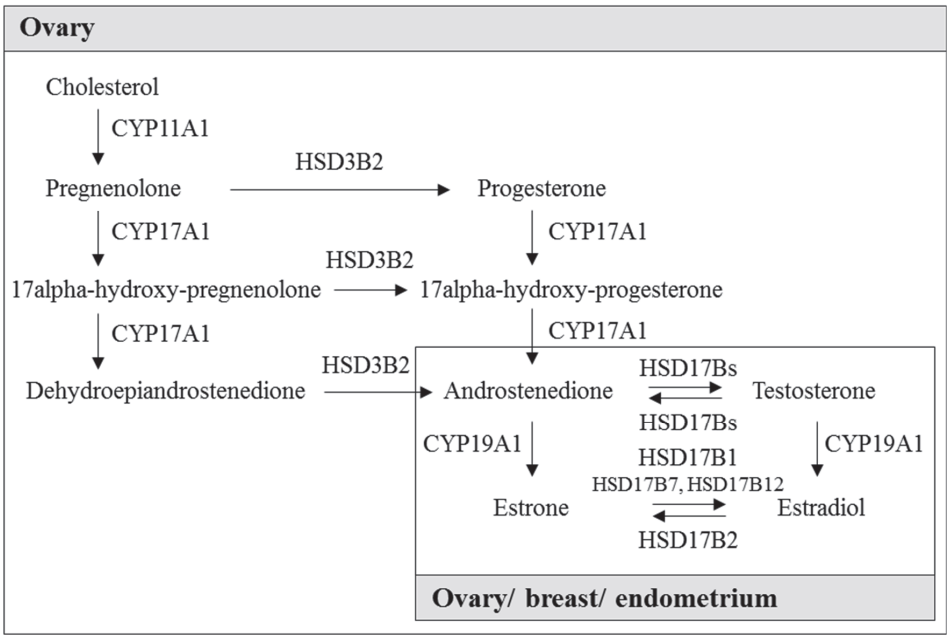

Figure 1. Ovarian estrogen biosynthesis from cholesterol in premenopausal women. The last steps of estrogen biosynthesis (separated by box) can also occur in estrogen target tissues such as the breast and endometrium. HSD17B1 is the main enzyme responsible for E1 conversion to E2; in addition, HSD17B7 and HSD17B12 can be involved in the activation of E1.

2.2.1 Estrogen biosynthesis in postmenopausal women

Although the ovaries are the main source of circulating estrogens in premenopausal women, other peripheral sources of estrogen production exist in the body, and these become the principal sites of estrogen synthesis after the cessation of ovarian function in menopause. These sources are the mesenchymal cells of the adipose tissue, skin, osteoblasts in bone, vascular endothelial and aortic smooth muscle cells, as well as several sites in the brain (Simpson, 2003). These extragonadal sites of estrogen biosynthesis have several critical features that differ from those of the ovaries. Principally, the estrogen synthesized within these tissues, particularly in bone, breast and brain, have significant biological influence locally as they act in a paracrine or intracrine manner (Haynes et al., 2010; Labrie et al., 2000a; Pasqualini et al., 1996; Simpson et al., 2000). Another important feature is that these extragonadal sites are dependent on circulating precursor C19 steroids for synthesizing C18 steroids, such as estrogens. In postmenopausal women, the principal source of C19 steroids is the adrenal cortex, which produces A-dione, DHEA and DHEA sulfate (DHEA-S). However, the secretion of these steroids and their plasma concentrations decrease markedly with advancing age (Labrie et al., 1997). Finally, A-dione, DHEA and DHEA-S are converted to active estrogens in tissue by the enzymes HSD17Bs, steroid sulfatase (STS), aromatase, HSD3B and 5alpha-reductase (SRD5A) (Labrie et al., 2000b; Simpson et al., 2000). In addition, local estrogen concentration is regulated by sulfotransferases (SULTs), which deactivate estrogens to estrogen sulfates (E1-S and E2-S) (Rižner, 2016). The concentration of biologically active estrogens produced by ovary in premenopausal women is also controlled by in pre-receptor level in peripheral tissues by these enzymes and aberrant expression of the enzymes can enhance the development of estrogen-dependent diseases such as endometrial (Lépine et al., 2010; Rižner, 2013) and breast cancer (Eliassen et al., 2006). A summary of estrogen biosynthesis in peripheral tissues is presented in Figure 2.

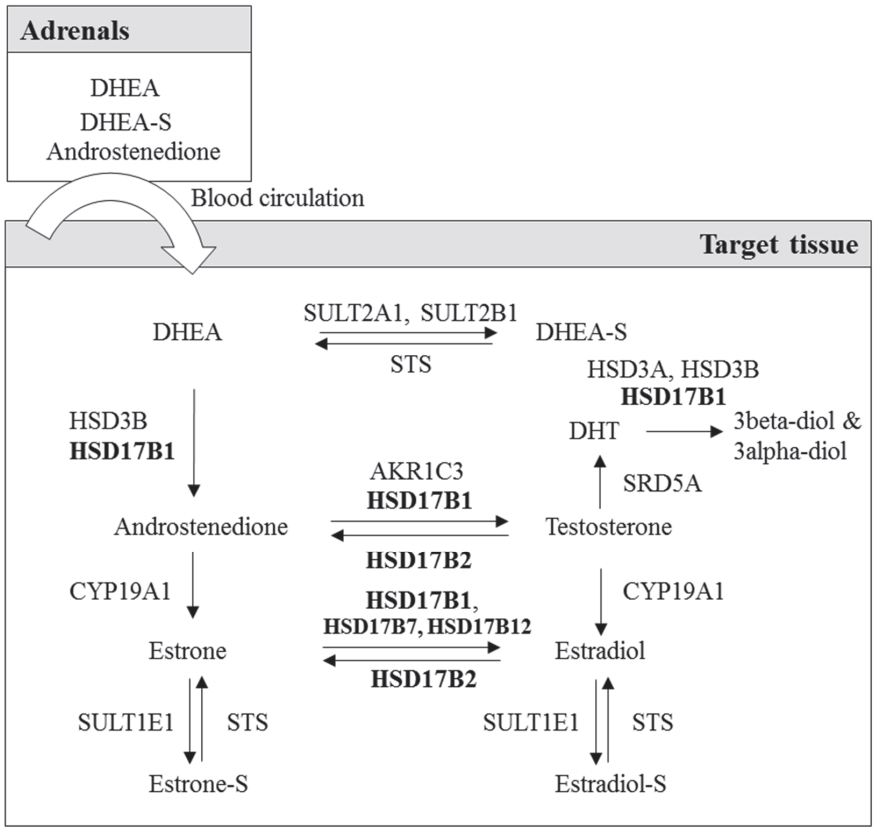

Figure 2. Estrogen biosynthesis in peripheral tissues in postmenopausal women. HSD17Bs enzymes are involved in the activation and deactivation of steroids in target tissues (indicated in bold). HSD17B1 is the main enzyme responsible for E1 conversion to E2.

2.3 HSD17B enzymes

Hydroxysteroid (17beta) dehydrogenases (HSD17Bs) are enzymes that activate or deactivate steroid hormones by catalyzing the conversion between the low active 17-ketosteroids and the highly active 17beta-hydroxysteroids and thus regulate the intracellular concentration of highly active steroid hormones. Fifteen HSD17B enzymes have been discovered in mammals to date, and these enzymes have been named according to the order in which they were discovered (Day et al., 2008a; He et al., 2016). All of these enzymes belong to the short-chain dehydrogenase/reductase (SDR) family, with the exception of HSD17B5, which belongs to the aldo-keto-reductase (AKR) family (Persson et al., 2009). These enzymes can be divided into steroid-activating enzymes by based on their preference for a reductive reaction at position 17 in the steroids and use of

NAD(P)H as a cofactor (types 1, 3, 5, 7 and 12) (Day et al., 2008a; He et al., 2016; Lukacik et al., 2006; Moeller and Adamski, 2009), and steroid-deactivating enzymes. The steroid-deactivating enzymes prefer an oxidative reaction at the same position and the use of NAD^+ as a cofactor (types 2, 4, 6, 8, 9, 10, 11, and 14) (Day et al., 2008a; He et al., 2016; Lukacik et al., 2006; Moeller and Adamski, 2009). An HSD17B9 homologue has been identified only in rats. HSD17B types 13 and 15 have been identified, but little is known about the oxidative/reductive preferences of these enzymes (Day et al., 2008a). Although HSD17Bs share the same protein fold at the steroid hormone binding sites, there are differences in non-conserved amino acid sequences that affect the functionality of the enzymes (Baker, 2001; Lukacik et al., 2006; Prehn et al., 2009). Different HSD17Bs have variable substrate preference; steroid substrates include E1, E2, testosterone, 3beta-diol and DHT, but other substrates are also possible. Additionally, these enzymes are expressed in different tissues and subcellular locations, leading to different physiological functions (He et al., 2016; Moeller and Adamski, 2009). Under different conditions, such as hypoxia, metabolic stress, and insulin resistance, changes in the cofactor NAD(P)/NAD(P)H balance might affect HSD17B activities (Sherbet et al., 2007). Furthermore, some of these enzymes also possess functions other than regulating reductive/oxidative reactions. For example, HSD17B7 is involved in cholesterol biosynthesis (Jokela et al., 2010), and HSD17B12 is involved in fatty acid metabolism (Kemiläinen et al., 2016).

2.4 HSD17B1 enzyme

Human HSD17B1 was the first cloned HSD17B; its sequence was identified by cloning the enzyme using antibodies against the protein purified from the placenta (Luu The et al., 1989; Peltoketo et al., 1988). The HSD17B1 gene was determined to be located in the region q.12.1 of chromosome 17 by gene mapping (Luu The et al., 1989; Peltoketo et al., 1988). This enzyme contains 327 amino acids and acts as a homodimer with two identical subunits with a molecular mass of 34.5 kDa (Lin et al., 1992; Peltoketo et al., 1988). Two mRNA species have been identified from human placenta, 1.3 kilobases (kb) and 2.3 kb and differ only in their 5'-noncoding regions (Luu The et al., 1989; Miettinen et al., 1996). The 1.3-kb transcript has been shown to be the major transcript responsible for functional HSD17B1 activity in cells, while the 2.3-kb transcript is constitutively detected in various target tissue-derived cell lines that display low levels of reductive activity (Miettinen et al., 1996). Furthermore, the 1-kb transcript in human uterine leiomyoma has been reported, but the function of this transcript is not known (Kasai et al., 2004).

Human HSD17B1 enzyme preferentially catalyzes the reductive reaction from E1 to E2 and functions as an estrogen-activating enzyme (Poutanen et al., 1993). The action of HSD17B1 in target tissues is illustrated in Figure 3. HSD17B1 can bind to both triphosphate cofactors NADHP and NADH at the molecular level but has a higher specificity toward NADHP than toward NADH, which are rich in cells that mainly direct the reduction of E1 to E2 (Jin and Lin, 1999). Kinetic studies demonstrate that the enzyme shows approximately 240-fold higher specificity towards E1 reduction than towards E2 oxidation under physiological conditions (Jin and Lin, 1999). However, in mouse and rat, the enzyme is also able to convert A-dione to testosterone at least as efficiently as it converts E1 to E2 (Nokelainen et al., 1996; Puranen et al., 1997). The human and rat enzymes possess highly similar K_m values for reductive estrogenic activity, but the K_m value for A-dione to testosterone conversion is markedly higher for human HSD17B1 than for the rat enzyme *in vitro* (Puranen et al., 1997). Altogether, there is a difference in substrate specificity between the human and rodent HSD17B1 enzymes; however, the catalytic properties of the human and rodent enzymes are similar (Puranen et al., 1997). More recently, it has been shown that human HSD17B1 is able to significantly reduce A-dione to testosterone *in vivo* (Saloniemi et al., 2007, 2009). Other metabolic functions have also been discovered as HSD17B1 is able to reduce DHEA to A-diol (Lin et al., 1992). Furthermore, it can metabolize DHT to 3beta-diol and 3alpha-diol in HSD17B1-transfected breast cancer cells. These formed diols can bind to ESR2 and, to some degree, to ESR1 (Aka et al., 2010).

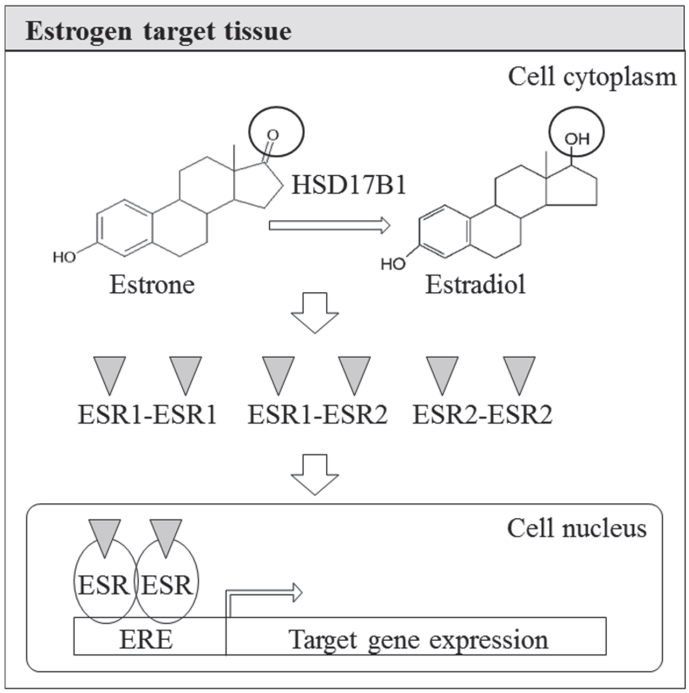

Figure 3. The action of HSD17B1 in target tissues through classical ESR activation in premenopausal women. E1 produced by the ovary is transported to estrogen target tissues, such as the breast or endometrium, and E1 is converted to E2 by cytoplasmic HSD17B1. Estradiol binds to ESRs and regulates estrogen-dependent gene transcription by binding to the ERE region of DNA.

Human HSD17B1 is expressed in the granulosa cells of the ovary (Ghersevich et al., 1994a; Sawetawan et al., 1994) and in syncytiotrophoblast cells of the placenta (Dupont et al., 1991; Fournet-Dulguerov et al., 1987) where it plays an essential role in the synthesis of estrogens. In the human ovary, HSD17B1 is localized only in the granulosa cells of developing follicles, which range from primary follicles with a single layer of granulosa cells and preantral follicles with multiple layers of granulosa cells to large antral follicles (Sawetawan et al., 1994). Already in fetal tissue the expression of HSD17B1 is detected in the granulosa cells of the developing ovary. The first observation of the enzyme is reported to occur at week 17 of gestation, and after week 22, the expression increases towards birth (Vaskivuo et al., 2005). HSD17B1 has not been detected in granulosa cells of primordial follicles, theca interna, theca externa or surrounding stroma (Sawetawan et al., 1994). In the corpus luteum, HSD17B1 is localized only in granulosa-luteal cells (Sawetawan et al., 1994).

In rat, Hsd17b1 expression in granulosa cells is up-regulated during follicular development in growing Graafian follicles; thereafter, enzyme expression is

downregulated during luteinization. Finally, there is no HSD17B1 expression in rat luteinized follicles or corpora lutea (Ghersevich et al., 1994b). Similarly, as in humans, the stroma and theca cells do not express Hsd17b1 enzyme in the ovary (Ghersevich et al., 1994a). An Hsd17b1 mouse model deficient of the mouse Hsd17b1 enzyme, showed severe subfertility because of defective luteinization of granulosa cells in the ovary (Hakkarainen et al., 2015). *Hsd17b1* knockout mice failed to initiate pseudopregnancy, although these mice showed a normal estrus cycle indicating that Hsd17b1 enzyme is essential for pregnancy initiation but not for menstrual cycle (Hakkarainen et al., 2015). The difference between humans and mice is that in female mice, *Hsd17b1* expression is mainly found in granulosa cell in the ovaries and in very low concentrations in other tissues (Hakkarainen et al., 2015; Nokelainen et al., 1996), whereas in humans a high expression of HSD17B1 is observed in the placenta (Dupont et al., 1991; Fournet-Dulguerov et al., 1987) in addition to the ovary (Ghersevich et al., 1994a; Sawetawan et al., 1994).

Under healthy conditions, human HSD17B1 is also expressed at low levels in estrogen-dependent peripheral tissues such as the breast and endometrium, where it regulates local E2 concentration (Dassen et al., 2007; Gunnarsson et al., 2001; Mäentausta et al., 1991; Söderqvist et al., 1998). HSD17B1 protein is present in normal breast epithelial cells in all phases of the menstrual cycle (Gunnarsson et al., 2001; Söderqvist et al., 1998). In the endometrium, very low levels of mRNA expression (Dassen et al., 2007) and protein expression (Mäentausta et al., 1991) of HSD17B1 have been detected in healthy tissue. Specifically, HSD17B1 has been shown to appear in surface epithelial and glandular cells during the early and midluteal phase of the menstrual cycle, to gradually disappear in the late luteal phase, and to be absent in the follicular phase (Mäentausta et al., 1991). However, some studies have not been able to detect HSD17B1 in normal endometrium (Delvoux et al., 2009; Utsunomiya et al., 2001). In addition to classical estrogen-dependent tissues, low levels of HSD17B1 have been detected in bone (Sasano et al., 1997) and skeletal muscle (Aizawa et al., 2007).

2.4.1 The regulation of HSD17B1

In the ovary, HSD17B1 and aromatase are expressed in parallel during the latest stages of follicular maturation in cultured human granulosa-luteal cells (Ghersevich et al., 1994a). FSH increases the mRNA and protein expression of both aromatase and HSD17B1 in human granulosa cells (Oktem et al., 2017). However, in contrast, in one study a treatment with gonadotrophins did not affect HSD17B1 protein concentration or HSD17B1 activity in human granulosa cells

(Ghersevich et al., 1994a). In the rat ovary, Hsd17b1 expression is regulated by gonadotropins (FSH, hCG), thus FSH increases enzyme expression and activity via cAMP, and the effect of FSH is enhanced by estrogens and androgens (Ghersevich et al., 1994b, 1994c). FSH induces Hsd17b1 expression via the protein kinase A (PKA)-dependent pathway and the induction is modulated by protein kinase C (PKC) -dependent inhibition (Kaminski et al., 1997). A downregulator of E2 synthesis, epidermal growth factor (EGF) suppresses the FSH effect and, thus decreases Hsd17b1 expression in rat granulosa cells (Ghersevich et al., 1994b; Kaminski et al., 1997; Mason et al., 1990). Similarly, transforming growth factor alpha (TNFalpha), a member of EGF family, downregulates Hsd17b1 expression by inhibiting gonadotropin action in cultured rat granulosa cells (Ghersevich et al., 2001). A ovarian function modulator, transforming growth factor beta1 (TGF-beta1), strongly enhances Hsd17b1 expression in rat granulosa cells (Kaminski et al., 1997) but, in contrast, decreases Hsd17b1 expression in bovine granulosa cells (Zheng et al., 2008). In addition, immunological factors influence HSD17B1 expression, a cytokine interleukin-1beta (IL-1beta) has been observed to downregulate Hsd17b1 expression in cultured rat granulosa cells (Ghersevich et al., 2001). Furthermore, interleukin-6 (IL-6) and basic fibroblast growth factor (bFGF) have been observed to increase HSD17B1 activity in the human ovarian cancer cell line A2780 (Speirs et al., 1993a).

Ovarian folliculogenesis and steroidogenesis are also regulated by factors which belong to the protein family of TGFbeta such as activin and inhibin. Activin increases FSH production, while inhibin, which is produced in highest by preovulatory follicles, suppress FSH secretion through endocrine negative feedback. In addition, a factor affecting follicle formation and steroidogenesis is follistatin which decreases FSH production (Knight et al., 2012). Activin has been shown to enhance FSH-stimulated aromatase activity in granulosa cells (Ghersevich et al., 2000). Similarly to aromatase, activin increased Hsd17b1 activity and mRNA expression in rat granulosa (Ghersevich et al., 2000) and gonadotrope cells (Bak et al., 2009). Follistatin significantly diminished the stimulatory effect of activin on Hsd17b1 expression, whereas inhibin had no effect on Hsd17b1 activity (Ghersevich et al., 2000).

HSD17B1 expression has been correlated with ESR and progesterone receptor, suggesting the regulation of HSD17B1 by these steroids (Källström et al., 2010; Suzuki et al., 2000). Progesterone induce HSD17B1 expression in breast cancer cells, particularly in T-47D cells, (Poutanen et al., 1990, 1992a; Sivik and Jansson, 2012) and in glandular and surface epithelial cells of the human endometrium (Mäentausta et al., 1991), thus, HSD17B1 is under control of progesterone. The expression of HSD17B1 in endometrium is also positively associated with concentration of serum progesterone and inversely correlated with progesterone

receptor (Mäentausta et al., 1991). In contrast, in endometrial adenocarcinoma, the plasma progesterone concentrations are inversely correlated with HSD17B1 staining and positively correlated with progesterone receptor staining (Mäentausta et al., 1992). Additional regulatory factors affecting HSD17B1 expression in human endometrium by increasing the expression are TGFalpha in endometrial glandular epithelial EMI cells (Salama et al., 2009) and interferon-beta (IFN-beta) in human endometrium explants by increasing the enzymatic activity (De Cicco Nardone et al., 1996).

The expression of HSD17B1 is downregulated by E2 in ESR1-positive breast cancer cells via ESR1, suggesting E2-mediated negative feedback control of the enzyme (Hilborn et al., 2017b). In addition, the expression of HSD17B1 enzyme is also regulated post-transcriptionally by microRNAs (miRNAs), as demonstrated in healthy breast tissue in premenopausal women in which a number of miRNAs (miR-17, miR-26a, miR-106b) were predicted to target HSD17B1 expression (Sieuwerts et al., 2011). The negative feedback of the end product is also observed as slightly decreased expression of HSD17B1 by DHT in breast cancer cells (Aka et al., 2010; Hilborn et al., 2017b; Zhang et al., 2015). Other factors affecting HSD17B1 regulation by increasing the expression in breast cancer cells are TGFalpha in MCF-7 cells (Duncan and Reed, 1995), insulin-like growth factor type I and II (IGF-I and IGF-II) in MCF-7 cells (Singh and Reed, 1991), retinoic acids in T-47D cells (Piao et al., 1997a), interleukin-6 (IL-6) in MCF-7 cells (Speirs et al., 1993b), and interleukin-8 (IL-8) in primary epithelial breast cell cultures (Speirs et al., 1998). MiRNA-17, which has been postulated to be oncogenic, upregulated HSD17B1 expression, while miRNA-210 and miRNA-7-5p resulted in mixed results and uncharacterized miRNA-1304-3p reduced HSD17B1 expression in breast cancer cells (Hilborn et al., 2017b).

In placental choriocarcinoma JAR and JEG-3 cells, EGF and TGFalpha increases HSD17B1 protein concentrations and HSD17B1 catalytic activity (Lewintre et al., 1994). The expression and activity of HSD17B1 is also increased by retinoic acids in placental cells (Piao et al., 1997a; Zhu et al., 2002). In addition, the expression is regulated by hypoxia-induced miRNAs (miR-210 and miR-518c) in human preeclamptic placentas, where the expression of the *HSD17B1* gene was suppressed (Ishibashi et al., 2012).

The cell-specific regulation of *HSD17B1* gene expression is controlled by an enhancer which contains several interacting subunits (Piao et al., 1995). The gene has a silencer element with a GATA motif and a proximal promoter region with competing Sp1 and AP-2 sites, and it is possible that there are still unidentified regions that control gene expression (Piao et al., 1997b). Interactions of the factors binding to these elements and the concentrations and activation/inactivation of

these factors ultimately control the tissue-specific expression of the HSD17B1 gene (Piao et al., 1997b). Table 1 summarizes the regulation of HSD17B1.

Table 1. Regulation of HSD17B1 expression

Regulator	Tissue/Cell type	Effect	Reference
	Ovary		
Activin	Rat/mouse granulosa cells	↑	Ghersevich et al., 2000; Bak et al., 2009
Androgens	Rat granulosa cells	↑	Ghersevich et al., 1994a, 1994b
bFGF	Rat granulosa cells	↑	Kaminski et al., 1997
EGF	Rat granulosa cells	↓	Ghersevich et al., 1994a
E2	Rat granulosa cells	↑	Ghersevich et al., 1994a, 1994b
FSH	Human granulosa cells	↑	Oktem et al., 2017
FSH (&hCG)	Rat granulosa cells	↑	Ghersevich et al., 1994a, 1994b; Kaminski et al., 1997
Follistatin	Rat granulosa cells	↓	Ghersevich et al., 2000
IL-1beta	Rat granulosa cells	↓	Ghersevich et al., 2001
IL-6	Human ovarian cancer cells	↑	Speirs et al., 1993b
TNFalpha	Rat granulosa cells	↓	Ghersevich et al., 2001
TGF-beta1	Rat granulosa cells	↑	Kaminski et al., 1997
TGF-beta1	Bovine granulosa cells	↓	Zheng et al., 2008
	Placenta		
EGF	Human choriocarcinoma cells	↑	Lewintre et al 1994
miR-210,-518c	Human placenta	↓	Ishibashi et al., 2012
TNFalpha	Human choriocarcinoma cells	↑	Lewintre et al., 1994
Retinoic acids	Human choriocarcinoma cells	↑	Piao et al., 1997; Zhu et al., 2002
	Endometrium		
IFN-beta	Human endometrium	↑	De Cicco Nardone et al., 1996
Progesterone	Human endometrium	↑	Mäentausta et al., 1991
Progesterone	Human endometrial carcinoma	↓	Mäentausta et al., 1992
TNFalpha	Human endometrial cells	↑	Salama et al., 2009
	Breast		
Dihydrotestosterone	Human breast cancer cells	↓	Aka et al., 2010; Zhang et al., 2015; Hilborn et al., 2017b
E2	Human breast cancer cells	↓	Hilborn et al., 2017b
IGF-I and IGF-II	Human breast cancer cells	↑	Singh and Reed, 1991
IL-6	Human breast cancer cells	↑	Speirs et al., 1993a
IL-8	Human breast epithelial cells	↑	Speirs et al., 1998
miR-17	Human breast cancer cells	↑	Hilborn et al., 2017b
miR-1304-3p	Human breast cancer cells	↓	Hilborn et al., 2017b
Progesterone	Human breast cancer cells	↑	Poutanen et al., 1990, 1992; Sivik and Jansson, 2012
Retinoic acids	Human breast cancer cells	↑	Piao et al., 1997
TNFalpha	Human breast cancer cells	↑	Duncan and Reed, 1995
	Pituitary		
Activin	Mouse gonadotrope cells	↑	Bak et al., 2009

2.5 Breast cancer

Estrogen is essential for normal postpubertal mammary development; however, prolonged exposure to estrogens increases the risk of breast cancer (Key et al., 2002). Breast cancer is the most common cancer in women worldwide, and approximately 60–70% of human breast cancers are ESR1-positive. Estrogens promote carcinogenesis of ESR1-positive breast cancer by binding to and activating the ESRs (Castoria et al., 2010). In particular, estrogens induce proliferation and survival in breast cancer cells (Fox et al., 2009; Missmer et al., 2004; Raafat et al., 1999). The age at menarche and the time of pregnancy have the greatest influence on postmenopausal breast cancer risk, indicating that early life is the most sensitive period. It is believed that prolonged exposure to estrogens underlies the increased risk of breast cancer (Dall and Britt, 2017). In addition, obesity is associated with higher postmenopausal breast cancer incidence, likely due to increased aromatase activity in adipose tissue leading to increased local and circulating estrogen concentrations (Engin, 2017; Siiteri, 1987). Estrogen affects tumor initiation by the induction of enzymes and proteins involved in nucleic acid synthesis and by the activation of oncogenes, such as *c-myc* (Clemons and Goss, 2001). Estrogens can also affect breast carcinogenesis indirectly by stimulating the secretion of prolactin and the production of growth factors such as TGFalpha and EGF and non-growth-factor peptides (Clemons and Goss, 2001).

Breast cancers are traditionally classified into luminal A (ESR1 and progesterone receptor positive, low proliferation rate), luminal B (ESR1 and progesterone receptor positive, high proliferation rate), HER2-overexpressing, and triple-negative carcinomas (TNCs) that do not express ESR1, progesterone receptor or HER2 (Korhonen et al., 2004). The most common cancer is ductal carcinoma, accounting for approximately 80% of breast cancers, followed by lobular carcinoma, which accounts for 10% of breast cancers (Korhonen et al., 2004). Ductal carcinomas are known to develop in stages spanning from the normal terminal duct lobular unit (TDLU) to ductal carcinoma *in situ* (DCIS) and finally to invasive ductal carcinoma (IDC). DCIS is defined as a non-invasive cancer because it does not spread beyond the duct into surrounding breast stroma, but it is believed to be a direct precursor to IDC (Burstein et al., 2004). In the transition from normal breast tissue to DCIS, the number of ESR1-positive cells increases, while at the same time the number of ESR2-positive cells decreases. Eventually, in IDC, fewer than 10% of tumor cells express ESR2 (Huang et al., 2015).

Studies have shown that patients with ESR1-positive breast cancers have a better prognosis because these tumors tend to be lower grade and have less aggressive phenotypes. Even patients with metastatic tumors that expressed ESR1 often had significantly better survival than patients with ESR1-negative tumors. The better

prognosis is most likely due to the fact that most patients with ESR1-positive tumors also have an increased probability of responding to the established endocrine therapies (Louie and Sevigny, 2017). The adjuvant therapy include drugs targeting ESR1 such as selective estrogen receptor modulators (tamoxifen), aromatase inhibitors (AI) (anastrozole, letrozole or exemestane), LH-RH agonists (leuprolide, goserelin), pure selective estrogen receptor downregulators (SERDS) (fulvestrant) and oophorectomy (Nicolini et al., 2017). Most patients with ESR1-positive breast cancer are treated with ESR antagonists or aromatase inhibitors (Castoria et al., 2010). Aromatase inhibitors, used in breast cancer therapy, can eliminate the production of estrogen, while antiestrogens bind to ESR and antagonize its activity. Both of these strategies are successfully used in breast cancer patients; approximately 50% of patients with ESR1-positive breast cancer benefit from the adjuvant endocrine treatment (Jeselsohn et al., 2014). However, a significant fraction of treated patients will develop a metastatic disease and resistance to these therapies (Castoria et al., 2010; Chan et al., 2016; Jeselsohn et al., 2014).

Several studies have indicated that breast tumors have a higher intratumoral estrogen concentration than normal breast tissue (Chetrite et al., 2000; van Landeghem et al., 1985; Stanczyk et al., 2015) and plasma (Haynes et al., 2010; Pasqualini et al., 1996; Stanczyk et al., 2015). The mechanism responsible for maintaining high intratumoral estrogen concentrations is likely controlled by estrogen-metabolizing enzymes aromatase, STSs and HSD17Bs, which in the breast control the local estrogen concentration by activating and inactivating circulating steroids (Chetrite et al., 2000).

2.5.1 HSD17B1 in breast cancer

Human HSD17B1 is linked to increased E2 biosynthesis in breast cancer. This reaction occurs in target cells where the estrogenic effect is exerted via the ESR. Estrogens, particularly E2, are known to stimulate breast tumor growth (Lipovka and Konhilas, 2016). Despite the low levels of circulating estrogens in postmenopausal patients, the tumor tissue concentrations of these steroids are several-fold higher than those in plasma, suggesting accumulation of E1 and E2 in breast tumor tissue (Chetrite et al., 2000; Pasqualini et al., 1996). Interestingly, HSD17B1 mRNA expression levels and intratumoral E2/E1 ratios of breast cancers were observed to be higher in postmenopausal women than in breast cancers in premenopausal women (Miyoshi et al., 2001). These results demonstrate that the increased conversion from E1 to E2 catalyzed by HSD17B1 may play an important role in the maintenance of high intratumoral E2 levels in

postmenopausal patients (Miyoshi et al., 2001). The expression of HSD17B1 in T-47D breast cancer cells has been positively correlated with E1 reduction to E2, E2 levels, and cell proliferation; thus, HSD17B1 upregulates breast cancer cell growth by E2 synthesis (Aka et al., 2010). The increased growth of ESR1-positive breast cancer cells is at least partly mediated by the endogenous estrogen-responsive gene pS2, which is affected by HSD17B1 expression (Aka et al., 2010). While deletion of HSD17B1 in breast cancer T-47D cells has been observed to result in changes in E2-dependent cancer-related genes, a lack of HSD17B1 has also been related to inflammatory pathways (Aka et al., 2017). HSD17B1 has been demonstrated to regulate the growth of MCF-7 breast cancer cells transfected with HSD17B1 by modulating the expression of genes and proteins related to cell growth, such as BRCA2 and PCNA (Aka et al., 2012a). Furthermore, the corresponding study showed that HSD17B1 overexpression is associated with an increase in MCF-7 cell migration (Aka et al., 2012a).

2.5.2 HSD17B1 in breast cancer in postmenopausal women

HSD17B1 has been detected in breast epithelium and benign breast diseases and cancers in variable quantities (Ariga et al., 2000; Poutanen et al., 1992b; Sasaki et al., 2010). In postmenopausal breast cancer specimens, HSD17B1 has been observed to occur in 16–61 % of DCIS and IDC patients (Oduwole et al., 2004; Poutanen et al., 1992b; Sasano et al., 1996; Suzuki et al., 2000). In DCIS, a precursor lesion of IDC, both estrogen and mRNA expression of HSD17B1 were observed to be higher than in non-neoplastic breast tissue (Shibuya et al., 2008). Similarly, increased expression of HSD17B1 enzyme was detected in ESR1-positive tumors of postmenopausal breast carcinoma patients treated with the aromatase inhibitor exemestane (Chanplakorn et al., 2010). This increment may represent the compensatory response of breast carcinoma tissue to estrogen depletion (Chanplakorn et al., 2010). In epidemiological studies, a high expression of HSD17B1 predicts late recurrence of breast cancer among ESR-positive patients, and high expression levels of HSD17B1 indicate an increased risk for late relapse (Gunnarsson et al., 2001, 2005). An amplification of *HSD17B1* is related to decreased breast cancer survival in ESR-positive breast cancer patients (Gunnarsson et al., 2003, 2008). Overall, patients with tumors expressing HSD17B1 mRNA or protein have significantly shorter overall and disease-free survival than other patients do (Oduwole et al., 2004; Salhab et al., 2006), suggesting that the enzyme has a prognostic significance in breast tumor progression. The progression of breast cancer is also observed through increased levels of HSD17B1 correlated with tumor grade. In one study, an increased level of HSD17B1 mRNA was associated with grade 2 tumors with moderately

differentiated tissue compared with grade 1 tumors or normal breast tissue (Salhab et al., 2006). The expression of HSD17B1 has also been correlated with COX-2, which is considered a marker of more aggressive and less differentiated breast tumors (Gunnarsson et al., 2006).

2.5.3 HSD17B1 in breast cancer in premenopausal women

Studies in postmenopausal breast cancer patients have showed a wide range of HSD17B1 expression in tumor tissues, while in premenopausal breast cancer patients treated with tamoxifen HSD17B1 enzyme is found in up to 99% of specimens (Källström et al., 2010). The expression level of HSD17B1 could serve as a predictive marker for the efficiency of tamoxifen treatment, as patients with a low expression of HSD17B1 in ESR-positive breast cancer did benefit from adjuvant tamoxifen treatment (Källström et al., 2010). In addition, a low level of HSD17B1 is associated with a reduced risk of breast cancer recurrence in premenopausal patients treated with tamoxifen (Källström et al., 2010).

2.5.4 Polymorphisms in HSD17B1 gene

HSD17B1 polymorphic variants may play a role in cancer susceptibility. Numerous investigations have been conducted to assess the association between HSD17B1 and different single nucleotide polymorphisms (SNPs) in various cancers (Ashton et al., 2010; Feigelson et al., 2006; Gaudet et al., 2008; Justenhoven et al., 2008; Kato et al., 2009; Obazee et al., 2013; Plourde et al., 2008). One of the most studied SNPs is Ser312Gly polymorphism in the *HSD17B1* gene. Studies have been carried out to assess the association of *HSD17B1* Ser312Gly polymorphism with susceptibility to different types of tumors in diverse ethnic populations, including endometrial cancer, breast cancer, prostate cancer, uterine leiomyoma, and lung cancer. To date, however, no significant association has been found between *HSD17B1* Ser312Gly polymorphism and estrogen-related cancer risk (Shi et al., 2016). In contrast, Ser312Gly polymorphism has indicated a decreased overall risk of cancer among Caucasians in a subgroup analysis by ethnicity (Shi et al., 2016). However, one study succeeded to associate a genetic risk of HSD17B1 312 Gly allele polymorphism with premenopausal breast cancer risk in African American women (Kato et al., 2009).

2.5.5 Other HSD17B enzymes in breast cancer

The net production of active E2 is the result of a balance between the synthesis and the deactivation of E2. E2 activation is also mediated through HSD17B enzymes other than HSD17B1 in breast tissue. Reductive HSD17B enzymes HSD17B7 and HSD17B12 can convert E1 to E2 as well. In breast cancer specimens HSD17B7 was detected in 47% of patients and HSD17B12 in 83% of patients (Song et al., 2006). Knocking down of HSD17B7 expression in breast cancer cell lines resulted in a marked reduction in proliferation, suggesting that it may play a role in breast cancer (Zhang et al., 2015). The expression of HSD17B12 has been found to be higher in breast carcinoma specimens than in normal tissue (Song et al., 2006). However, a study involving breast cancer cells indicated that the conversion of E1 to E2 correlates with HSD17B1, not HSD17B12, expression (Day et al., 2008b). In the cell study, only T-47D cells, which exhibit endogenous HSD17B1 expression, were capable of converting E1 to E2 efficiently, despite the high level of expression of HSD17B12 in all cell lines tested (MCF-7, MDA-MB-231 and 293-EBNA), while the other cells used did not express HSD17B1 (Day et al., 2008b). The relationship between HSD17B12 and breast cancer does not necessarily involve intratumoral E2 biosynthesis but is rather correlated with the production of fatty acids (Nagasaki et al., 2009). Moreover, HSD17B5 as an E1-activating enzyme is also overexpressed in a wide variety of cancers, including breast cancer (Byrns et al., 2011), and overexpression of this enzyme has been shown to lead to worse prognosis (Oduwole et al., 2004).

The precise ratio of E1 to E2 in tumors is controlled by HSD17B1 and HSD17B2, which is an E2-deactivating enzyme (Zhang et al., 2012). HSD17B2 has been detected in normal breast tissue, but the expression has been observed to decrease in breast carcinoma samples in postmenopausal women (Ariga et al., 2000; Suzuki et al., 2000). In premenopausal breast cancer patients, HSD17B2 was expressed in 74% of specimens (Källström et al., 2010). By contrast, in postmenopausal women, HSD17B2 immunostaining was detected in 10% of breast cancer tumors (Gunnarsson et al., 2001) and mRNA expression in 25% of breast cancers (Oduwole et al., 2004). However, a controversial result showed protein expression of HSD17B2 in over 90% of postmenopausal breast cancer specimens (Jansson et al., 2009). In ESR-positive cancer, the absence of HSD17B2 combined with a high expression of HSD17B1 indicated an increased risk of late relapse in breast cancer (Gunnarsson et al., 2001), suggesting a protective role of HSD17B2 in breast carcinogenesis. These results demonstrate that the E2/E1 ratio is controlled by both HSD17B1 and HSD17B2, although HSD17B1 is the first determining factor in the cellular environment (Jansson et al., 2006a; Zhang et al., 2012). For other oxidative HSD17Bs enzymes, increased expression levels of HSD17B14 have been associated with longer disease-free survival among breast cancer patients (Jansson

et al., 2006b), and tumors expressing high levels of HSD17B14 have been shown to reduce the number of local recurrences in tamoxifen-treated ESR-positive breast cancer patients (Sivik et al., 2012).

2.6 Endometrial cancer

Endometrial cancer is the most common gynecologic malignancy in developed countries. Overall, up to 80% of all endometrial cancers are type I endometrioid adenocarcinomas, which generally develop through endometrial hyperplasia and typically have a good prognosis (Emons et al., 2000; Kurman and McConnell, 2010). Type II tumors are usually serous papillary-, clear cell-, or squamous carcinomas and in most cases develop from atrophic endometrial tissue in aged women (Emons et al., 2000). Risk factors for increased endometrial cancer incidence have been associated with early menarche and late menopause, suggesting an association with greater lifetime exposure to estrogens during reproductive years (Emons et al., 2000; Kaaks et al., 2002; Sherman, 2000). In fact, epidemiological studies have confirmed that elevated levels of E1 and E2 in plasma increase the endometrial cancer risk of postmenopausal women (Audet-Walsh et al., 2011). Other hormone-related factors associated with this risk are parity and use of exogenous estrogens without progestin for oral contraception or postmenopausal hormone replacement therapy (Emons et al., 2000; Kaaks et al., 2002). Furthermore, type I endometrial cancer is associated with environmental risk factors such as obesity and diabetes (Kaaks et al., 2002), which are related to Western lifestyle norms. In these diseases, increased body adiposity may increase the risk by aromatization of androgen into estrogens in adipose tissue and consequently increase the plasma concentration of estrogens (Kaaks et al., 2002; Siiteri, 1987). In fact, elevated plasma A-dione and testosterone have been shown to increase endometrial cancer risk in pre- and postmenopausal women (Audet-Walsh et al., 2011).

Type I endometrial cancer tumors develop from proliferative endometrium, which transforms through endometrial hyperplasia and atypical hyperplasia before achieving well-differentiated endometrioid carcinoma (Emons et al., 2000; Kurman and McConnell, 2010). The development of endometrial hyperplasia and endometrial cancer is positively correlated with increased estrogen exposure (Emons et al., 2000), thus, these carcinomas are mainly considered estrogen-dependent tumors (Kurman and McConnell, 2010). The endometrial changes in endometrial hyperplasia are classified into two categories based on the presence or absence of cytological atypia, and the categories are further divided based on their architectural complexity (simple and complex hyperplasia). The most important

prognostic indicator of the progression from hyperplasia to carcinoma is cytological atypia (Kurman and McConnell, 2010).

Estrogen action is mediated primarily by ESR1 and ESR2 in the endometrium, and the ratio of these receptors plays a role in the carcinogenesis of endometrial cancer (Jeon et al., 2006; Jongen et al., 2009). Higher levels of ESR1 than ESR2 are reported in specimens of diseased endometrium and a shift in the ratio of the two ESR subtypes is associated with endometrial carcinogenesis. The expression of ESR1 increases in the progression from normal proliferation to simple and complex hyperplasia, whereas ESR2 expression is not affected during this process (Hu et al., 2005). In atypical hyperplasia and adenocarcinoma, the expression levels of both ESR1 and ESR2 are reduced (Hu et al., 2005). Nevertheless, most endometrial adenocarcinomas express ESR1, and the ESR2/ESR1 ratio is decreased compared with that of normally proliferative tissue (Hu et al., 2005). In the early stages of endometrial carcinogenesis, patients with ESR1-positive tumors have a better overall survival (Jongen et al., 2009). In addition, a powerful prognostic factor for endometrial cancer progression is progesterone receptor type A (Arnett-Mansfield et al., 2001). Progesterone is a natural antagonist of estrogen, controlling cell proliferation in the endometrium; thus, progesterone decreases the risk of endometrial cancer (Arnett-Mansfield et al., 2001; Jongen et al., 2009). The absence of progesterone receptor type A in endometrioid cancer is an independent prognostic factor for the recurrence of disease (Jongen et al., 2009).

2.6.1 HSD17B1 in endometrial cancer and other estrogen-dependent disorders in uterus

Endometrial cancer is usually diagnosed after menopause, when follicular steroidogenesis has ceased. The peripheral tissues become an important source of estrogens in postmenopausal women. In fact, in postmenopausal women with type I endometrioid cancer, circulating levels of serum E1, E2, and E1-S are higher than in healthy controls (Audet-Walsh et al., 2011; Ito et al., 2006; Lépine et al., 2010). In addition, postmenopausal patients with endometrioid cancer have 10–40 times higher E2 levels in tumor tissues than those measured in serum (Ito et al., 2006) and higher E2 levels in tumor than in healthy tissue (Berstein et al., 2003), thus local estrogen metabolism helps to maintain the high levels of hormone in tumor tissues (Chetrite et al., 2000; Ito et al., 2006; Pasqualini et al., 1996).

In ESR-positive type I endometrial cancer, increased HSD17B1 expression level has been observed in postmenopausal women analyzed by gene expression and protein levels (Cornel et al., 2012). This increased expression of HSD17B1 in patients with endometrial cancer was associated with poorer prognosis than that of

the remaining women (Cornel et al., 2017) and polymorphism of *HSD17B1* gene was associated with cervical cancer (Lutkowska et al., 2017). Furthermore, increased HSD17B1 expression has been reported in endometriotic tissue, leading to higher estrogen synthesis in endometriosis patients (Colette et al., 2013; Dassen et al., 2007; Delvoux et al., 2014; Smuc et al., 2008). Similarly, polymorphism of *HSD17B1* is associated with an increased risk of endometriosis (Hu et al., 2012; Lamp et al., 2011). Moreover, HSD17B1 has been discovered in leiomyomas, which are benign tumors derived from the myometrium (Kasai et al., 2004).

2.6.2 Other HSD17B enzymes in endometrial cancer and estrogen-dependent uterine disorders

Unbalanced estrogen metabolism may lead to increased estrogen synthesis in endometriosis or in other uterine disorders. By converting E2 to E1, HSD17B2 an E2-deactivating enzyme controls the amount of active E2 in tissue (Ito et al., 2006; Utsunomiya et al., 2001). In healthy endometrium, HSD17B2 expression is increased in the secretory phase compared with that in the proliferative phase because of increased progesterone action, thus decreasing the local E2 concentration (Dassen et al., 2007; Huhtinen et al., 2012b). In one study, HSD17B2 expression was detected to gradually decrease in the transition from a normal endometrium (secretory phase) to hyperplasia, with carcinoma ultimately showing the lowest expression of the enzyme, as HSD17B2 was detected in 75% of endometrial hyperplasias and in 37% of endometrial cancers (Utsunomiya et al., 2001). In addition, the level of HSD17B2 mRNA in endometrial carcinoma is inversely correlated with the intratumoral E2 concentration (Ito et al., 2006). These results suggest that HSD17B2 is involved in the regulation of intratissue estrogen levels in the normal endometrium and disruption of this control may be related to the development of endometrial disorders (Colette et al., 2013; Dassen et al., 2007; Ito et al., 2006; Utsunomiya et al., 2001). The same phenomenon, *i.e.,* the suppression of HSD17B2 expression, has been observed in endometriosis patients (Colette et al., 2013; Dassen et al., 2007; Huhtinen et al., 2012b). Similarly to HSD17B2, other HSD17B enzymes catalyzing oxidative reactions, HSD17B6 (Huhtinen et al., 2012b) and HSD17B4 (Dassen et al., 2007) have shown downregulated expression in endometriosis lesions. The expression of other enzymes possessing HSD17B reductive activity has been reported to be increased in certain tissues, such as HSD17B12 in endometrial tumors (Lépine et al., 2010) and HSD17B7 in endometriotic tissue (Dassen et al., 2007; Smuc et al., 2008), thus participating in E2 upregulation.

2.7 HSD17B1 inhibitors

The use of inhibitors of HSD17B1 has been proposed as a treatment for estrogen-dependent diseases, such as breast and endometrial cancer and endometriosis, by lowering E2 concentrations in diseased tissues and thus inhibit estrogen-dependent proliferation of tumor cells (Day et al., 2008a; He et al., 2016; Hilborn et al., 2017a). The inhibitor could be given alone or in combination with other breast cancer treatments currently in use. There are two main primary forms of inhibitors under development: steroidal and nonsteroidal (Poirier, 2011). Most of the HSD17B1 inhibitors described in the literature to date are based on the steroid scaffold, with expansion at positions 6, 15, 16, and 17 in the steroid structure (Ayan et al., 2012; Cadot et al., 2007; Allan et al., 2006; Tremblay et al., 2005). The common structural feature of inhibitors is that they aim to bind to the active site. One of these inhibitors is a phenol, which can bind to His221 and Glu282 residues of the protein and have a hydrophobic structure, that inhabits the hydrophobic area at the active site (Allan et al., 2006; Poirier, 2011). This subgroup also includes different E2 and E1 derivatives (Cadot et al., 2007; Laplante et al., 2008a; Tremblay et al., 2005). Fewer reports have addressed non-steroidal inhibitors of HSD17B1 (Henn et al., 2012), although non-steroidal inhibitors could provide an improved pharmacokinetic profile, as steroid based inhibitors are quite hydrophobic (Allan et al., 2008).

Several of these HSD17B1 inhibitors have been tested *in vitro* by measuring the proliferation of cells (Ayan et al., 2012; Cadot et al., 2007; Laplante et al., 2008b), the estrogenic activity of compounds (Ayan et al., 2012; Cadot et al., 2007; Henn et al., 2012; Laplante et al., 2008b) or binding to ESR1 (Ayan et al., 2012; Henn et al., 2012). Diverse steroidal inhibitors with modifications of E2 sidechains were reported to inhibit the proliferation of T-47D breast cancer cells induced by E1 with inhibition efficiency of 61%–80% (Ayan et al., 2012; Cadot et al., 2007; Laplante et al., 2008b). Furthermore, inhibitors demonstrated reduced inhibitory activity in T-47D cells (Ayan et al., 2012; Cadot et al., 2007; Henn et al., 2012; Laplante et al., 2008b) and low binding affinity to ESR1 (Ayan et al., 2012; Henn et al., 2012).

In vivo validation is less frequently used in the development of inhibitors. One of the difficulties associated with inhibitor screening is the low inter-species homology of the HSD17B1 enzyme, as mouse Hsd17b1 is only 63% homologous to human HSD17B1 at the amino acid level, which reflects different substrate affinities (Day et al., 2008a; Nokelainen et al., 1996). *In vivo* studies of HSD17B1 inhibitors have been mainly performed with mouse xenografts. These studies have used estrogen-dependent breast cancer cells to form tumors in immunodeficient mice in the presence of E1, and the effect of HSD17B1 inhibitors has been

evaluated by measuring tumor volume (Ayan et al., 2012; Day et al., 2008b; Husen et al., 2006a, 2006b). Tumor size has been successfully reduced through treatment with HSD17B1 inhibitors, demonstrating that HSD17B1 inhibition markedly diminishes the estrogen-dependent growth of breast cancer cells (Ayan et al., 2012; Day et al., 2008b; Husen et al., 2006a, 2006b). Moreover, marmoset monkeys with induced endometriosis have been used for HSD17B1 inhibitor testing, showing decreased endometriosis-related symptoms after HSD17B1 inhibitor treatment (Arnold and Einspanier, 2013). One transgenic mouse model has been developed for testing HSD17B1 inhibitors. The mice were shown to express human HSD17B1 under mouse mammary tumor virus (MMTV) promoter, and the efficacy of inhibitors was tested by measuring the enzyme activity *in vivo*. With a potent HSD17B1 inhibitor compound tested the highest inhibitory effect was 85% in male and 33% in female mice (Lamminen et al., 2009).

2.7.1 Other HSD17B inhibitors

The development of inhibitors for reductive enzyme HSD17B5 as a potential therapeutic for hormone-dependent breast cancer and prostate cancer has been under investigation. HSD17B5 catalyzes the reduction of E1 to E2 (Byrns et al., 2010) and that of A-dione to testosterone (Byrns et al., 2010; Laplante and Poirier, 2008), which can promote the proliferation of hormone-responsive breast and prostate cancers, respectively. HSD17B5 can also limit the formation of anti-proliferative prostaglandins by catalyzing the reduction of prostaglandin H2 to PGF2alpha and PGD2 to 9alpha,11beta-PGF2 (Byrns et al., 2011). Several different compounds have been studied as inhibitors of HSD17B5, including non-steroidal anti-inflammatory drugs, steroid hormone analogues, flavonoids, cyclopentanes, and benzodiazepines (Byrns et al., 2011). Additionally, inhibition of HSD17B3 has been explored to treat hormone-dependent prostate cancer by blocking the conversion of A-dione to testosterone. Some studies have indicated increased expression of HSD17B3 in cancerous prostate biopsies compared with that in normal prostate tissue (Day et al., 2008a; Koh et al., 2002). Presently, there is less research concerning the development of inhibitors of the remaining, less well characterized HSD17Bs. However, the inhibition of HSD17B10 is being studied for the treatment of Alzheimer's disease (Day et al., 2008a).

3 AIMS OF THE STUDY

The aim of the present study was to increase our understanding of the role of human HSD17B1 in development estrogen-dependent diseases, particularly in endometrial and breast cancer. Because HSD17B1 is expressed in both of these cancers, it is hypothesized that HSD17B1 affects the estrogen concentration in cancer tissue. Thus, HSD17B1 is considered a promising drug target in these diseases. However, it is essential to better understand the physiological role of HSD17B1 *in vivo*, which will aid in the development and utilize of convenient mouse models for *in vivo* efficacy testing of HSD17B1 inhibitors. For these purposes, we applied transgenic female mice expressing human HSD17B1 enzyme (HSD17B1TG mice).

The specific aims of the present study were as follows:

1) To study the role of HSD17B1 in estrogen biosynthesis (I).

2) To define the role of HSD17B1 in the development of endometrial disorders and in mammary carcinogenesis (I, III).

3) To apply HSD17B1TG mice in HSD17B1 inhibitor screening (I, II, III).

4 MATERIALS AND METHODS

4.1 Ethical statement and animal handling (I-III)

Animal care and use were conducted in accordance with the Finnish Act on Animal Experimentation and EU laws, guidelines, and recommendations. All studies were approved by the Finnish Animal Ethics Committee (2007-01367, 2010-04888, 257/04.10.07/2013, 10605/04.10.07/2016). Mice were housed under controlled conditions (12 h light/dark cycle at 21 ± 1°C), and they had free access to soy-free RM3 chow (Special Diet Service, Whitman Essex, UK) and tap water. All surgical operations (pellet or pump implantations, gonadectomies and mammary transplantations) were performed under isoflurane (Piramal Healthcare, Northumberland, UK) or 2,5% tribromoethanol (Avertin; Sigma-Aldrich) anesthesia. The mice also received both preoperative and postoperative analgesia for 3 days (0,15–0,5 mg/kg buprenorphine, Temgesic; Schering Plough) and carprofen (5 mg/kg, Rimadyl, Pfizer) postoperatively. To obtain tissue samples, mice were terminally anesthetized with 2,5% tribromoethanol or CO_2 asphyxiation and blood was withdrawn from the heart, followed by euthanasia by cervical dislocation.

4.2 HSD17B1TG mice (I-III)

Transgenic mice expressing the human HSD17B1 gene under the CMV-enhanced chicken beta-actin promoter (HSD17B1TG mice) were previously generated (Saloniemi et al., 2007) and used in this study. Heterozygous females with the FVB/N genetic background of the line with the strongest universal expression (line 013) were used in these studies. In addition, estrogen reporter mice (ERELuc mice; Lemmen et al., 2004) bearing the transgene with 3 estrogen-responsive elements and a TATA box ahead of the luciferase reporter gene were used in studies II and III. In these studies, HSD17B1TG males and ERELuc females were crossbred, and the resulting Bi-TG females heterozygous for both transgenes in the hybrid FVB/N × C57BL6J background were used for experiments. The model shows constant expression of human HSD17B1 and ERE-driven luciferase reporter gene. In these Bi-TG mice, estrogens bind to ESRs which leads to translocation of the hormone/receptor complex into the nucleus, resulting in ERE-driven luciferase reporter gene expression (Figure 4).

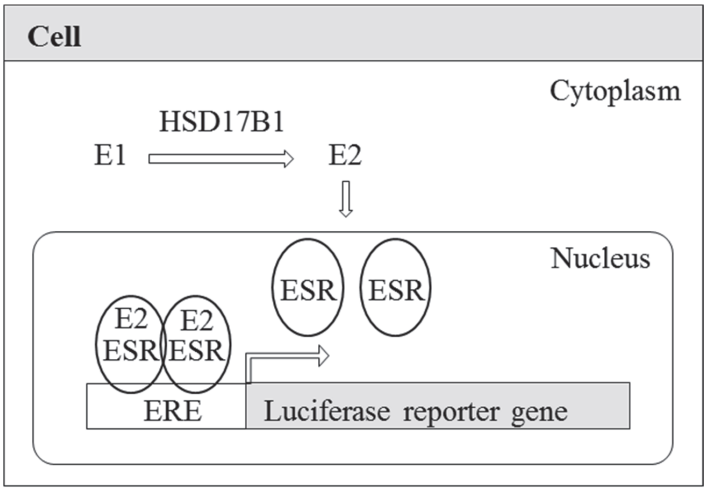

Figure 4. In Bi-TG mice luciferase reporter gene is transcribed after activated ESRs are bound to ERE sequence.

As previously described by Saloniemi et al. (2007), HSD17B1TG mice undergo several androgen-dependent phenotypic changes at the age of 4 months, such as lack of upper nipples, male-like anogenital distance (AGD), lack of vaginal opening, partially unseparated vagina and urethra, and a phenotype resembling human benign ovarian serous cystadenoma. The serum concentrations of sex steroids (E2 and testosterone) were normal at adulthood. However, at fetal age, the intratissular testosterone and E2 concentrations were significantly increased in HSD17B1TG fetuses, as measured at embryonic day 17.5.

4.3 Histology and immunohistochemistry (I-III)

The mouse tissues collected after euthanasia were fixed in 4% paraformaldehyde (PFA, 12 h−24 h) or 10% formalin (at least 24 h) at room temperature. After being washed in ethanol, the tissues were embedded in paraffin and cut into 5 µm sections and stained with hematoxylin and eosin or processed for immunohistochemistry. For analysis, all stained slides were scanned with a Pannoramic 250 Flash II digital slide scanner (3DHISTECH).

Sections for immunohistochemistry were rehydrated and antigen retrieval was performed by boiling in 10 mM citrate buffer (pH 6,0) or 10 mM TRIS-EDTA buffer (pH 9,0). Endogenous peroxidase activity was blocked by incubating sections in H_2O_2 (1%−3%) at room temperature. Primary antibodies were incubated overnight at +4°C for 1 h in RT (anti-cytokeratin 19). All primary antibodies used in this study are listed in the Table 2. All primary antibodies were

anti-human, excluding anti-mouse Ki-67 antibody. Primary antibodies were detected by using an anti-mouse and anti-rabbit Dako HRP-labeled EnVision+ system (Dako). Visualization was performed using a DAB+ substrate (Dako). The primary antibody for Ki-67 antigen was detected by biotinylated anti-rat IgG (Vector) and visualized by a Vector Vectastain Elite ABC-kit (Vector). All sections were counterstained with Mayer´s hematoxylin. The total number of stained nuclei was determined by using ImageJ (ver.1.48), and the percentage of these Ki-67 positive nuclei was used for statistical analyses. Over 200 endometrial epithelial (II) and mammary epithelial cells (cytokeratin 19 positive cells, III) from different fields were counted for Ki-67. The expression of ESR1 and progesterone receptor were scored according to the Allred scoring method (Allred et al., 1998). Briefly, immunostaining was rated by an intensity score ranging from 0 (negative) to 3 (strong staining) and by a proportion score ranging from 0 (0% positive) to 5 (100% positive).

Table 2. Primary antibodies used in the experiments

Primary antibody	Concentration	Manufacturer
anti-NCL-PCNA made in mouse (PC10)	1:500	Novo-castra Laboratories Ltd
anti-Ki-67 made in rat (TEC-3)	1:25(II), 1:500(III)	Dako
anti-ESR1 made in mouse (1D5)	1:100	Dako
anti-progesterone receptor made in rabbit (A0098)	1:100	Dako
anti-keratin 5 made in rabbit (EP1601Y)	1:100	Thermo Scientific
anti-alpha actin made in mouse (1A4)	1:1000	Santa Cruz Biotechnology
anti-cytokeratin 19 made in rabbit (EPR1579Y)	1:500	Epitomics
anti-calponin made in rabbit (EP798Y)	1:2000	Epitomics
anti-HSD17B1 made in mouse	1 µg/ml	Developed by our group

4.4 Determination of HSD17B1 activity *in vivo* (I, III)

HSD17B1TG and WT females were slowly injected *i.v.* with radioactive [^{3}H]-E1 (61 µg/kg, 2,5 µl/g, 555,000 cpm/µl, ~1,6 Mbq/mouse, Perkin Elmer) dissolved in ethanol:saline (20:80 by vol.) into 4-month-old mice. The mice were terminally anesthetized, and 15 minutes after substrate injection, blood was withdrawn from the heart. After euthanasia, tissues were dissected, snap-frozen in liquid nitrogen and stored at −80°C. The frozen tissues were homogenized by Ultra-Turrax in 500 µl ice-cold 50 mM Tris-HCl buffer (pH 7,4), and extracted twice with isopropylether. The ether was evaporated under nitrogen flow and the steroids were dissolved in acetonitrile-water (48:52 by vol.). Finally, the sample [^{3}H]-E1 and [^{3}H]-E2 were separated with HPLC (Waters™ 2695, Waters Corporation) and

counted by an on-line beta-counter (Packard Flow Scintillation Analyzer). The HSD17B1 activity was based on the percentage of [^{3}H]-E1 converted to [^{3}H]-E2.

4.5 Hormonal and inhibitor treatments

4.5.1 Mouse uterotrophic assay (I, II)

The standard uterotrophic assay is a well-defined measure for evaluating the estrogenic activity of a compound *in vivo,* as estrogens increase uterus weight in a dose-dependent manner. In general, the assay utilizes young adult females after ovariectomy with sufficient time for uterine tissue to regress or immature females before puberty. The effect of increased estrogen action in uterus is observed as increased uterus weight (Clode, 2006). Accordingly, the properties of HSD17B1 were studied by comparing the uterus response in immature HSD17B1TG mice and wild-type (WT) mice by administering vehicle (corn oil, Sigma-Aldrich), 1 µg/kg/d of E1 (Sigma-Aldrich), or 50 µg/kg/d of E2 (Sigma-Aldrich) *i.p.* in a 50 µl volume. Injections were carried out once a day from the age of 15 days to 19 days. On day 20, the mice were sacrificed and the uterus was dissected and weighted. The assay was also used to study the efficacies of 2 different HSD17B1 inhibitors, an estrone C-15 derivative (compound 21 in Messinger et al., 2009, EC-15) and a novel steroidal core inhibitor (SC), using the Bi-TG mice. At the age of 15 days, Bi-TG and ERELuc control females were injected *s.c*. with vehicle or 0,3 µg/kg/day of E1 or 0,3 µg/kg/day E1 together with HSD17B1 inhibitors (25 mg/kg/day) in 100 µl of corn oil (Sigma-Aldrich) for 5 days. After cervical dislocation, the wet weights of the uteri were recorded. Half of the uteri were snap-frozen in liquid nitrogen and used later for luciferase activity measurement *ex vivo*, and the other half were fixed and used for histology and immunohistochemistry.

4.5.2 Superovulation treatment (I)

HSD17B1TG females were injected *i.p.* with 5 IU of pregnant mare serum gonadotropin (PMSG, Sigma-Aldrich) in 100 µl of PBS. Second injection of 5 IU of hCG (Schering-Plough), *i.p.* in 100 µl of PBS was carried out after 49 hours. To mimic normal estrous cycle, two superovulation treatments were carried out in a period of 8 days. After two treatments, mice were euthanized and ovarian and uterine samples were collected and used for histological analyses.

4.5.3 Progestin treatment (I)

Adult HSD17B1TG mice at the age of 4 to 5 months were treated with medroxyprogesterone acetate MPA pellets (16 mg/kg/d) or vehicle pellets (Innovative Research of America) for 2 weeks. The pellets were inserted subcutaneously to anesthetized mice. After 2 weeks, mice were euthanized, and ovarian and uterine samples were collected and used for histological analyses.

4.5.4 Inhibitor treatment (I)

A specific HSD17B1 inhibitor (compound number 49; Messinger et al., 2009) was delivered (10 mg/kg/d) in dimethyl sulfoxide/propanediol (1:1 by vol., Merck) to HSD17B1TG mice for 6 weeks by subcutaneous minipumps (Alzet #2004). After 3-week of dosing, the pumps were replaced with the new ones. At the age of 5,5 months mice were euthanized to obtain ovarian and uterine samples for analysis.

4.5.5 Testing of the efficacy of HSD17B1 inhibitors on ERELuc luciferase reporter activity in Bi-TG mice (II)

The effect of HSD17B1 inhibitors on activation of ESRs in the presence of E1 was studied in 2-month-old gonadectomized female Bi-TG and ERELuc mice. Two weeks after gonadectomy, the mice were treated subcutaneously with either vehicle, 0,3 µg/kg/day of E1 or 0,3 µg/kg/day of E1 + 20 mg/kg/d of HSD17B1 inhibitor (a steroidal core inhibitor; SC) *s.c.* in 100 µl corn oil for 5 consecutive days. The mice were then euthanized and tissues were collected, snap-frozen in liquid nitrogen and stored at −80°C until the luciferase activity in liver was analyzed *ex vivo*.

4.5.6 Measuring of luciferase activity ex vivo (II, III)

Bi-TG mice crossbred from ERELuc-reporter mice and HSD17B1TG mice were used for assessing E1 activation. Frozen tissues from the Bi-TG and ERELuc females were homogenized by an Ultra-Turrax in 500 µl of lysis buffer (25 mM Tris acetate, pH 7,8, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM dithiotreitol), containing protease inhibitor (Complete Mini-proteinase inhibitor, Roche Diagnostics). The homogenate was centrifuged for 30 min at 1000g, and luciferase activity was determined from the supernatant with a Luciferase assay kit (BioThema, Handen) following the manufacturer's instructions. The light

produced by luciferase enzyme was measured with a Victor Multilabel Counter (PerkinElmer), and the luciferase activity was normalized against the protein concentration in the sample determined by a Protein Assay Kit (BCA Protein Assay Kit, Pierce Chemical) according to the manufacturer's instructions.

4.6 Mammary gland transplantations (III)

In transplantation experiments, 18- to 20-day old FVB/N female mice were used as hosts and 20-day to 6-month-old HSD17B1TG and WT females were used as donors. Under isoflurane anesthesia, the developing mammary parenchyma of the host was removed by clearing the mammary fat pad between nipples 4 and 5 and the inguinoabdominal lymph node. A piece of mammary epithelium from the HSD17B1TG donor was transplanted onto the right side and WT epithelium was transplanted onto the left side of the remaining mammary fat pad of each host. The hosts were sacrificed 4 months later and the transplanted mammary glands were divided into three parts and processed for histological and morphological analyses and RNA expression.

4.7 Morphological analysis of mammary gland (III)

For morphological analysis, mammary glands were spread on a glass slide and fixed in Carnoy's fixative (acetic acid-ethanol) for several days at +4°C. The slides were rinsed with ethanol and tap water and stained with carmine-alum for 3−4 days. Stained slides were dehydrated in a series of ethanol washes (70−100% ethanol), cleared in xylene, and finally mounted onto glass slides in Permount.

4.8 HSD17B1 activity measurement in tissues *ex vivo* (III)

HSD17B1 activity in transplanted HSD17B1TG and WT mammary glands were confirmed by analyzing the conversion of labelled [^{3}H]-E1 to [^{3}H]-E2. The tissues were homogenized in 10 mM KH_2PO_4 (pH 7,5) containing 1 mM EDTA, protease inhibitor (Complete Mini, Roche Diagnostics GmbH), 0,01% BSA and 10% glycerol. Protein concentrations of the homogenates were determined by using BCA protein Assay Kit (Pierce Chemical) according to manufacturer´s instructions. Five µg of protein was mixed with [^{3}H]-E1 (PerkinElemer, final [^{3}H]-E1 concentration in the reaction 6,3 nM) and 1,4 mM NADPH, these were incubated in 37°C for 40 minutes. Steroids were extracted from samples twice with diethylether (Merck), redissolved into 48% acetonitrile in water. The [^{3}H]-E1 and

[^{3}H]-E2 were separated by HPLC (Waters™ 2695, Waters Corporation) and counted by beta-counter (Packard Flow Scintillation Analyzer).

4.9 Hormone measurements (III)

Blood samples from adult HSD17B1TG mice were stored at +4°C for 24h and serum was separated by centrifugation and stored at −20°C. Serum prolactin concentration was measured by RIA with a previously published method (Bergendahl et al., 1989).

4.10 Microarray (III)

Gene expression profile of transplanted HSD17B1TG and WT mammary gland was performed from tissues isolated from 4-months-old mice (three mice per group). RNA was extracted from tissues by TRIzol method (Bioline Ltd) according to manufacturer´s instructions. The RNA was treated with Deoxyribonuclease I (Invitrogen) as instructed. The DNase treated RNA was further purified using NucleoSpin®RNA XS Kit (Macherey-Nagel). Purified RNA was amplified with Illumina® TotalPrep™ RNA Amplification Kit (Applied Biosystems). Generated cRNA sample were hybridized into Illumina's MouseWG-6 v2 Expression Bead Chips according to Illumina® Whole-Genome Gene Expression Direct Hybridization Assay Guide. Analysis of the arrays was performed with IPA® software (Ingenuity Systems, Inc.) Microarray and analysis were performed by The Finnish Microarray and Sequencing Centre (Turku).

4.11 Analysis of human HSD17B1 expression in transplanted mammary glands (III)

DNase-treated RNA was used in cDNA synthesis carried out via a DyNAmo cDNA Synthesis Kit (Thermo Scientific). The cDNA was diluted 1:100 for quantitative reverse transcriptase PCR (RT-qPCR). To analyze the expression of human *HSD17B1* and mouse *Hsd17b1* genes in transplanted mammary glands, RT-qPCR analysis was performed using DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes). All samples were run in triplicate reactions. Mouse Ppia (Tm 63°C) was used as an endogenous control to equalize the amount of RNA in samples. Primer sequences and RT-qPCR conditions for analyzing the expression of *HSD17B1* (Tm 56,4°C) are described by Saloniemi et al. (2007), and the PCR reactions included 34 cycles.

4.12 Estrogen receptor antagonist and HSD17B1 inhibitor treatment of mice with transplanted mammary gland tissue (III)

Five-month-old female mice transplanted with WT and HSD17B1TG mammary gland epithelium were treated *s.c.* with ESR antagonist (ICI 182,780, 17 mg/kg injected twice a week, Selleckhem) or with an HSD17B1 inhibitor EC-15 (compound 21 in Messinger et al., 2009) provided by Forendo Pharma Ltd., Turku, Finland). Mice were injected daily with 10 mg/kg of either treatment provided subcutaneously in 10% DMSO and 90% Phosal® 50PG. A subgroup of mice in the ICI 182,780 treatment group was also treated with 5 mg/kg carprofen (Rimadyl, Pfizer). After 4 weeks of treatment, the mice were sacrificed and mammary gland tissues were collected for analysis.

4.13 Statistical analyses (I-III)

Statistical analyses were performed using the SigmaStat 3.1 program (SYSTAT Software Inc.) or GraphPad Prism 6 or 7 (GraphPad Software, Inc.). Statistically significant differences ($p \leqslant 0.05$) between the control and the treatment group were determined by Student's t-test (normally distributed data) or the Mann-Whitney U test (not normally distributed data). When several groups were compared, one-way ANOVA followed by Tukey's post hoc test (normally distributed data) or Kruskal-Wallis one-way ANOVA of Ranks followed by Dunn's multiple comparisons test (non-normally distributed data) were used. The data are expressed as means ± SEM.

5 RESULTS

5.1 Estrogen-induced endometrial hyperplasia in HSD17B1TG mice (I)

5.1.1 Endometrial hyperplasia in HSD17B1TG mice (I)

Histological analysis of the uterine tissue of HSD17B1TG females revealed that the mice developed endometrial hyperplasia by the age of 4 months with 100% prevalence. At 4 months of age, 25% of the endometrial hyperplasia detected had atypical nucleoli, whereas the rest of the samples (75%) presented with typical hyperplasia of the luminal and glandular epithelium (I, Figure 1A-D and Table 1). The proliferation of the hyperplastic glands was confirmed by immunostaining for PCNA (I, Figure 1E-G). Although 25% of animals developed atypical hyperplasia, progression to endometrial carcinomas was not observed by the age of 12 months. These mice also showed anovulatory ovaries caused by inefficient luteinization of ovarian follicles (I, Figure 1 H-I).

5.1.2 Increased HSD17B1 activity in vivo in HSD17B1TG mice (I, III)

Conversion of E1 to E2 was studied by injecting radioactive E1 into HSD17B1TG and WT mice *i.v.* and by measuring the proportions of radioactive signal obtained from E1 and E2 after 15 minutes post administration. The data revealed that the E2/E1 ratio was significantly higher (1,5 to 4 times higher) in all the tissues examined (uterus, serum, liver, heart and mammary gland) in HSD17B1TG female mice compared with that in the WT tissues, except in the ovaries (I, Figure 3; III, Figure 2A). In HSD17B1TG female mammary tissue, a significant proportion (57–85%) of the administered E1 was converted to E2, while in WT mammary tissue, only 6–11% of E1 was found to be converted to E2. The similar E2/E1 ratios in WT and HSD17B1TG ovaries is likely explained by high levels of endogenous Hsd17b1 in the ovaries.

5.1.3 HSD17B1TG mice as a model for human endometrial hyperplasia (I)

Endometrial hyperplasia detected in the HSD17B1TG mice could, as in humans, be a result of persistent anovulation and decreased cyclic progesterone secretion.

To test the consequences of ovulation for the phenotype, standard gonadotropin stimulation with PMSG and hCG injections was provided in a 4-day intervals to mimic the normal estrous cycle in mice. Induction of ovulation reversed the endometrial hyperplasia in HSD17B1TG mice (I, Figure 4A-B and Table 3), indicating that the endometrial phenotype was a result of ovarian dysfunction. Treatment with a high dose of MPA for 2 weeks also reversed the endometrial hyperplasia, but ovulation was not induced by the progestin treatment (I, Figure 4C-D and Table 3). These data suggest that the endometrial hyperplasia in the HSD17B1 mice shares an endocrine dependence similar to that observed for human diseases. Thus, we analyzed whether HSD17B1 inhibitors could also serve as treatment option for endometrial hyperplasia of the HSD17B1TG mice. To this end, HSD17B1TG females were treated with a specific HSD17B1 inhibitor, compound number 49 reported by Messinger et al. (2009), for 6 weeks. Interestingly, the inhibitor treatment completely reversed the hyperplastic morphology of the glandular endometrial epithelium but, as expected, did not induce luteinization of ovarian follicles in HSD17B1TG mice (I, Figure 5 and Table 3); thus, the inhibitor behaved similarly to progestin. However, the endometrial luminal epithelial cells remained proestrous-like in 50% of animals treated with the inhibitor, which was often associated with focal endometrial inflammation. Surprisingly, the expression levels of proliferation marker PCNA were similar between the inhibitor- and vehicle-treated groups as analyzed by immunohistochemistry. In summary, the data indicate that endometrial hyperplasia in HSD17B1TG mice is the sum of the effects of ovarian dysfunction and increased local E2 production in the uterus.

5.2 HSD17B1 expression leads to enhanced estrogen response in adult cycling females (II, III)

In this study, we used Bi-TG mice (ERELuc mice crossbred with HSD17B1TG mice) to combine two estrogen responsive assays *in vivo*, the mouse uterotrophic assay and a luciferase reporter gene assay for studying the estrogen response. In the first study, luciferase activity was measured in several tissues of 2-month-old female mice without an external substrate added. The results showed increased reporter gene activity in a variety of tissues of adult Bi-TG female mice compared with that in the control ERELuc females. The tissues with increased activity included, for example, mammary gland (III, Figure 2B), muscle, kidney, liver and uterus tissue (II, Figure 1). In the Bi-TG ovary, the luciferase activity was similar to that measured in the ERELuc mice ovary, likely due to the high expression of the endogenous *Hsd17b1* in the ovaries (Hakkarainen et al., 2015). Furthermore, increased estrogen action was observed in the uterus, as significantly increased

uterus weight (wet weight 798 mg) was measured in the Bi-TG compared with that in the ERELuc mice (wet weight 98 mg) at the age of 2 months (II, Figure 2A-B). However, the effect of the transgene expression on the uterus weight was stronger in these Bi-TG mice in the FVB/N × C57BL6J hybrid background compared with that measured in mice in the FVB/N genetic background. Similarly to the HSD17B1TG mice in the FVB/N background, the Bi-TG females in the hybrid genetic background also developed the endometrial hyperplasia phenotype (II, Figure 2C).

5.2.1 Reporter gene activity in the liver of Bi-TG females executes as a sensitive readout for HSD17B1 inhibitor testing (II)

As the Bi-TG females had the highest luciferase activity in the liver, we further studied the potential use of the liver luciferase reporter gene activity as a readout for HSD17B1 inhibitor action. The effect of a steroidal core HSD17B1 inhibitor, SC, was then tested in ovariectomized adult female mice. In the Bi-TG mice, a dose of 0,3 μg/kg/day of E1 for 5 days significantly increased the luciferase activity in the liver, while the increase was significantly reduced by a dose of 20 mg/kg/day HSD17B1 inhibitor (SC) (II, Figure 3). As expected, a similar treatment in ERELuc mice did not cause any increase in luciferase activity.

5.3 Immature uterus responses in HSD17B1TG mice (I,II)

5.3.1 HSD17B1 enhances estrogen action in immature mouse uterus (I)

Human HSD17B1 expression enhances uterine growth, a sign of estrogen exposure, when exposed to weak estrogen. This effect was demonstrated by a study in which immature HSD17B1TG females and WT littermates were treated for 5 days with vehicle, 1 μg/kg/d of E1, or 50 μg/kg/d of E2, used as a positive control. The E1-induced increase in uterus weight was twice as high in HSD17B1TG animals as in WT mice (I, Figure 2 and Table 2). A summary of uterus weights is presented in Table 3. As expected, E2 also markedly stimulated uterine growth, and the magnitude of this growth response was identical between WT and HSD17B1TG mice.

5.3.2 HSD17B1 inhibitors reduce E1-induced uterus response in immature Bi-TG female mice (II)

The uterotrophic assay in immature mice was also used as a readout to test the effect of HSD17B1 inhibitors (EC-15 and SC) in Bi-TG mice after E1 administration. The uterotrophic assay was first performed with two doses, 0,3 and 1 µg/kg/day of E1, to optimize the dose for 5 days. In this study, the uterus weight of both Bi-TG mice and ERELuc mice increased with E1 dose in a dose-dependent manner, and the lower dose was chosen for further studies. We then tested the efficacy of HSD17B1 inhibitors (25 mg/kg/day) to reduce the E1-induced increase in uterus weight, and the results showed that EC-15 markedly decreased the uterus weight. However, SC-treated mice showed only a tendency toward lower uterus weight compared with mice treated with the same dose of E1. A summary of uterus weights is presented in Table 3.

Table 3. Uterus weights in uterotrophic assays in immature mice

	Vehicle	E1 0,3 µg/kg	E1 1 µg/kg
	Uterus weight (mg ± SEM)		
WT	9,8 ± 0,38		11,8 ± 0,79
HSD17B1TG	11,1 ± 0,39		19,5 ± 0,78***
ERELuc	10,2 ± 1,2	12,4 ± 0,4	17,2 ± 2,8
Bi-TG	13,6 ± 1,7	23,7 ± 2,3**	33,9 ± 2,6**
Bi-TG EC-15	13,2 ± 1,1	15,6 ± 1,0*	28,2 ± 2,8
Bi-TG SC	13,8 ± 1,2	18,6 ± 1,7	30,0 ± 1,5

*** p<0,001 Compared to the WT mice treated with E1

** p≤0,01 Compared to the ERELuc treated with E1

* p≤0,05 Compared to the Bi-TG mice treated with E1

To further confirm the diminished uterine estrogen response after HSD17B1 inhibitor treatment, the luciferase activity was measured *ex vivo* in these mice. In these studies, we observed that the EC-15 treatment significantly reduced E1-induced luciferase activity and uterus weight in the uterus of the Bi-TG mice compared with mice treated with E1, while SC tended to reduce luciferase activity and uterus weight but the response was not statistically significant (II, Figure 4). Interestingly, the activity of the luciferase was consistent with the uterine weights of the non-treated mice as well, as the luciferase activity was significantly increased in the Bi-TG female uterus compared with that in the uterus of the ERELuc mice treated with E1.

To assess the mechanisms through which HSD17B1 inhibitors affect proliferation, immunohistochemical staining for Ki-67 was performed for the uteri using specimens from the uterotrophic assay. Indeed, the E1 treatment (0,3 µg/kg/day) markedly increased the number of Ki-67-positive nuclei in the Bi-TG mice, both

in the glandular and luminal epithelial cells of the uterus (II, Figure 5). Additionally, the treatment with the HSD17B1 inhibitors (EC-15 and SC, 25 mg/kg/d) significantly decreased the number of nuclei stained in the glandular but not luminal epithelium. However, EC-15 showed a tendency toward a reduced number of stained nuclei in the luminal epithelium as well. The treatment with E1 did not affect Ki-67-positive staining in the luminal or glandular cells of the uterus in ERELuc mice.

5.4 HSD17B1 expression leads to inflammation-assisted myoepithelial breakage in mammary glands (III)

5.4.1 HSD17B1TG females have stimulated mammary gland and increased incidence of mammary cancer (III)

HSD17B1TG mice showed enlarged mammary gland ducts filled with secretion by 4 months of age (III, Figure 1A). By the age of 10 months, HSD17B1TG females displayed enhanced lobuloalveolar development resembling the mammary phenotype observed in mid-pregnancy (III, Figure 1B). The mammary gland ducts contained a milk-like secretion and lipid-filled droplets. The lactating mammary phenotype was further enhanced in 18-month-old female HSD17B1TG mice (III, Figure 1C). In HSD17B1TG mice, unlike in WT mice, serum prolactin concentrations increased with aging (III, Figure 1D). By the age of 18 months, three out of six HSD17B1TG females developed mammary gland tumors, while no tumors were observed at the same age in WT mice. Some of the tumors developed histological features similar to those of human ductal carcinoma, gradus I or II. Tumors in HSD17B1TG females contained areas with the lactating phenotype and inflammatory cells.

5.4.2 Mammary gland-restricted HSD17B1 expression induces formation of mammary lesions, epithelial cell proliferation and intratissue E2 production (III)

The effect of local HSD17B1 expression in the mammary glandular epithelium was studied by transplanting WT and HSD17B1-expressing mammary epithelium to WT mammary glands devoid of endogenous parenchyma. The morphology of the glands 4 months after transplanting the HSD17B1-expressing epithelium revealed lesions formed at the sites of ducts and alveoli, while no lesion formation in glands with WT epithelium were found. Histological analysis of the HSD17B1

expressing mammary lesions revealed massive periductal inflammatory cell infiltration into and around certain ducts while no inflammatory cell infiltration was observed in glands of WT epithelial transplants (III, Figure 3A). Increased HSD17B1 expression and activity in mammary glands with HSD17B1 expressing epithelium was confirmed (III, Figure 3B-C). Furthermore, the expression of the proliferation marker, Ki-67, was significantly increased in the transplanted HSD17B1-expressing mammary epithelial cells compared with that in WT epithelial cells (III, Figure 4A-B). However, ESR1 and progesterone receptor expressions were similar in transplanted HSD17B1TG and WT mammary epithelium (III, Figure 4A, C-D). Despite the increased epithelial cell proliferation, the mice with local HSD17B1-expression in mammary parenchyma did not develop mammary tumors over the 18-months follow-up period.

5.4.3 HSD17B1-driven periductal mastitis associates with disruption of luminal epithelial cell layer and breakdown of continuous myoepithelium (III)

The altered structure of the HSD17B1-induced lesions were further analyzed with epithelial and basal/myoepithelial cell markers. Immunohistochemical staining of luminal epithelial cell marker keratin 19 (KRT19) and basal/myoepithelial cell markers keratin 5 (KRT5), calponin (CNN1) and actin (ACTB) revealed a link between HSD17B1 induced periductal inflammation and focal disruption of the epithelial luminal cell and myoepithelial cell layers at the site of inflammatory cell infiltration in the lesions (III, Figure 5A-D). Furthermore, IPA analysis of microarray data obtained from mammary glands with transplanted WT and HSD17B1TG epithelium revealed changes in gene expressions linked to inflammatory disorder and connective tissue disorder (Table 4).

Table 4. Microarray analysis

IPA analysis of microarray data

Diseases and disorders	p-value	# molecules
Inflammatory Response	1,03E-33 - 1,16E-05	144
Connective Tissue Disorders	2,06E-20 - 6,53E-06	84
Inflammatory Disease	2,06E-20 - 1,16E-05	121
Skeletal and Muscular Disorders	2,06E-20 - 6,53E-06	89
Immunological Disease	9,70E-20 - 1,15E-05	127

5.4.4 ESR antagonist reduces mammary lesions (III)

The role of estrogen signaling in the HSD17B1-induced inflammatory lesions was investigated by treating the mammary gland transplanted mice with an antiestrogen ICI 182,780 or with HSD17B1 inhibitor, EC-15. The ICI 182,780 treatment markedly reduced the number of established mammary gland lesions and ameliorated the histological phenotype, while the HSD17B1 inhibitor tended to decrease the lesion number but did not reach statistical significance (III, Figure 6A, C). ICI 182,780 reduced mammary epithelial cell proliferation significantly, while the extents of epithelial cell proliferation in vehicle- and HSD17B1-inhibitor-treated mice were similar (III, Figure 6B, C). Carprofen treatment had no effect on lesion formation.

6 DISCUSSION

6.1 The role of HSD17B1 in estrogen biosynthesis

In the present study, we analyzed the role of HSD17B1 in estrogen activation *in vivo* by using HSD17B1TG female mice. Several human HSD17Bs are capable of converting E1 to E2, including HSD17B1 (Miettinen et al., 1996; Poutanen et al., 1993), HSD17B7 (Törn et al., 2003) and HSD17B12 (Luu-The et al., 2006). However, the role of these enzymes in extra-gonadal tissues in E2 formation is controversial. The expression of HSD17B1 is low in peripheral tissues but the catalytic efficacy is markedly higher than that of HSD17B7 or HSD17B12 (Day et al., 2008b; Luu-The et al., 2006), suggesting a central role of HSD17B1 enzyme in E2 formation in peripheral tissues. A study with T-47D breast cancer cells demonstrated that HSD17B1 played the most significant role on the conversion from E1 to E2 compared with other reductive HSD17Bs. The conversion of E1 to E2 was decreased significantly by 78,5% after knocking down of HSD17B1, but after knocking down of HSD17B7 the conversion decreased only 3,1% and knocking down of HSD17B12 the conversion decreased 1,0% (Zhang et al., 2015).

In vivo studies have further provided evidence of a role of human HSD17B1 in E1 activation. Xenografting HSD17B1-transfected MCF-7 cells into immunodeficient mice resulted in enhanced estrogen-dependent growth of tumors in the presence of E1 (Husen et al., 2006a). However, the human enzyme is not fully specific to estrogenic substrates, but it can also activate androgens as previously shown in HSD17B1TG female mice presenting a masculinized phenotype by enhanced conversion of A-dione to testosterone (Saloniemi et al., 2007, 2009). Furthermore, the enzyme can catalyze the formation of A-diol from DHEA and the formation of both 3beta-diol and 3alpha-diol from DHT (Aka et al., 2010). 3Beta-diol and 3alpha-diol can activate ESR2 and, to some degree, also ESR1, affecting the estrogen signaling of these steroids (Aka et al., 2010). However, the substrate preference also depends on the availability of substrates and cofactors in the tissues, whereas the amount of substrates depends on menopausal status and the combination of steroid-metabolizing enzymes expressed within the tissue (Day et al., 2008a; Lukacik et al., 2006). During fetal life, excess androgenic precursors are available, resulting in increased A-dione to testosterone conversion and subsequent masculinization of HSD17B1TG females (Saloniemi et al., 2007). However, estrogens have the strongest affinities to HSD17B1, whereas DHT and DHEA have significantly weaker affinities (Han et al., 2017). In this study, increased local E2 formation from administered E1 was observed in all extra-gonadal HSD17B1TG tissues. In addition, the dose of 1 µg/kg/day of E1 used in

the study was sufficient to markedly increase the estrogen-dependent growth of the uterus in immature HSD17B1TG mice, while a similar response was not observed in WT mice. This result further demonstrated the ability of HSD17B1 to enhance estrogen action in target tissues.

6.2 The role of HSD17B1 in endometrial hyperplasia

In this study, we showed that expression of human HSD17B1 in mice enhanced estrogen action in the uterus and resulted in endometrial hyperplasia and anovulation. Endometrial hyperplasia is shown to be a precursor lesion of endometrial carcinoma and is highly dependent on the estrogen/progestin balance (Emons et al., 2000; Lax, 2004). Estrogens and their metabolites are more strongly correlated to endometrial cancer patients than to healthy postmenopausal women (Audet-Walsh et al., 2011). Particularly, HSD17B1 mRNA and protein levels are higher in ESR1-positive low-grade endometrial cancer than controls, whereas the expression level of HSD17B7 and HSD17B12 do not vary between groups (Cornel et al., 2012). In addition, the increased estrogen levels in the ectopic lesions of endometriosis patients was associated with elevated levels of HSD17B1 but not of the other HSD17B enzymes (Delvoux et al., 2014).

Continuous exposure to estrogens in the absence of sufficient levels of progestins also promotes the development of endometrial cancer (Emons et al., 2000; Lax, 2004). As in humans, endometrial hyperplasia and carcinoma can be induced by continuous estrogen exposure in rodents (Gunin et al., 2005). However, in HSD17B1TG mice endometrial carcinomas were not observed; thus mechanisms other than overexpression of HSD17B1 are required for uterine carcinogenesis. To further support the role of estrogen in uterine carcinogenesis, mice with an ESR1 deficiency were resistant to estrogen in the uterus, which remained hypoplastic after E2 treatment (Couse and Korach, 2001), while mice lacking progesterone receptor responded abnormally to estrogen and progestin treatments and developed endometrial hyperplasia (Lydon et al., 1995). Interestingly, in HSD17B1TG mice, endometrial hyperplasia closely resembled the human disease, and the hyperplasia phenotype was restored by ovulation induction or by exogenous progestin treatment that likely normalized the estrogen-progestin ratio indicating that this ratio is essential in the development of endometrial hyperplasia, as in humans.

Treatment with an HSD17B1 inhibitor also reversed the hyperplastic endometrial glandular morphology but only partially blocked the proliferation of luminal epithelial cells. The inhibitor treatment was not sufficient to induce ovulation in these mice. One reason for the insufficiency of ovulation induction could be failure

in the programming of the hypothalamus-pituitary-gonadal axis and a lack of a luteinizing hormone surge to induce ovulation (Robinson, 2006).

6.3 HSD17B1TG mice as a model for HSD17B1 inhibitor screening

HSD17B1 enzyme is detected and overexpressed in estrogen-dependent tumors such as those associated with uterine and breast cancers (Chanplakorn et al., 2010; Cornel et al., 2012; Gunnarsson et al., 2003; Shibuya et al., 2008; Suzuki et al., 2000). Activation of E1 in target tissues increases the intratumoral concentration of E2 independently of the serum concentration (Cornel et al., 2012; Miyoshi et al., 2001; Shibuya et al., 2008). Inhibition of this enzyme is therefore an attractive target for the treatment of estrogen-dependent diseases. Aromatase inhibitors have been successfully used as an endocrine therapy in postmenopausal breast cancer by lowering estrogens, and HSD17B1 inhibitors are expected to lower E2 concentrations in target tissues. Bone loss is a known side effect of aromatase inhibitors because of the nearly complete depletion of circulating estrogens (Ribnikar et al., 2017; Sasano et al., 1997). In contrast, HSD17B1 expression is very low in bone; thus, HSD17B1 inhibitors would possibly have a weaker effect on bone than aromatase inhibitors would (Dong et al., 2009; Purohit et al., 1992). In inhibitor development, high HSD17B1 inhibition and low inhibition of its physiological counterpart HSD17B2 are essential for effectively reducing E2. In addition, the inhibitors must not activate ESR1 to avoid stimulation of tumor growth. Consequently, it is necessary to develop an *in vivo* test system for the evaluation of active compounds.

Most validation studies characterizing candidate HSD17B1 inhibitors have been conducted with purified proteins of HSD17B1 or *ex vivo* using HSD17B1-transfected cell lines or cell lines naturally expressing HSD17B1 (Ayan et al., 2012; Cadot et al., 2007; Henn et al., 2012; Laplante et al., 2008a). The most studied ESR1-positive breast cancer cell lines T-47D and MCF-7 naturally express HSD17B1 enzyme, however, the expression in MCF-7 cells (87 thousand copies of mRNA/µg RNA) is lower than in T-47D cells (697 thousand copies of mRNA/µg RNA) (Aka et al., 2012b). This leads to the use of T-47D cells in several studies (Ayan et al., 2012; Cadot et al., 2007; Henn et al., 2012; Laplante et al., 2008a), moreover, the transfection of *HSD17B1* is also applied to get higher gene expression (Husen et al., 2006a, 2006b).

The risk of unanticipated side effects of the inhibitors *in vivo*, when using only *ex vivo* studies for inhibitor testing, has to take into account. The effect of inhibitors on other targets is not necessarily known, inhibitors could, for example, modulate lipid pathways (Prehn et al., 2009). The analysis of metabolomics before *in vivo*

studies would be useful in analyzing the side effects of drug responses beforehand (Prehn et al., 2009). Studies conducted in mice, however, should appreciate the fact that sex steroid metabolism is different in mice than in humans (Baker et al., 2015; Nokelainen et al., 1996). Furthermore, the structure of the substrate binding pocket of rodent and human enzymes varies to some extent, although the amino acid sequence in the binding pocket is quite similar among different species (Möller et al., 2010; Puranen et al., 1997). This variation may lead to diverse substrate preferences among different species and to alteration in the potency of inhibitors in different species. The rodent enzymes has the largest differences compared with the human enzymes when tested with different inhibitors (Möller et al., 2010) as mouse Hsd17B1 is 63% homologous to human HSD17B1 at the amino acid level (Nokelainen et al., 1996).

In vitro assays are useful in screening different drug compounds. The potential efficacy of compounds as well as their safety must be tested in preclinical *in vivo* models before entering clinical trials. The majority of assays conducted to test HSD17B1 inhibitors *in vivo* have been based on cancer cell xenografts. For example, HSD17B1 inhibitors have been tested with estrogen-dependent T-47D and HSD17B1-transfected MCF-7 human breast cancer cells xenografted to nude mice (Ayan et al., 2012; Day et al., 2008b; Husen et al., 2006a, 2006b). In addition, endometriosis marmoset monkeys have been used for testing the efficacy of HSD17B1 inhibitors, showing that endometriosis-related behavior was decreased by inhibitor treatment (Arnold and Einspanier, 2013). Efficacy studies for HSD17B1 inhibitors have also been carried out using human tissues *ex vivo*. In these studies, tissues from endometriosis patients, eutopic and ectopic endometrial tissue lysates from uterus, were used. The inhibitor used decreased the production of E2 from E1 and corrected the metabolic balance in endometriotic tissue (Delvoux et al., 2014). Xenograft studies and, in particular, monkey studies are laborious and expensive to perform. Therefore, faster and easier preclinical test models would be beneficial and would facilitate the *in vivo* screening of lead compounds for further optimization.

Transgenic mice are highly beneficial for preclinical HSD17B1 inhibitor screening *in vivo*, as most of the inhibitors available are human-specific (Abdelsamie et al., 2015; Möller et al., 2010). One of the mouse models developed for testing HSD17B1 inhibitors is based on transgenic mice expressing human HSD17B1 under MMTV-promoter. The model was used to test HSD17B1 inhibitors by injecting E1 and HSD17B1 inhibitors *i.v.* and then measuring the level of E1 to E2 conversion in blood circulation within 2 minutes (Lamminen et al., 2009). Although the model is fast, the weakness of this model is the short exposure time of inhibitors, and because of the 10-minutes to 1-hour exposure times, the effect of the inhibitors on the estrogen-dependent growth of uterus weight or on the

proliferation of the endometrial epithelial cells cannot be analyzed. The advantages and disadvantages of *in vivo* models used to test HSD17B1 inhibitors are summarized in Table 5.

Table 5. Advantages and disadvantages of *in vivo* models used for HSD17B1 inhibitor testing

In vivo model	Advantages	Disadvantages	References
Breast cancer T-47D cell xenografts	Human derived tumor	Laborous, slow	Ayan et al., 2012; Day et al., 2008a
Breast cancer transfected MCF-7 cell xenografts	Human derived tumor, high expression of *HSD17B1*	Laborous, slow	Husen et al., 2008a, 2008b
Marmoset endometriosis monkey	Similar cycle profile as humans	Expensive, availability	Arnold and Einspanier 2013
MMTV-HSD17B1 transgenic mouse *i.v.* injection	Rapid	Only one endpoint	Lamminen et al., 2009
HSD17B1 expressing transgenic mouse			the present study
Immature uterine growth assay	Several endpoints, rapid	Precise E1 dosing	
Endometrial hyperplasia	Similar to human disease	Long dosing	
Mammary stricted expression	Tissue spesific expression	Long dosing, laborous	

In the present study, the suitability of HSD17B1TG mice for HSD17B1 inhibitor testing was analyzed by using both uterus weight and epithelial cell proliferation as readouts. Furthermore, the endometrial hyperplasia present in HSD17B1TG mice was partly restored by HSD17B1 inhibitor treatment. Additionally, we aimed to develop a model for screening HSD17B1 inhibitors quickly and easily. For this purpose we applied estrogen reporter mice, ERELuc mice (Lemmen et al., 2004), and crossbred them with HSD17B1TG mice to study the influence of human HSD17B1 expression on ESR activation in the absence and presence of E1 supplementation and the effect of HSD17B1 inhibitors in these mice. Previous studies in male ERELuc mice have shown high induction of luciferase gene expression in bone, kidney, liver, adrenal, prostate, colon and esophagus after E2 administration, and the ESR-dependence of reporter gene expression was confirmed by treating mice with ESR-antagonist ICI 182,780, which resulted in reduced luciferase activity (Lemmen et al., 2004). Experiments in Bi-TG mice generated in the present study showed that ESR activation was increased in several

Bi-TG tissues studied in human HSD17B1-expressing mice. A significant increase in luciferase activity was detected in the mammary gland, kidney, liver and uterus of adult females without any exogenous E1 treatment, in line with data showing a high induction of luciferase in liver after E2 administration (Lemmen et al., 2004). The uterus weight of adult Bi-TG mice was dramatically increased compared with that of ERELuc mice and HSD17B1TG mice, and the endometrial hyperplasia phenotype was present, similarly to HSD17B1TG mice. Both HSD17B1TG and Bi-TG mice in the hybrid FVB/N x C57BL6J background showed a greater increase in uterus weight after E1 administration than HSD17B1TG mice in the FVB/N background. This result is in line with previous data showing that mice in the C57BL6J background are more responsive to estrogens than those in the FVB/N background (Roper et al., 1999).

The advantage of using immature Bi-TG mice was that several related but distinct endpoints of estrogen action were determined and the assay was fast and relatively inexpensive. In this study, one of the two inhibitors tested reduced the uterus weight response and luciferase activity measured for uterus *ex vivo*. Furthermore, the inhibitor decreased the number of the positive nuclei determined by Ki-67 staining in the uterus, particularly in glandular compartments. These results indicate that the HSD17B1 inhibitor reduced HSD17B1-dependent ESR-activation and endometrial epithelium proliferation. The uterotrophic assay with immature mice was more sensitive than the assay with ovariectomized adult female mice used in these studies. However, in adult ovariectomized mice, we showed that liver luciferase activity *ex vivo* was increased with E1 administration, and treatment with the HSD17B1 inhibitor restored luciferase activity to the basal level. Thus, liver luciferase activity is a sensitive assay for testing inhibitors as well. Unfortunately, the *in vivo* imaging techniques applied were not sufficiently sensitive to distinguish the difference.

6.4 The role of HSD17B1 in mammary carcinogenesis

Epidemiological studies indicate that HSD17B1 participates in breast carcinogenesis (Gunnarsson et al., 2001, 2003, 2005, 2008; Oduwole et al., 2004; Salhab et al., 2006). HSD17B1 is specially expressed in neoplastic breast epithelial cells, which imitate breast cancer progression (Fu et al., 2010). However, the exact mechanisms underlying the manner in which HSD17B1 affects the development and progression of mammary epithelial lesions or cancer are not clear. In this study, we investigated the effect of HSD17B1 expression on mouse mammary glands by using transgenic mice expressing human HSD17B1. HSD17B1TG females showed a pregnancy-resembling phenotype in the mammary glands. The

lactating phenotype could be caused by the observed increase in serum prolactin concentrations in these mice. In HSD17B1TG mice 50% of females developed mammary tumors at old age, showing histological similarities to human breast cancer. Prolactin has been shown to act as a mammary oncogene, and it can potentiate neoplastic processes with other factors, such as estrogen, in mice (Arendt and Schuler, 2008; Rulli et al., 2002). Moreover, higher levels of prolactin are associated with increased breast cancer risk in women (Tworoger and Hankinson, 2008; Wang et al., 2016). This trend is also observed in rodents, in which elevated prolactin is well known to increase mammary gland tumors (Fernandez et al., 2010; Harvey, 2012). In addition to elevated prolactin levels, ESR activation in the mammary gland was increased in our mouse model, likely affecting tumorigenesis. Estrogens have shown to contribute, in particular, to breast carcinogenesis in postmenopausal women (Stanczyk et al., 2015) by increasing the proliferation of mammary epithelial cells (Mallepell et al., 2006). The phenotypic changes observed in HSD17B1TG female mammary glands are presumably linked to the increase in both prolactin levels and estrogen signaling.

Mammary-specific HSD17B1 expression resulted in lesions at the sites of ducts and alveoli with periductal inflammatory cell infiltration. Estrogens are known to affect the proliferation of mammary epithelial cells (Mallepell et al., 2006), but they also induce leucocyte infiltration into the mammary glands (Aupperlee et al., 2014). HSD17B1-induced lesions with inflammation and epithelial cell proliferation were dependent on ESR signaling, and their formation was inhibited by antiestrogen ICI 182,780 treatment, suggesting that lesion formation and proliferation are dependent on ESR activation in these mice. The mice demonstrated that the local expression of HSD17B1 in the mammary glands is sufficient to induce lesion formation. It has been shown that HSD17B1 affects cancer-related gene expression and growth by activating ESR1 in breast cancer cells (Aka et al., 2017). Mammary-specific expression of HSD17B1 did not lead to cancer formation, indicating that in addition to HSD17B1, other factors are needed for cancer development. Overexpression of aromatase in transgenic female mice resulted in increased estradiol tissue levels in the mammary glands, mammary gland hyperplasia and other preneoplastic changes but was not sufficient for mammary tumor development, demonstrating the need for factors other than estrogen for mammary carcinogenesis (Kirma et al., 2001; Tekmal et al., 1996).

The periductal mastitis in mammary glands exhibiting HSD17B1 expression was associated with disruption of the luminal and myoepithelial cell layers at the sites of inflammatory cell infiltration. Infiltration of immune cells into the breast stroma and tumors is one of the earliest events in breast cancer carcinogenesis (Yeong et al., 2017). Human ESR-positive intraductal breast tumors show frequent focal disruptions in the myoepithelial cell layer, which are considered to be a

requirement for cancer cells to invade (Man and Nieburgs, 2006). In normal mammary glands, myoepithelial cells suppress tumor growth and invasion by maintaining tissue polarity and integrity (Gudjonsson et al., 2005). Myoepithelial disruption in mammary glands might be a result of immune cell infiltration, which can physically disrupt the basement membrane (Man et al., 2003). Two mechanisms by which chronic inflammation and tissue damage may affect tumor initiation is alteration of the epithelial stem cell niche and expansion of the stem or progenitor cell pool because of factors secreted by inflammatory cells (Polyak and Kalluri, 2010). A phenotype similar to mammary-restricted HSD17B1 expression has been reported in P-cadherin knockout mice, which are deficient of the myoepithelial cell adhesion molecule P-cadherin (Radice et al., 1997). These mice develop hyperplasia and dysplasia of the mammary epithelium accompanied by abnormal lymphocyte infiltration (Radice et al., 1997). The *HSD17B1* gene has been shown to affect inflammatory signaling pathways and the expression of genes involved in cell growth and connective tissue integrity (Aka et al., 2017) and breast cancer cell migration (Aka et al., 2012a). The hypothetical effect of increased HSD17B1 expression in the mammary gland epithelium and breast carcinogenesis based on our results is presented in Figure 5.

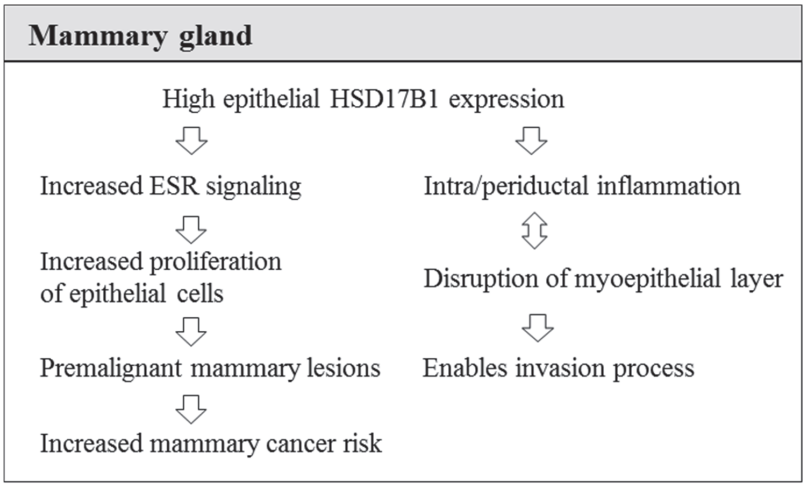

Figure 5. The hypothetical effect of increased HSD17B1 expression on mammary gland epithelium and mammary carcinogenesis.

7 CONCLUSIONS

Our study of HSD17B1TG female mice shows that human HSD17B1 is involved in the activation of a less potent estrogen, E1, to a potent estrogen, E2, *in vivo* and is involved in estrogen-related alterations observed in the uterus and mammary glands.

I) Human HSD17B1 expression increases E1-induced growth of the uterus in immature mice, demonstrating the ability of HSD17B1 to enhance estrogen action in target tissues. The human HSD17B1 enzyme is not fully estrogen-specific but plays a central role in estrogen activation in target tissues.

II) HSD17B1TG mice developed endometrial hyperplasia which was reversed by HSD17B1 inhibitor treatment. Thus, inhibition of HSD17B1 is one of the possible approaches to decreasing endometrial estrogen production, which is essential for the development of endometrial hyperplasia and cancer.

III) Bi-TG mice expressing both HSD17B1 and luciferase estrogen reporter gene (ERELuc) serve as a convenient model for testing HSD17B1 inhibitors *in vivo*.

IV) Local expression of human HSD17B1 in mammary gland leads to the development of inflammation-associated mammary gland lesions with a disruption of the luminal epithelial and myoepithelial cell layers at the site of inflammatory cell infiltration. Treatment with the antiestrogen ICI 182,780 decreased the mammary lesion number in these mice. These results suggest that HSD17B1 may play a role in inflammation-assisted mammary carcinogenesis via ESR-related mechanisms.

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