

**SEX STEROID METABOLISM
IN THE PLACENTA AND
THE BREAST**

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Abstract

The biosynthesis and metabolism of sex steroids are controlled by a series of steroidogenic enzymes. In the placenta and the breast, 3 β -hydroxysteroid dehydrogenase type 1 (3 β -HSD1) is essential for the synthesis of all steroid hormones by catalyzing pregnenolone to progesterone (P) or dehydroepiandrosterone (DHEA) to androstenedione (A-dione). P450 aromatase (P450arom) converts androgens to estrogens and is therefore critical for estrogen production. 17 β -hydroxysteroid dehydrogenases (17HSDs) are a group of enzymes responsible for the interconversion between low-activity 17-ketosteroids and high-activity 17 β -hydroxysteroids, thus acting as key enzymes modulating the biosynthesis and metabolism of both estrogens and androgens.

In situ hybridization assays showed that 3 β -HSD1, P450arom and 17HSD1, 2, 5 and 7 are expressed in early and mid-gestation placentas. Abundant expressions of 3 β -HSD1, P450arom and 17HSD1 were seen in syncytiotrophoblast (ST) cells. Signals of these three enzymes were also detected in some column cytotrophoblast (CCT) cells. 17HSD2 and 5 were located in intravillous stromal (IS) cells, whereas 17HSD7 mRNA was present in all types of placental cells. This suggests that the human placenta produces not only P and estrogens, but also androgens. Moreover, the placenta possesses a function, by the action of 17HSD2, to protect the fetus and the maternal body from excessive sex steroid influence.

In tubal pregnancy, P450arom and 17HSD1 were found in ST cells, implying an estrogen biosynthesis mechanism similar to that in normal intrauterine pregnancy. In both JEG-3 choriocarcinoma cell line and cultured normal human cytotrophoblast (CTB) cells, retinoic acids were shown to promote the enzyme activity as well as mRNA expression of P450arom and 17HSD1, and hence their action on the biosynthesis of E₂.

The mRNA expressions of 17HSD1, 2 and 5 in 794 breast carcinoma specimens were analyzed and correlated with ER α , ER β , PR, Ki67, c-erbB2 and clinical parameters. 17HSD1, 2 and 5 were detected in epithelial cells in normal and malignant breast tissues. In breast cancer specimens, the positive cases for 17HSD1, 2 and 5 were 16%, 25% and 65%, respectively. 17HSD1 was found to be an independent prognostic marker of the progression of breast cancer.

Keywords: 17 β -hydroxysteroid dehydrogenase, breast cancer, estrogens, placenta, progesterone

To my family

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Oulu, February 2004

Yan Li

Abbreviations

Hormones and steroids

17-OH-P	17 α -hydroxy-4-pregnene-3,20-dione
20-OH-P	20 α -dihydroprogesterone, 20 α -hydroxy-4-pregnen-3-one
3 α A-diol	5 α -androstane-3 α ,17 β -diol
3 β A-diol	5 α -androstane-3 β ,17 β -diol
5 α A-dione	5 α -androstane-3,17-dione
A-diol	androstenediol, 5-androstene-3 β ,17 β -diol
A-dione	androstenedione, 4-androstene-3,17-dione
ADT	androsterone, 3 α -hydroxy-5 α -androstan-17-one
cholesterol	5-cholesten-3 β -ol
DHEA	dehydroepiandrosterone, 3 β -hydroxy-5-androsten-17-one
DHEA-S	dehydroepiandrosterone sulfate
DHP	dehydroprogesterone
DHT	dihydrotestosterone, 17 β -hydroxy-5 α -androstan-3-one
E ₁	estrone, 3-hydroxy-1,3,5(10)-estratriene-17-one
E ₂	estradiol, 1,3,5(10)-estratriene-3,17 β -diol
E ₃	estriol, 1,3,5(10)-estratriene-3,16 α ,17 β -triol
P	progesterone, 4-pregnene-3,20-dione
T	testosterone, 17 β -hydroxy-4-androsten-3-one

Others

17HSD	17 β -hydroxysteroid dehydrogenase
17HSD 1–11	17HSD type 1–type11
20 α -HSD	20 α -hydroxysteroid dehydrogenase
3 β -HSD	3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase
AF	transcriptional activation function
ATP	adenosine-5'-triphosphate
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine-3',5'-monophosphate

cDNA	complementary DNA
CCT	column cytotrophoblast
CT	cytotrophoblast
CTB	cultured cytotrophoblast
DBD	DNA binding domain
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
ERE	estrogen-responsive element
EVCT	extravillous cytotrophoblast
FV	floating villi
<i>HSD17B</i>	gene for 17HSD
IS	intravillous stromal
kb	kilobase
kDa	kilodalton
K_m	Michaelis-Menten constant
LBD	ligand binding domain
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
NAD^+	nicotinamide-adenine dinucleotide
$NADP^+$	nicotinamide-adenine dinucleotide phosphate
nt	nucleotide
P450arom	cytochrome P450 aromatase
P450c17	17 α -hydroxylase/C ₁₇₋₂₀ lyase
P450scc	cholesterol side chain cleavage enzyme
PBS	phosphate-buffered saline
PR	progesterone receptor
RA	retinoic acid
9cRA	9- <i>cis</i> -retinoic acid
atRA	<i>all-trans</i> -retinoic acid
SDR	short-chain dehydrogenase/reductase
SERM	selective estrogen receptor modulator
SHBG	sex hormone binding globulin
ST	syncytiotrophoblast
TAM	tamoxifen

List of original publications

This thesis is based on the following articles, which are referred to in text by their Roman numbers:

- I Zhu SJ, Li Y, Li H, Wang YL, Xiao ZJ, Vihko P & Piao YS (2002) Retinoic acids promote the action of aromatase and 17 β -hydroxysteroid dehydrogenase type 1 on the biosynthesis of 17 β -estradiol in placental cells. *J Endocrinol* 172: 31-43.
- II Li Y, Qin L, Xiao ZJ, Wang YL, Herva R, Leng JH, Lang JH, Isomaa V & Piao YS (2003) Expression of P450 aromatase and 17 β -hydroxysteroid dehydrogenase type 1 at fetal-maternal interface during tubal pregnancy. *J Steroid Biochem Mol Biol* 87: 241-246.
- III Li Y, Isomaa V, Pulkka A, Herva R, Peltoketo H & Vihko P. Expression of 3 β -hydroxysteroid dehydrogenase type 1, P450 aromatase and 17 β -hydroxysteroid dehydrogenase types 1, 2, 5 and 7 in human early and mid-gestation placenta. Submitted
- IV Oduwole O, Li Y, Isomaa V, Mäntyniemi A, Pulkka A, Kurkela R, Soini Y & Vihko P. 17 β -hydroxysteroid dehydrogenase type 1 is an independent prognostic marker in breast cancer. Submitted

Contents

Abstract	
Acknowledgements	
Abbreviations	
List of original publications	
1 Introduction	15
2 Review of the literature	17
2.1 Physiological functions of estrogens	17
2.2 Overview of estrogen action	18
2.2.1 Estrogen signalling pathways	18
2.2.2 Structure of ER α and ER β	19
2.2.3 Interaction between ERs and selective estrogen receptor modulators (SERMs)	20
2.3 Estrogen biosynthesis pathway in human steroidogenic tissues	20
2.4 Estrogen biosynthesis and metabolism in peripheral tissues	22
2.5 Metabolism of sex steroids in the human placenta	22
2.6 Estrogens in the breast and in breast cancer	23
2.7 17 β -hydroxysteroid dehydrogenases	25
2.7.1 Human 17HSD type 1	25
2.7.2 Human 17HSD type 2	26
2.7.3 Human 17HSD type 5	27
2.7.4 Human 17HSD type 7	28
2.7.5 Other human 17HSD isoenzymes	28
2.8 Aromatase	29
2.9 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3 β -HSD)	30
2.10 20 α -hydroxysteroid dehydrogenase (20 α -HSD)	30
3 Outlines of the present study	32
4 Materials and methods	33
4.1 Tissue samples (I-IV)	33
4.2 Cell culture (I)	33
4.3 RNA isolation and Northern blot analysis (I)	33
4.4 Reverse transcription polymerase chain reaction (RT-PCR) (I)	34

4.5 Measurement of aromatase and 17HSD activities (I)	34
4.6 Detection of E ₂ by radioimmunoassay (I).....	34
4.7 Immunohistochemistry (II, III, IV).....	35
4.8 <i>In situ</i> hybridization (II, III, IV)	35
4.9 Statistical analyses (I, IV).....	35
5 Results	37
5.1 Expression of P450arom and 17HSD1 in the feto-maternal compartment in intrauterine and tubal pregnancy (II, III)	37
5.2 Effects of retinoic acids (RAs) on the expression of P450arom and 17HSD1, and E ₂ biosynthesis in cultured placental cells (I)	37
5.3 Expression of 3β-HSD1, 20α-HSD, 17HSD types 2, 5 and 7 mRNAs in human early and mid-gestation placenta (III).....	38
5.4 Expression of 17HSD types 1, 2, 5, ERα, ERβ, PR, Ki67 and <i>c-erbB2</i> in normal and cancerous breast tissues (IV)	39
6 Discussion	40
6.1 P450arom and 17HSD1 in the feto-maternal compartment in intrauterine and tubal pregnancy (II, III)	40
6.2 Influence of RAs on the expression of P450arom and 17HSD1 in placenta cells (I).....	41
6.3 3β-HSD1, P450arom and 17HSD1 in placental CCT cells (III).....	42
6.4 17HSD2, 5 and 7 in the placenta (III).....	43
6.5 Steroid metabolism in breast cancer: 17HSD type 1, an independent prognostic factor in breast cancer (IV)	43
6.6 Conclusions	45
References	

1 Introduction

Steroid hormones are essential for the maintenance of physiological homeostasis, and their actions are therefore central to human life. Imbalances in steroid hormone action underlie the onset and progression of some diseases threatening human health. Estrogens are sex steroid hormones critical to reproduction and female development. They also affect the functioning of a large number of other systems, such as the skeletal, cardiovascular, central nervous and immune systems.

Estrogens have three main molecular forms: estrone (E_1), estradiol (E_2) and estriol (E_3). E_2 has much higher biological potency than E_1 and E_3 , and E_2 is thus the physiologically most significant estrogen. The cellular effects of estrogens are mediated by estrogen receptors (ERs), which have been recognized in two forms: $ER\alpha$ and $ER\beta$. As members of the nuclear receptor superfamily, $ER\alpha$ and $ER\beta$ have structures common to nuclear receptors, including the N-terminal or A/B domain, the DNA-binding or C domain and the ligand-binding or D/E/F domain. In human, the identities between $ER\alpha$ and $ER\beta$ in their N-terminal, DNA-binding and ligand-binding domains are 30%, 96% and 53%, respectively. Also, $ER\alpha$ and $ER\beta$ are present in various tissues and play different roles in mediating the estrogen effect (Nilsson *et al.* 2001).

Estrogens are mainly produced in the ovary in women with regular menstrual cycle, but are supplied by the placenta in pregnant women. Two types of cells contribute to the biosynthesis of estrogens in the ovary. In theca cells, cholesterol is sequentially converted to androstenedione (A-dione) by a series of steroidogenic enzymes, whereas in granulosa cells, aromatase catalyzes A-dione to E_1 , which is subsequently converted to E_2 by 17 β -hydroxysteroid dehydrogenase type 1 (17HSD1). In the placenta, E_2 biosynthesis occurs in syncytiotrophoblast (ST) cells, where dehydroepiandrosterone (DHEA) derived from fetal and maternal adrenals is used as the main precursor molecule for E_2 production. In this pathway, 3 β -hydroxysteroid dehydrogenase type 1 (3 β -HSD1) catalyzes DHEA to A-dione, which is subsequently converted to E_2 by aromatase and 17HSD1. In addition, E_2 is inactivated by 17HSD2 in the placenta (Mustonen *et al.* 1998a).

Estrogens are considered obligatory for the proliferation of normal breast epithelial cells and involved in the development of breast cancer (Anderson *et al.* 1998, Zumoff 1998). The current opinion supports the idea that the intracellular E_2 concentration is not only affected by the endocrine supply, but also largely modulated by the local

biosynthesis and metabolism. The breast possesses a complete set of enzymes, including 3β -HSD1, P450 aromatase (P450arom) and 17HSD1, for local E_2 production using precursor DHEA, which is abundantly available in blood circulation. On the other hand, 17HSD2, an E_2 -inactivating enzyme responsible for the oxidation of E_2 to E_1 , is also expressed in the mammary gland (Söderqvist *et al.* 1998, Miettinen *et al.* 1999). 17HSD5 is another 17HSD enzyme expressed in the mammary gland, which mainly catalyzes the reduction of A-dione to testosterone (T) (Dufort *et al.* 1999, Penning *et al.* 2001), and 17HSD7, which is capable of converting E_1 to E_2 (Törn *et al.* 2003). Co-ordinated action of various steroidogenic enzymes maintains the E_2 homeostasis in the breast. Overexpression of E_2 biosynthesis enzyme(s) or decreased expression of E_2 -inactivating enzyme(s) may elevate the E_2 level in the breast and consequently promote abnormal cell growth.

2 Review of the literature

2.1 Physiological functions of estrogens

Estrogens mainly exist in three molecular forms: estrone (E_1), estradiol (E_2) and estriol (E_3). Since the biological potency of E_2 is much higher than that of the other types, E_2 is physiologically the most important estrogen. The classical roles of estrogens in female reproduction and development have been well defined. In the ovary, E_2 stimulates the proliferation of granulosa cells and the growth of follicles (Goldfien & Monroe 1991, Adashi 1996, Robker & Richards 1998). In the uterus, E_2 in combination with progesterone (P) regulates the cyclic growth of the endometrium (Couse & Korach 1999, Mertens *et al.* 2001). Estrogens together with P are also crucial for the maintenance of pregnancy and fetal development (Pepe & Albrecht 1995, Albrecht *et al.* 2000).

In recent years, knowledge about the role of estrogens in male reproduction has increased. It has been reported that male patients with defective estrogen production have reduced fertility (Morishima *et al.* 1995). Moreover, impaired spermatogenesis was found in male aromatase knockout mice (ArKO) (Robertson *et al.* 1999). On the other hand, inadequate influence of estrogens may also bring about severe negative effects on the male system. For example, exposure to E_2 during fetal development results in multiple irreversible damages in the structure and function of the testis and epididymis (Aceitero *et al.* 1998, Atanassova *et al.* 1999).

In addition to their roles in reproduction and female development, estrogens affect a large number of other physiological systems. In skeleton, for example, estrogens are important for maintaining bone mass in adult women by suppressing bone remodelling and keeping a balance between osteoblastic and osteoclastic activities (Jilka *et al.* 1992, Turner *et al.* 1994). Furthermore, estrogen replacement therapy remarkably decreases osteoporosis in postmenopausal women (Gallagher 1999). It was shown in other studies that estrogens are also important for the development and maintenance of the male skeleton (Smith *et al.* 1994, Carani *et al.* 1997). In addition, a large number of studies have pointed out the influence of estrogens on the cardiovascular system (Mendelsohn & Karas 1999), central nervous system (Fink *et al.* 1996, Mendelsohn & Karas 1999, Stahl 2001) and immune system (Olsen & Kovacs 1996, Cutolo *et al.* 2002).

2.2 Overview of estrogen action

2.2.1 Estrogen signalling pathways

It is well established that estrogens synthesized in the ovary are released into blood circulation and are mostly bound to sex hormone binding globulin (SHBG) and albumin, from which they are released and enter the target cells by passive diffusion for the action. This has been classically described as the endocrine regulation of estrogens. Later, the paracrine and intracrine pathways of estrogen action were discovered. In the former pattern, estrogens locally produced in cells of one type modulate the neighbouring cell type. In the latter form, intracellularly produced estrogens directly regulate the same cells (Labrie *et al.* 1997).

In the 1950s, nothing was known about the intracellular molecular mechanisms of estrogen action. In the early 1960s, a finding on the specific binding of E₂ in the uterus led to the conclusion that the biological effects of estrogen are mediated by a receptor protein (Jensen & Jacobsen 1962). After that, extensive efforts have been undertaken to understand the biochemical characteristics and functions of the receptor. In the mid-1980s, cloning of an estrogen receptor (ER) was reported (Green *et al.* 1986, Greene *et al.* 1986). In 1996, another ER named ER β was discovered in rat prostate (Kuiper *et al.* 1996). Thereafter, the previously found ER has been called ER α .

ER α and ER β belong to the steroid/thyroid/vitamin D nuclear receptor superfamily. In the absence of ligand, ERs are associated with several proteins in the nucleus, such as heat shock proteins, to form a protein complex (Pratt 1990, Pratt & Toft 1997). Once they are bound by an estrogen ligand, a conformational change occurs in their structure, which triggers the dissociation of the heterocomplex, dimerization of the receptor, interaction between the receptor and DNA, recruitment of co-activators or other transcription factors and formation of a preinitiation complex in the target genes (Nilsson *et al.* 2001).

Before the identification of ER β , it was thought that ER α interacts with DNA as a homodimer. Now, it is becoming clear that ER α and ER β bind to DNA not only as homodimers, but also as heterodimers (Cowley *et al.* 1997, Pettersson *et al.* 1997). The DNA sequence interacting with ERs is called the estrogen response element (ERE), which comprises two half sites of the core sequence AGGTCA, which are oriented in a palindromic order with a spacing of three base pairs, i.e. AGGTCAnnnTGACCT. It was recently found that ERs can also regulate gene transcription without direct contact with DNA. One example is that ER α and ER β interact with the transcription factor Fos/Jun on the AP1 response element and affect gene transcription (Gaub *et al.* 1990, Umayahara *et al.* 1994, Paech *et al.* 1997). In another example, ER α modulates transcription by interacting with the transcription factor Sp1, which binds to a GC-rich Sp1 site in some genes (Batistuzzo de Medeiros *et al.* 1997, Porter *et al.* 1997, Qin *et al.* 1999). The action of ERs may be also ligand-independent, in which case extracellular regulators, such as growth factors, or cyclic adenosine monophosphate (cAMP) activate the intracellular kinase pathways, such as the mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways. These lead to the phosphorylation and

activation of ER, resulting in an impact on cell growth and proliferation (Smith 1998, Migliaccio *et al.* 1998, Castoria *et al.* 1999).

Accumulating data suggest that estrogens may act through a non-genomic pathway, which results in a very rapid effect (Migliaccio *et al.* 1984). This involves ERs in the vicinity of the cell membrane and cytoplasm (Mendelsohn 2000, Simoncini *et al.* 2000, Kousteni *et al.* 2001). An example is that, in endothelial cells, estrogen-mediated membrane effects trigger the MAPK pathway (Collins & Webb 1999). Furthermore, there is likely to be some cross-talk between the genomic and nongenomic actions of estrogens (Revelli *et al.* 1998, Katzenellenbogen & Katzenellenbogen 2000, McDonnell & Norris 2002).

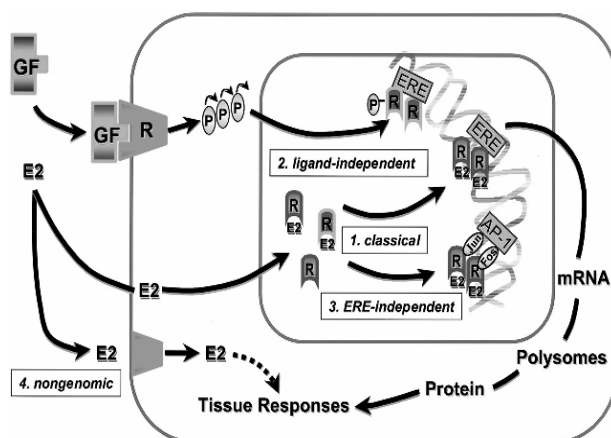


Fig. 1. The multifaceted mechanisms of estrogen receptor signalling. Adapted from Hall *et al.*, *J Biol Chem* 276: 36869-36872, 2001, with the permission of American Society for Biochemistry and Molecular Biology.

2.2.2 Structure of $ER\alpha$ and $ER\beta$

Nuclear receptors share a common structure architecturally characterized by the N-terminal or A/B domain, the DNA-binding or C domain and the ligand-binding or D/E/F domain. The N-terminal domain of $ER\alpha$ and $ER\beta$ contains a hormone-independent transcriptional activation function, AF-1 (Lees *et al.* 1989, Tora *et al.* 1989, Tsai & O'Malley 1994). This region is involved in protein-protein interaction and transcriptional activation, though its length and amino acid composition vary markedly between different nuclear receptors. In human, for example, $ER\alpha$ and $ER\beta$ share only 30% identity in the N-terminal domain (Ogawa *et al.* 1998).

The DNA-binding domain (DBD) is highly conserved in the nuclear receptor superfamily. It possesses two zinc fingers, which are essential for receptor dimerization and DNA binding (Kumar & Chambon 1988, Tsai & O'Malley 1994, Franco *et al.* 2003,

Meza *et al.* 2003). Human ER α and ER β have 96% identity in their DBDs. Especially the P box, which determines DNA recognition and specificity, is identical in the two receptors. Thus, ER α and ER β are considered to have the same DNA-binding property with regard to specificity and affinity (Nilsson *et al.* 2001 and refs. therein).

The D region of the ligand-binding domain (LBD) is also called the hinge region and serves as a linker between DBD and LBD. The E/F region of LBD is relatively large with complex functions. ER α and ER β have similar binding affinities for 17 β -estradiol, with Kd values of 0.2 and 0.6 nM, respectively (Kuiper *et al.* 1996, Tremblay *et al.* 1997). Apart from ligand binding, the E/F region is involved in nuclear localization, receptor dimerization and transcriptional regulation (Tsai & O'Malley 1994, Horwitz *et al.* 1996). LBD also contains AF-2, which is ligand-dependent, in contrast to AF-1 (Webster *et al.* 1988, Lees *et al.* 1989). In addition, the F region is suggested to play a role in modulating the effects of the agonist/antagonist (Montano *et al.* 1995).

2.2.3 Interaction between ERs and selective estrogen receptor modulators (SERMs)

In addition to E₂, a number of compounds are able to bind the ERs, which are now referred to as SERMs. This class of molecules have been demonstrated to display remarkable differences in their stimulatory and/or inhibitory effects on various estrogen target tissues, acting as agonists in some tissues but as antagonists in others (Grese & Dodge 1998, McDonnell 1999). Tamoxifen (TAM), the most widely used compound in the treatment of breast cancer, was originally considered an antiestrogen, but has since been more properly designated as a SERM. Induction of apoptosis is a major mechanism of the observed anti-tumour effect of TAM. In ER-positive tumours, TAM inhibits AF-2 activity and consequently, functions as an antagonist in the environment where AF-2 is required. However, in the case where AF-1 of ER α is the dominant activator, TAM possesses partial agonist activity (Tzukerman *et al.* 1994). In ER-negative tumours, the effect of TAM is mediated by other signalling pathways, such as protein kinase C and MAPK (Mandlekar & Kong 2001).

2.3 Estrogen biosynthesis pathway in human steroidogenic tissues

In human, three organs are specialized for the biosynthesis of steroid hormones: the gonads, the adrenal cortex and the placenta during pregnancy. Regardless to the place of production, the *de novo* biosynthesis of all steroid hormones starts from a common precursor, cholesterol, and involves similar biochemical reaction cascades. First, cholesterol is converted to pregnenolone by a cholesterol side-chain cleavage enzyme (P450scc) (Duque *et al.* 1978). Then, pregnenolone is oxidized at position C₃ by 3 β -hydroxysteroid dehydrogenases/ Δ^5 - Δ^4 isomerases (3 β -HSDs), and thus progesterone (P) is formed (Luu-The *et al.* 1989a, Rheaume *et al.* 1991). Subsequent 17 α -hydroxylation of pregnenolone and P by 17 α -hydroxylase/C₁₇₋₂₀ lyase (P450c17) results in the formation

of 17-hydroxypregnenolone and 17-hydroxyprogesterone (17-OH-P), respectively. P and 17-OH-P locate on the branching point of the steroid production cascade, since they can be eventually converted to either sex steroids or corticosteroids by the action of various steroidogenic enzymes. Towards estrogen biosynthesis, 17-hydroxypregnenolone and 17-OH-P are converted to dehydroepiandrosterone (DHEA) and androstenedione (A-dione), respectively, by the lyase activity of P450c17 (Hedin *et al.* 1987, Sasano *et al.* 1989). Formation of A-dione can be also achieved by 3β -oxidation of DHEA through 3β -HSDs. Because of the existence of sulfotransferases in steroidogenic tissues, for example, in adrenal cortex, pregnenolone, 17-hydroxypregnenolone and DHEA can be modified to their sulfate forms, pregnenolone-S, 17-hydroxypregnenolone-S and DHEA-S, respectively (Martel *et al.* 1994). The final steps of estrogen production are controlled by P450arom and 17HSDs. Aromatase catalyzes the aromatization of androgens to estrogens, such as A-dione to E_1 or T to E_2 , whereas 17β -HSDs are responsible for the interconversion between 17-ketosteroids, like A-dione and E_1 , and 17β -hydroxysteroids, for example, T and E_2 (Simpson *et al.* 1994, Peltoketo *et al.* 1999b and refs. therein).

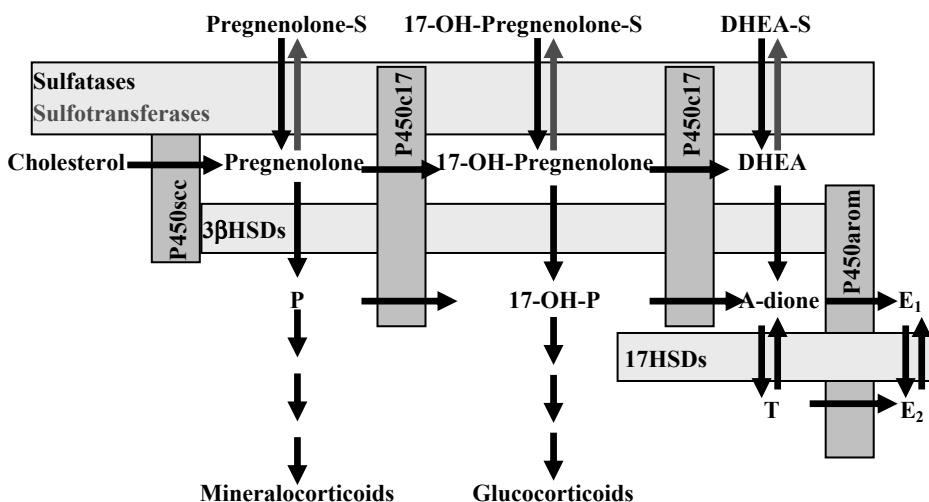


Fig. 2. Pathways of the steroid hormone biosynthesis.

In women, the ovary is the main source of circulating estrogens before menopause. This process requires the participation of two types of cells. Theca cells produce A-dione, which is then converted to estradiol in granulosa cells. This process is postulated in the “two-cell theory” (Hillier *et al.* 1994). During pregnancy, the placenta replaces the ovary as the endocrine organ for steroid supply (Simpson & Macdonald 1981). Besides the gonads, the adrenal cortex is an active steroidogenic organ for both females and males. It supplies DHEA, DHEA-S and A-dione as the endpoint molecules of sex steroids (Kroboth *et al.* 1999).

2.4 Estrogen biosynthesis and metabolism in peripheral tissues

In men and post-menopausal women, both circulating and local estrogens in target tissues are mainly supplied by peripheral tissues. For example, adipose tissue is a major estrone storage resource in post-menopausal women. Unlike steroidogenic tissues, the peripheral tissues are suggested not to be capable of performing *de novo* estrogen production from cholesterol because they do not contain the enzymes catalyzing the upstream reactions in the steroid biosynthesis pathway. Thus, adrenal-derived C₁₉ steroids, such as DHEA, DHEA-S and A-dione, serve as precursors for the formation of estrogens in peripheral tissues (Labrie *et al.* 1994, Longcope 1996). The adrenal cortex secretes large amounts of C₁₉ steroids, which are present abundantly in circulation. Peripheral estrogen synthesis, which has been found in, for example, the adipose tissue, skin, bone, vascular endothelium, hypothalamus and muscle, is affected mainly by steroidogenic enzymes of three types: aromatase, estrogenic 17HSDs and estrone sulfatase. The former two types of enzymes play the same roles in peripheral tissues as they do in steroidogenic tissues with regard to estrogen biosynthesis (Folkerd & James 1983, Sasano & Harada 1998). Estrone sulfotransferase catalyzes E₁ to its sulfate form, E₁-S, which exists as a reservoir for the formation of E₁ after hydrolysis by estrone sulfatase (Santner *et al.* 1986, Pasqualini *et al.* 1992).

Under normal physiological conditions, estrogens need to be metabolized for the maintenance of hormonal homeostasis. This is critical to the target cells with regard to protection from unwanted estrogen effects or the aftercare subsequent to estrogen action (Chatterjee *et al.* 1994). Predominantly, estrogens are inactivated in the liver, where E₂ is oxidized to E₁ mainly by 17HSD2 (Wu *et al.* 1993, Adamski *et al.* 1995, Mustonen *et al.* 1997, Biswas & Russel 1997, Fomitcheva *et al.* 1998). This mechanism is applicable to other peripheral tissues as well, such as the breast, endometrium, small intestine, colon, gastric tissue, kidney, pancreas and prostate (Miettinen *et al.* 1999, Oduwole *et al.* 2002, 2003a, b, Härkönen *et al.* 2003). The metabolic process of E₂ also involves a number of other biochemical pathways, including hydroxylation, conjugative metabolism and formation of catechol estrogens (Zhu & Conney 1998 and refs. therein). The existence of multiple metabolic systems protects the peripheral tissues from excessive action of estrogens by providing an optimal estrogen level.

2.5 Metabolism of sex steroids in the human placenta

The placenta is a unique organ of the feto-maternal unit, which possesses a crucial role in the maintenance of pregnancy and fetal development (Pepe & Albrecht 1995, Albrecht *et al.* 2000). After implantation, the trophoblast cells of the placenta undergo differentiation towards either the villous or the extravillous trophoblast pathway (Kaufmann *et al.* 1992, Loke & King 1995). Villous trophoblasts coat the floating villi (FV), which are responsible for hormone production and fetal-maternal material exchange. In an early placenta, FV are composed of fibrovascular stroma coated by double-layered trophoblast epithelium. The inner layer is constructed of villous cytotrophoblast (VCT) cells, while

the outer layer consists of syncytiotrophoblast (ST) cells, i.e. multinucleated giant cells that are differentiated from VCT cells.

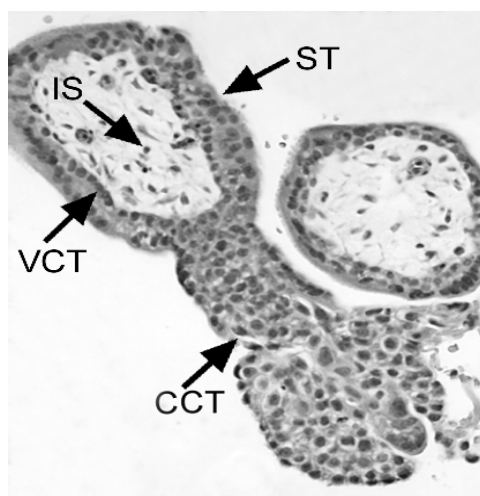


Fig. 3. Structure of the floating villi (FV) in the human placenta. ST, syncytiotrophoblast; VCT, villous cytotrophoblast; CCT, column cytotrophoblast; IS, intravillous stroma.

In humans, ovarian function begins to decline after fertilization, and the placenta gradually becomes the central site of estrogen biosynthesis during pregnancy. After seven weeks of gestation, nearly all circulating estrogens are synthesized by the placenta (Simpson & MacDonald 1981). Differently from the ovary, however, the human placenta is not able to perform *de novo* estrogen synthesis from cholesterol, because it lacks the 17α -hydroxylase/ C_{17-20} lyase (P450c17) enzyme. Instead, the placenta employs fetal and maternal adrenal-derived precursor molecules, such as DHEA and DHEA-S, to synthesize estrogens (Albrecht & Pepe 1990). In ST cells, the main cell type for placental hormone production, steroid precursors are desulfonated by steroid sulfatase and thereafter converted to estrogens by sequential reactions catalyzed by 3β -HSD1, P450arom and 17HSDs (Langer & Engel 1958, Siiteri & MacDonald 1966, Ishii-Ohba *et al.* 1986, Albrecht & Pepe 1990, Salido *et al.* 1990, Miller 1998, Peltoketo *et al.* 1999b, 2003).

2.6 Estrogens in the breast and breast cancer

It is well established that estrogens are obligatory for the development of normal breast and involved in the induction and progression of breast cancer. One fact is that the female mammary gland undergoes a surge of cell divisions during puberty. There is also cyclic proliferation and involution in the breast during the menstrual cycle throughout adult life (Russo *et al.* 1999). It has been shown that the growth of cycling epithelial cells of the mammary gland is stimulated by increased amounts of E_2 percutaneously administered to

premenopausal women for 10-13 days (Chang *et al.* 1995). Also, the multiplication of normal breast cells cultured *in vitro* is increased by the addition of E₂ to culture (Mauvais-Jarvis *et al.* 1986). Moreover, the phenotypes of ER α and ER β knockout mice indicate that ER α is critical to the growth and development of mammary gland whereas ER β is involved in terminal differentiation of glandular epithelium (Couse & Korach 1999, Förster *et al.* 2002). In mice lacking P450arom, an enzyme obligatory for estrogen biosynthesis, the development of breast in an adult female is similar to that in a prepubertal female (Fisher *et al.* 1998).

Breast cancer is the most common malignancy and the leading cause of cancer mortality in women (Parkin *et al.* 1999), and a low incidence of breast cancer is also seen in men (English *et al.* 2000, Omari-Alaoui *et al.* 2002). It was already reported more than 100 years ago that bilateral oophorectomy leads to remission of breast cancer in premenopausal women (Beatson 1896). This was the first indication of the association between breast cancer and estrogens. Over the recent years, multiple risk factors, such as estrogen exposure, diet, genetics and environmental factors, have been identified in the epidemiology of carcinogenesis and the progression of breast cancer (Vihko & Apter, 1986, 1989, William & Creasman 2002 and refs. therein). A positive correlation between the plasma estrogen concentration and the breast cancer risk has been observed in postmenopausal women (Toniolo *et al.* 1995, Berrino *et al.* 1996, Hankinson *et al.* 1998). The E₂ concentration was also found to be significantly higher in breast tumours than in normal breast tissue (Vermeulen *et al.* 1986, Pasqualini & Chetrite 2002). Moreover, the tumour/plasma ratio of E₂ increases significantly in postmenopausal women, who are the population at the highest risk for breast cancer (Siiteri 1987). On the other hand, the levels of E₁ and E₂ in plasma are similar in healthy women and breast cancer patients, both before and after menopause (Pasqualini & Chetrite 1996). This suggests that the tumour levels of estrogens are independent of the plasma concentration and menopausal stage. Recent study on large clinical trials of SERMs on breast cancer patients further pointed out that estrogens are the risk factor for breast cancer (Grese & Dodge 1998, McDonnell 1999, O'Regan & Jordan 2002). In addition, TAM reduces the risk of breast cancer in healthy pre- and postmenopausal women (Fisher *et al.* 1998). Overall, there is a large body of epidemiological and clinical evidence to establish E₂ as a risk factor for breast cancer.

About 90% of breast cancer cases are invasive ductal or lobular carcinomas that develop from the epithelial cells of the terminal ductal lobular units (Russo & Russo 1998). Experimental data have strongly suggested that estrogens stimulate the proliferation of epithelial cells in both normal and neoplastic breast tissues (Russo & Russo 1997, 1998, Dickson & Lippman 2000). Estrogens induce the development of mammary cancer in a rodent model, whereas they also exert both direct and indirect effects on the proliferation of cultured breast cancer cells (Lupulescu 1995, Miettinen *et al.* 1996b, Creasman 2002).

The development of breast cancer is a multistage process concurrent with the change in cell sensitivity during the progression from normal status to hyperplastic, hormone-sensitive cancer and, finally, to hormone-insensitive cancer (King 1991). The transition from hormone dependency to hormone independency of breast cancer may involve the activation of an oncogene and a loss of either estrogen receptors or estrogen responsiveness in other genes (Lippman & Dickson 1989). More than 50% of breast

cancer patients with ER α and progesterone receptor (PR)-positive tumours respond to hormonal therapy, while a similar response is seen in less than 10% of the receptor-negative tumours (McGuire *et al.* 1991). Therefore, ER α , in conjunction with PR, is thought to be a prognostic marker of breast cancer. Although ER β appears to be the more abundant form of the two ERs in human normal and breast cancer tissues (Gustafsson & Warner 2000), its importance in breast cancer has not been fully evaluated yet.

2.7 17 β -hydroxysteroid dehydrogenases

17 β -hydroxysteroid dehydrogenases (17HSDs) consist of a group of enzymes that catalyze the interconversion between 17-ketosteroids and 17 β -hydroxysteroids (Peltoketo *et al.* 1999a, b, 2003). Both estrogens and androgens possess their highest biological activities in the 17 β -hydroxy form, such as E₂ and T. Thus, 17HSD enzymes play key roles in regulating the action of sex steroid hormones by controlling the formation and inactivation of the active forms of estrogens and androgens. To date, at least 11 different 17HSDs, named 17HSD type 1-11, have been characterized from different species, among which the types 6 and 9 have not been cloned from human (Peltoketo *et al.* 1999b, Adamski & Jakob 2001). These enzymes differ in their amino acid composition, tissue distribution, subcellular localization, substrate specificity, catalytic properties and co-factor preferences. The diverse actions of 17HSDs meet the normal physiological requirement with an accurately controlled system of the supply and inactivation of active sex steroid hormones.

2.7.1 Human 17HSD type 1

Human 17HSD type 1, which belongs to the short-chain dehydrogenase/reductase (SDR) superfamily, is the first cloned member of 17HSDs (Peltoketo *et al.* 1988) and has been characterized most extensively. As a cytosolic protein, native 17HSD type 1 exists as a homodimer (Burns *et al.* 1972, Nicholas & Harris 1973, Lin *et al.* 1992), and each monomer is composed of 327 amino acids with a calculated molecular mass of 34 853 dalton (Peltoketo *et al.* 1988). 17HSD type 1 preferably catalyzes the reduction of low-activity E₁ to high-activity E₂. In cultured cells, it also converts A-dione to T (Nokelainen *et al.* 1996, Puranen *et al.* 1997). Structural studies revealed that the amino acids 148 to 268 of the enzyme constitute a conserved region in the SDR family, being mainly responsible for the differences in substrate specificity (Puranen *et al.* 1997).

Human 17HSD type 1 is encoded by the HSD17B1 gene located in the chromosome region 17q12-21 (Winqvist *et al.* 1990, Luu-The *et al.* 1990, Peltoketo *et al.* 1992, Simard *et al.* 1993). HSD17B1 consists of six exons and five short introns and has two transcription starting sites located 9-10 and 971 nucleotides upstream of the translation initiation codon. This leads to two transcripts, 1.3 kb and 2.3 kb in size, differing in the length of their 5'-noncoding regions (Luu-The *et al.* 1989b, 1990). The 2.3 kb mRNA is constitutively expressed in most tissues and cell lines (Tremblay *et al.* 1989, Luu-The *et al.* 1990), whereas the 1.3 kb mRNA is expressed in cells containing 17HSD1 protein,

and its amount correlates with the concentration of the protein and 17HSD1 activity (Tremblay *et al.* 1989, Poutanen *et al.* 1992a, Miettinen *et al.* 1996a). Moreover, the transcription of the 1.3 kb mRNA is subject to regulation by various factors (Poutanen *et al.* 1992a, Ritvos & Voutilainen 1992, Tremblay & Beaudoin 1993, Lewintre *et al.* 1994a, b, Piao *et al.* 1997b).

The transcription of the 1.3 kb mRNA is controlled by three regulatory elements in the 5'-flanking region of the HSD17B1 gene, a proximal promoter located at -78 to +9, a cell-specific enhancer situated at -661 to -392, and a silencer between the promoter and the enhancer (Piao *et al.* 1995, 1997a). Transcription factor Sp1 interacts with a GC-box in the proximal promoter and is essential for basal transcription. However, GATA factors (GATA-2 and GATA-3) interact with their binding site adjacent upstream to the GC-box and, as a result, interrupt the binding of Sp1 (Piao *et al.* 1997a). In addition, a retinoic acid responsive element (RARE) has been found in the HSD17B1 enhancer from -503 to -487, and it mediates the effect of retinoic acids on gene transcription (Piao *et al.* 1995). No promoter element has been identified for the transcription of 2.3 kb mRNA.

The 17HSD1 mRNA and protein have mainly been found in the ovary and placenta. Their presence was also detected in the endometrium, mammary gland and certain choriocarcinoma and breast cancer cell lines (Luu-The *et al.* 1990, Poutanen *et al.* 1990, Mäentausta *et al.* 1990, 1991, Sawetawan *et al.* 1994, Miettinen *et al.* 1996a, Söderqvist *et al.* 1998, Miettinen *et al.* 1999). In the ovary, 17HSD1 is expressed in the granulosa cells of developing follicles and plays a critical role in E₂ biosynthesis during follicular development (Ghersevich *et al.* 1994a, Swetawan *et al.* 1994). In the placenta, 17HSD1 is present in ST cells, i.e. sites accountable for the production of placental hormones (Fournet-Dulguerov *et al.* 1987, Mäentausta *et al.* 1991, Sawetawan *et al.* 1994, Mustonen *et al.* 1998a, Takeyama *et al.* 1998). In choriocarcinoma cells, the expression of 17HSD1 is stimulated by growth factors, cAMP, TPA (activator for protein kinase C) and retinoic acids (Lewintre *et al.* 1994a, b, Piao *et al.* 1997b). In breast cancer cells, the expression of 17HSD1 is upregulated by several factors, such as interleukin 6 (IL-6), IL-1 β , insulin-like factors type I and type II, tumour necrosis factor α and progestins (Adams *et al.* 1991, Singh & Reed 1991, Poutanen *et al.* 1992, Duncan *et al.* 1994). In the endometrium and mammary gland, 17HSD1 is localized in epithelial cells (Mäentausta *et al.* 1991, Miettinen *et al.* 1999), and endometrial expression is most active during the early to mid-secretory phases (Mäentausta *et al.* 1990, 1991). In addition, 17HSD1 was found in estrogen-dependent tumours, including breast cancer tissue (Poutanen *et al.* 1992), and in approximately 50% of endometrial adenocarcinomas (Mäentausta *et al.* 1992).

2.7.2 Human 17HSD type 2

Human 17HSD type 2, which was cloned from human prostate (Wu *et al.* 1993), is a membrane-bound protein comprising 387 amino acids. It contains a transmembrane domain with a type 2 signal anchor motif at the N-terminal and a motif for endoplasmic reticulum retention at the C-terminal (Wu *et al.* 1993, Akinola *et al.* 1996). Immunostaining has identified 17HSD type 2 in the endoplasmic reticulum (Puranen *et*

al. 1999). This enzyme catalyzes the oxidation of 17β -hydroxysteroids to their low-activity forms, 17-ketosteroids, such as E_2 to E_1 , T to A-dione and dihydrotestosterone (DHT) to 5α -androstenedione. 17HSD type 2 is also capable of converting 20α -dehydroprogesterone (20-OH-P) to P (Wu *et al.* 1993, Puranen *et al.* 1999, Peltoketo *et al.* 1999b, 2003).

Human 17HSD2 mRNA, 1.4 kb in size, is widely present in various tissues, such as the placenta, endometrium, breast, liver, small intestine, kidney, pancreas, colon, prostate and sebaceous glands (Casey *et al.* 1994, Elo *et al.* 1996, Miettinen *et al.* 1996a, 1999, Moghrabi *et al.* 1997, Mustonen *et al.* 1997, 1998a, b, Thiboutot *et al.* 1998). In the endometrium, 17HSD type 2 is localized to the glandular epithelial cells (Casey *et al.* 1994, Mustonen *et al.* 1998a). In the liver, it has been found in hepatocytes (Takeyama *et al.* 1998). In the intestine, 17HSD type 2 is present in the surface epithelium of the villi (Mustonen *et al.* 1998b). In the colon, 17HSD type 2 mRNA is expressed predominantly in the surface epithelial cells, and some expression is also detected in the cryptal epithelial cells (Oduwole *et al.* 2002).

17HSD2 is thought to be a main sex steroid-inactivating enzyme to protect the target tissues from excessive hormone influence (Elo *et al.* 1996, Mustonen *et al.* 1998a, Peltoketo *et al.* 1999b). For example, 17HSD2 present in the placenta inactivates both E_2 and T to protect the fetus from excessive E_2 and to protect the mother with a male fetus from excessive T (Moghrabi *et al.* 1997, Mustonen *et al.* 1997, 1998a). 17HSD2 is believed to be involved in the pathogenesis of human disorders. For example, 17HSD2 expression is downregulated in colon cancer (English *et al.* 2000, Oduwole *et al.* 2002, 2003b), and the enzyme level is decreased in prostate cancer progression, which is associated with concurrently increased E_2 production and expression of E_2 -induced genes (Elo *et al.* 1996, Härkönen *et al.* 2003).

The gene encoding 17HSD type 2 is HSD17B2. The gene consists of seven exons and six introns and has been localized to the chromosome region 16q24 (Casey *et al.* 1994). Up to date, little is known about the regulation of 17HSD type 2 expression. In the endometrium, its expression level fluctuates in accordance with the blood P concentration (Casey *et al.* 1994). In endometrial cancer cells, the expression of 17HSD type 2 is increased by retinoic acids (Li *et al.* 2002).

2.7.3 Human 17HSD type 5

Unlike other 17HSDs, which belong to SDR, human 17HSD type 5 is a member of the aldoketo reductase (ARK) family (Dufort *et al.* 1999, Luu-The *et al.* 2001). Human 17HSD type 5 protein, which comprises 323 amino acids, is encoded by the HSD17B5 gene located at the chromosome region 10p14-15 (Lin *et al.* 1997, Rheault *et al.* 1999a). Originally, this enzyme was identified as 3α -HSD type 2 (Khanna *et al.* 1995), since it shares high homology with 3α -HSD type 1 (84%) and type 3 (86%) as well as 20α -HSD (88%) (Dufort *et al.* 1999). Human 17HSD type 5 has multiple enzymatic activities. It efficiently performs androgenic 17HSD function, catalyzing A-dione to T, and also has the 3α -HSD activity needed for the conversion of DHT to 5α -androstane- 3α , 17β -diol (3α A-diol) (Dufort *et al.* 1999). This enzyme also catalyzes the conversion of

prostaglandin (PG) D₂ to PGF_{2α} (Desmond *et al.* 2003). Recent studies have shown that 17HSD type 5 is a suppressor of nuclear receptor-regulated cell differentiation (Desmond *et al.* 2003). Further, it possesses 20α-HSD activity responsible for the reduction of P to 20-OH-P (Dufort *et al.* 1999, Luu-The *et al.* 2001).

Human 17HSD5 is expressed in the liver, testis, adrenal, prostate, ovary, mammary gland and endometrium (Dufort *et al.* 1999, Penning *et al.* 2001). In the prostate, it is mainly localized in the basal cells of glandular epithelium, in stromal fibroblasts and endothelial cells (El-Alfy *et al.* 1999). 17HSD5 is also present in the DU-145 and LNCaP prostate cancer cell lines and in MG-63 bone carcinoma cells (Dufort *et al.* 1999). In ovarian epithelial tumour, 17HSD type 5 has been found to be the predominant isoform of 17HSDs and, therefore, to modulate the ligand supply to the androgen and progesterone receptors in receptor-containing tumours (Blomquist *et al.* 2002).

2.7.4 Human 17HSD type 7

Human 17HSD7 is a membrane-associated protein encoded by the HSD17B7 gene located to the chromosome region 1q23 (Törn *et al.* 2003). The enzyme converts E₁ to E₂ and DHT to 5α-androstane-3β, 17β-diol (3βA-diol) with equal catalytic activity. In addition, 17HSD7 is able to catalyze P and 20-OH-P to 4-pregnen-3β-ol-20-one and 4-pregnen-3β,20α-diol, respectively (Törn *et al.* 2003). Human 17HSD7 mRNA is abundantly expressed in the adrenal gland, liver, lung and thymus. It is also clearly localized in the pituitary gland, prostate, kidney, lymph node, small intestine and trachea. Therefore, the enzyme might be involved in the local production of estrogenic metabolites in peripheral tissues (Törn *et al.* 2003).

2.7.5 Other human 17HSD isoenzymes

The human 17HSD3 enzyme is a microsomal protein containing 310 amino acids. It predominantly catalyzes the conversion of A-dione to T and, to a lesser extent, E₁ and DHEA to E₂ and A-dione, respectively (Geissler *et al.* 1994). 17HSD3 mRNA (1.3 kb) is exclusively expressed in the testis (Geissler *et al.* 1994). In addition, it has been detected in a Sertoli-Leydig cell tumour, adjacent theca lutein ovarian tissue (Barbieri & Gao 1997) and in adipose tissue (Corbould *et al.* 1998). It has been demonstrated that 17HSD3 plays an important role in the normal development of male genitalia. 17HSD3 deficiency results in male pseudohermaphroditism (Geissler *et al.* 1994), which is an autosomal and recessive disorder affecting males, whereas females are asymptomatic (Rösler *et al.* 1996, Mendonca *et al.* 1999).

Human 17HSD4, an 80 kDa peroxisomal protein with 736 amino acids, is expressed ubiquitously, being most abundant in the liver and kidney and least abundant in the brain and spleen (Adamski *et al.* 1995, Normand *et al.* 1995). Its 3 kb mRNA has been detected in several cancer cell lines, such as T47D, HEP-G2 and LNCaP (Adamski *et al.* 1995, Normand *et al.* 1995, Moller *et al.* 1999). 17HSD4 is a multi-functional enzyme. It converts E₂ to E₁ with regard to its 17HSD activity. Moreover, the N-terminal domain of

17HSD4 catalyzes the β -oxidation of fatty acids, such as 3-hydroxyacyl-CoA esters (Dieuaide-Noubhani *et al.* 1996, Leenders *et al.* 1996, Qin *et al.* 1997a). In addition, the central region catalyzes the hydration of intermediates of bile acid synthesis (Qin *et al.* 1997b). Furthermore, the C-terminal domain is assumed to be involved in the intracellular transport of sterols and lipids (Adamski *et al.* 1995, Normand *et al.* 1995, Leenders *et al.* 1996, Qin *et al.* 1997a). The mutations in the HSD17B4 gene lead to serious peroxisomal disorders (Van Grunsven *et al.* 1998).

Human 17HSD8 is capable of converting active estrogens and androgens to their low-activity forms. It is expressed abundantly in the liver and pancreas, moderately in the kidney and skeletal muscle, and weakly in the heart, placenta and lung (Maxwell *et al.* 1995, Kikuti *et al.* 1997, Fomitcheva *et al.* 1998). It is suggested that 17HSD8 plays a protective role in the target tissues against the action of sex steroids, especially in highly steroid-sensitive organs, such as the kidney (Fomitcheva *et al.* 1998).

Human 17HSD10 is a 108 kDa protein with four identical subunits (He *et al.* 1998), catalyzing the reduction of E_2 to E_1 and the β -oxidation of L-3-hydroxy-CoA to 3-ketoacyl-CoA (Yan *et al.* 1999). It also converts 3α A-diol to DHT and might thus play a role in the activation of androgens in the prostate (He *et al.* 2000a, b). In normal tissues, this enzyme is located to mitochondria (He *et al.* 2001, Shafqat *et al.* 2003), and it was also referred to as endoplasmic reticulum-associated amyloid β -binding protein during the pathogenesis of Alzheimer's disease (Yan *et al.* 1997). A high concentration of 17HSD10 is suggested to be a potential risk factor for Alzheimer's disease, because it weakens the protective effects of estrogens and generates aldehydes in neurons (He *et al.* 1999).

Human 17HSD11 was initially named as retSDR2 (Haeseleer & Palczewski 2000). This enzyme has been demonstrated to efficiently catalyze the conversion of 3α A-diol to androsterone (ADT) (Brereton *et al.* 2000). Therefore, 17HSD11 is suggested to be another enzyme involved in the control of androgen synthesis.

2.8 Aromatase

Aromatase is an enzyme complex comprising two microsomal proteins, P450arom and NADPH cytochrome P450 reductase. The latter is an essentially ubiquitous protein in the endoplasmic reticulum of different cell types. It transfers reducing equivalents from NADPH to any microsomal form of cytochrome P450, which it comes into contact with. P450arom is responsible for the recruitment of C_{19} androgens and converts them to estrogens in steroidogenic tissues, such as the ovary and placenta, as well as in some peripheral tissues, including the breast, bone, vasculature and brain (Simpson *et al.* 1994, 2002). Thus, aromatase plays a critical role in estrogen biosynthesis. In non-steroidogenic cells transfected with P450arom cDNA, androgens could be converted to estrogens (Corbin *et al.* 1988, Pompon *et al.* 1989). Furthermore, the dynamic change in aromatase activity is concurrent with the P450arom mRNA level (Evans *et al.* 1987). On the other hand, the reductase component of the aromatase complex is only subject to modest regulation (Steinkampf *et al.* 1987). Thus, the action of aromatase is basically determined by P450arom.

Human P450arom is encoded by the CYP19 gene on chromosome 15p21.2. It contains a 30 kb coding region and a 93 kb regulatory region (Simpson *et al.* 1994, Bulun *et al.* 2003). The coding region spans 9 exons beginning with exon II (Simpson *et al.* 2002). The expression of P450arom is tissue-specific, due to the alternative usage of distinct promoters in different tissues. In the placenta, for example, transcription of the CYP19 gene is controlled by the distal promoter I.1. In breast adipose tissue, however, gene transcription is governed by promoter I.4. It has been shown that the 5'-untranslated exons are spliced at a common intron/exon boundary upstream of the translational start site, which leads to identical protein translation in different tissues (Simpson *et al.* 1994, 2002 and refs. therein). However, different promoters bind various transcription factors, which lead to the dissimilar expression of P450arom in each tissue (Simpson *et al.* 1997, 2002).

2.9 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase (3 β -HSD)

Human 3 β -HSD enzymes are NAD⁺-dependent bifunctional membrane-bound single enzyme proteins. They are located in the endoplasmic reticulum and mitochondria, catalyzing the oxidation and isomerization of Δ^5 -3 β -hydroxysteroid precursors into Δ^4 -ketosteroids. Therefore, they play an essential role in the biosynthesis of all steroid hormones, including glucocorticoids, mineralocorticoids, progesterone, androgens and estrogens (Thomas *et al.* 1989, Cherradi *et al.* 1995). In addition, 3 β -HSDs catalyze the formation and/or degradation of 5 α -androstanes and 5 α -pregnanes, such as DHT and dihydroprogesterone (DHP) (Simard *et al.* 1996, Mason *et al.* 1997, Payne *et al.* 1997). In humans, there are two 3 β -HSD isoenzymes, type I and type II, encoded by HSD3B1 and HSD3B2 genes, respectively. Both genes are 7.8 kb in length, consist of four exons and three introns and are located to chromosome 1p13.1 (Lachance *et al.* 1990, Lachance *et al.* 1991). 3 β -HSD type I comprises 372 amino acids and is expressed in the placenta, skin, mammary gland, prostate, and several other normal and tumour tissues (Rheume *et al.* 1991, Dumont *et al.* 1992, Geldof *et al.* 1995, Gingras *et al.* 1999a, b). 3 β -HSD type II contains 371 amino acids and shares 93.5% identity with the type I enzyme. It is predominantly expressed in the adrenal, ovary and testis (Rheume *et al.* 1991, Lachance *et al.* 1992). Transient expression of these two isoforms shows that type I 3 β -HSD has a higher catalytic efficiency, using pregnenolone, DHEA and DHT as substrates, compared to 3 β -HSD type II (Rheume *et al.* 1991). In human placenta, 3 β -HSD type I is responsible for the conversion of pregnenolone and DHEA to P and A-dione, respectively (Thomas *et al.* 1989).

2.10 20 α -hydroxysteroid dehydrogenase (20 α -HSD)

Human 20 α -HSD belongs to the aldoketo reductase superfamily. It contains 323 amino acids, with molecular weight of 36 767 Da. It is NADP(H)-dependent and catalyzes the conversion of P into its inactive form, 20 α -hydroxy-progesterone (20 α -OH-P). In cooperation with other enzymes, such as 3 α -HSD type 3 and 17 β -HSD type 5, 20 α -HSD

plays an important role in protecting tissues from the action of P (Zhang *et al.* 2000, Couture *et al.* 2002). 20α -HSD mRNA has been detected in the liver, prostate, testis, adrenal, brain, uterus, mammary gland, endometrium and placenta (Zhang *et al.* 2000, Nakajima *et al.* 2003). In addition, 20α -HSD mRNA expression was found to increase in secretory-phase endometrium and in decidual tissue, which suggests that the enzyme down-regulates P at peri-implantation periods (Nakajima *et al.* 2003).

3 Outlines of the present study

Estrogens are essential for reproduction and female development and affect a large number of peripheral tissues. The extent of estrogen action is dependent on the expression level of ERs and the intracellular E₂ concentration in target cells. The latter is determined not only by the endocrine supply, but also by the local biosynthesis and inactivation of E₂ in target tissues. The present study aimed at better understanding of the action of the key steroidogenic enzymes governing the production and inactivation of E₂ in the placenta, a steroidogenic tissue, and in the breast, a target tissue of sex steroid hormone action.

The specific aims of this study were:

1. to examine the expression of P450arom and 17HSD1 in the feto-maternal compartment in intrauterine and tubal pregnancy.
2. to elucidate effects of retinoic acids on the expression of P450arom and 17HSD1 and E₂ biosynthesis in cultured placental cells.
3. to analyze expression of 3β-HSD1, 20α-HSD, 17HSD types 2, 5 and 7 mRNAs in human early and mid-gestation placenta.
4. to detect the expression of 17HSD types 1, 2 and 5 in a large number of normal and malignant breast tissues, to further evaluate the protein levels of ERα, ERβ, PR, Ki67 and *c-erbB2* and to identify the important molecule(s) associated with the progression of breast cancer.

4 Materials and methods

For detailed descriptions of the materials and methods, see the original articles I-IV.

4.1 Tissue samples (I-IV)

Samples of normal human placental tissues of 7-9 weeks' gestation were freshly collected at Beijing Haidian Hospital for the isolation of VCT cells. The fallopian tube samples at the mid-proliferative phase and the mid-secretory phase were collected from regularly cycling women undergoing hysterectomy because of uterine myoma at Peking Union Hospital (Beijing, China). All specimens were collected with the permission of the local ethics committee and following informed consents by the patients.

The placenta specimens from different gestational stages, feto-fallopian tube compartments from tubal pregnancies, and breast carcinoma specimens were collected at Oulu University Hospital (Oulu, Finland). The permission to use the human material was obtained from The National Authority for Medicolegal Affairs, Finland.

4.2 Cell culture (I)

The JEG-3 choriocarcinoma and T47D breast cancer cell lines were obtained from the American Type Culture Collection (Rochville, MA, USA) and cultured according to the supplier's instructions. Cytotrophoblast cells were isolated from human placenta of 7-9 weeks' gestation and cultured in FD medium (Hams Mixed Nutrients F12:DMEM=1:1).

4.3 RNA isolation and Northern blot analysis (I)

Total RNAs from cultured cells were isolated using TRIzol Reagent according to the manufacturer's instruction. 15 µg of each sample was resolved on 1% agarose gel, followed by transfer of RNA onto nylon membrane (Amersham Life Science). The UV

cross-linked membrane was separately probed by the ^{32}P -labelled 1.0-kb *EcoRI-SacI* fragment of human 17HSD1 cDNA (Piao *et al.* 1997b), the 1.4-kb *SacI* fragment of human P450arom cDNA (Ghersevich *et al.* 1994) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. After hybridization, the membrane was exposed to x-ray film (Fuji Photo Film, Tokyo, Japan) at -80°C . The autoradiographic signals were quantified by densitometry analysis with a Molecular Dynamics 300A computing densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

4.4 Reverse transcription polymerase chain reaction (RT-PCR) (I)

Total RNA (1.0 μg) of JEG-3 cells was reversed-transcribed using an oligo (dT) primer in a total volume of 20 μl . For PCR amplification, 2 μl of each RT product was added to a mixture (50 μl) containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl_2 , 0.25 mM dNTPs, 1 μM sense and antisense primers and 5U Taq DNA polymerase. The reaction was performed in 30 sequential cycles at 94°C for 20 sec, at 60°C for 30 sec and at 72°C for 30 sec, using the GenAmp PCR System 2400 (Perkin-Elmer Corp, Norwalk, CT, USA).

4.5 Measurement of aromatase and 17HSD activities (I)

Aromatase activity was assessed by measuring the tritiated H_2O produced by the specific release of tritium from $[\text{1}\beta\text{-}^3\text{H}]\text{A-dione}$ (Thompson & Siiteri 1974). In brief, JEG-3 cells, after exposure to various stimuli for the indicated time, were incubated in serum-free DMEM containing 1.0×10^5 cpm of $[\text{1}\beta\text{-}^3\text{H}]\text{A-dione}$ and 0.2 μM cold A-dione for 1 h at 37°C . The reaction was stopped by adding 0.5 ml of 30% trichloroacetic acid to the media, and the plates were then placed in an ice bath for 10 min. After that, the media were extracted with 5 ml of diethyl ether-ethyl acetate (9:1) twice, for 15 min each time. A 900 μl aliquot of the aqueous phase was measured in a scintillation counter.

Reductive 17HSD activity was determined by calculating the conversion rate of E_2 to E_1 . After the JEG-3 cells had been stimulated for 24 h, the media were removed from 6-well plates and 2 ml of serum-free medium containing 500 nM unlabelled E_1 and 2.5×10^6 cpm of $[\text{2,4,6,7-}^3\text{H}]\text{E}_1$ was added to each well. The cells were then incubated for 1 h at 37°C in cell culture conditions, and the subsequent steps followed the method described previously (Miettinen *et al.* 1996a).

4.6 Detection of E_2 by radioimmunoassay (I)

After treatment with various stimuli for 24 h, JEG-3 and CTB cells cultured in 24-well plates were washed twice with serum-free media (DMEM for JEG-3 cells and FD for CTB cells). Then, 1.0 ml of the same media containing 500 nM DHEA or A-dione was added to each well. The cells were incubated for 1 h at 37°C in cell culture conditions.

After that, the media were collected and the E₂ concentration was determined by radioimmunoassay as described previously (Zhuang & Li 1991).

4.7 Immunohistochemistry (II, III, IV)

Paraffin sections were deparaffinized with xylene and rehydrated in a descending ethanol series. Then they were retrieved in 10 mM citrate buffer (pH 6.0) at 95°C for 15 min. To eliminate endogenous peroxidase activity, the samples were treated with 1% hydrogen peroxide for 15 min. After being washed 3 times with 50 mM Tris hydrochloride buffer (TBS), the sections were incubated with primary antibodies at 4°C overnight. The subsequent procedures were carried out by using the DAKO ENVISION system (DAKO Co., Carpinteria, CA, USA) according to the manufacturer's instructions. Counterstaining with hematoxylin was performed before mounting the slides.

4.8 *In situ* hybridization (II, III, IV)

A 376-bp fragment (nt 1-376) of human 17HSD type 1 cDNA (GI: 4504500) (Peltoketo *et al.* 1988), and a 380-bp fragment (nt 191-570) of human 17HSD type 2 cDNA (GI: 4504502) (Wu *et al.* 1993), were constructed in pGEM-4Z vector (Promega). A 594-bp fragment (nt 407-1000) of human 17HSD5 cDNA (GI: 24497582) (Dufort *et al.* 1999), a 832-bp fragment (nt 39-870) of human 17HSD type 7 cDNA (GI: 7705420) (Krazeisen *et al.* 1999), a 732-bp fragment (nt 614-1345) of human P450arom cDNA (GI: 13904859) (Simpson *et al.* 1987), a 596-bp fragment (nt 123-718) of human 20 α -HSD cDNA (GI: 20149574) (Stolz *et al.* 1993) and a 706-bp fragment (nt 87-792) of human 3 β -HSD1 cDNA (GI: 287843) (Lorence *et al.* 1990), were cloned into a pCRII-TOPO (Invitrogen, Carlsbad, CA) vector, respectively. The linearized plasmids vectors were used as templates to transcribe [α -³⁵S]CTP-labelled sense and antisense RNA probes using riboprobe in an *in vitro* transcription system (Promega) with T7 or SP6 RNA polymerases. The protocol was as previously described (Mustonen *et al.* 1997). Full term placenta sections were used as controls.

The *in situ* hybridization results were evaluated semiquantitatively by dividing the signal intensity into four categories: - = no signal present; + = weak signal; ++ = moderate signal; and +++ = strong signal. The results were evaluated by two independent researchers.

4.9 Statistical analyses (I, IV)

Statistical significance was determined by analysis of variance or Student's *t*-test where appropriate. Categorical variables were analysed by Chi-square and Fisher's exact tests. Analysis of survival was performed using the Kaplan-Meier method and differences between survival curves were examined for significance using the log-rank test.

Multivariable analysis was performed with Cox-regression model to determine the independent prognostic value of variables. A two-sided P value of less than 0.05 was considered statistically significant in all cases.

5 Results

5.1 Expression of P450arom and 17HSD1 in the feto-maternal compartment in intrauterine and tubal pregnancy (II, III)

P450arom mRNA as well as 17HSD1 mRNA and protein were expressed in the ST cells of FV and in some CCT cells in normal intrauterine placentas during early and mid-gestation. In tubal pregnancy specimens, P450arom mRNA was found in ST cells, whereas 17HSD1 protein was present not only in ST cells, but also in a large portion of EVCT cells and 20% of CCT cells. In addition, the level of 17HSD1 protein expression in the epithelial cells of the fallopian tube is low at the mid-proliferative and mid-secretory stages, but significantly higher during tubal pregnancy.

5.2 Effects of retinoic acids (RAs) on the expression of P450arom and 17HSD1, and E₂ biosynthesis in cultured placental cells (I)

The effect of *all trans*-RA (*at*-RA) on aromatase activity and P450arom mRNA was studied in JEG-3 cells. After treatment of *at*-RA for 48 h, aromatase activity was significantly induced in a dose-dependent manner. Upon stimulation with 100 nM *at*-RA, enzyme activity increased 4.0-fold at 6 h, and the induction lasted for up to 48 h. Also, 8Br-cAMP, TPA (the protein kinase C activator) and IL-1 β increased aromatase activity, while 8Br-cAMP and TPA further enhanced the effect of *at*-RA. Moreover, Northern analysis showed that the mRNA expression of P450arom is induced by *at*-RA in a dose- and time-dependent fashion. In addition, 8Br-cAMP and TPA, either alone or together with *at*-RA, could remarkably elevate the expression level of P450arom mRNA.

Both *at*-RA and *9cis*-RA stimulated the conversion of DHEA or A-dione to E₂ over 2-fold in JEG-3 cells. Using DHEA as precursor, E₂ production in CTB cells was increased 2.0- and 2.8-fold by treatment of 100 nM *at*-RA and *9cis*-RA, respectively. Upon stimulation for 24 h, the expression of the 3.0-kb mRNA of P450arom in cultured CBT

cells was increased to 130% by 100 nM *at*-RA and to 170% by 100 nM *9cis*-RA, respectively. The expression of 17HSD1 mRNA was also elevated by the same treatment, but to a lesser extent.

The effects of the selective RAR α agonist Ro40 and the antagonist Ro41 on P450arom and 17HSD1 expression were also investigated in JEG-3 cells by Northern analysis. Treatment with 100 nM Ro40 for 24 h resulted in stronger induction of P450arom expression than that with 100 nM *at*-RA or *9cis*-RA. This stimulation was blocked by simultaneous administration of 1.0 μ M Ro41, which also decreased the effects of *at*-RA and *9cis*-RA. In addition to being induced by *at*-RA (9.6-fold), *9cis*-RA (8.5-fold) and Ro40 (6.8-fold), 17HSD1 expression was increased by Ro41 (3.6 fold). Furthermore, Ro41 in combination with *at*-RA and *9cis*-RA caused 28- and 24-fold induction, respectively. Co-treatment with Ro40 and Ro41 resulted in an additive increase in 17HSD1 expression. The stimulatory effect of Ro41 or Ro41 together with *at*-RA, *9cis*-RA and Ro40 was also reflected at the level of reductive 17HSD activity.

Reporter gene analysis further showed that in T47D cells, Ro41 (1 μ M) did not affect the activity of the HSD17B1 enhancer, which contains a RARE, but remarkably blocked the effect of *at*-RA (100 nM). In JEG-3 cells, 100 nM *at*-RA and 1 μ M Ro41 increased the enhancer activity to 260% and 130%, respectively, and additively elevated the enhancer activity to 300%.

RT-PCR analysis was performed to identify the presence of RXRs and RARs in JEG-3 cells. RAR α and RXR α were found, whereas no other members could be detected even though various amplification conditions were examined.

5.3 Expression of 3 β -HSD1, 20 α -HSD, 17HSD types 2, 5 and 7 mRNAs in human early and mid-gestation placenta (III)

In situ hybridization demonstrated that the mRNA of 3 β -HSD1 was expressed in placental ST cells and some CCT cells in early and mid-gestation. The expression level in CCT cells was lower than that in ST cells. On the other hand, 17HSD type 2 mRNA was located in IS cells, and the expression level increased from 7 weeks until 16 weeks of gestation and thereafter reached a plateau. The localization of type 5 mRNA was identical to that of 17HSD type 2, but signal intensity was weaker. 17HSD type 7 mRNA was present in all types of placental cells including ST, VCT, CCT and IS cells. No 20 α -HSD mRNA was detected in the early and mid-gestation placentas.

Table 1. The location of 3 β -HSD1, 17HSD2, 5, and 7 mRNAs in human early and mid-gestation placenta. ST, syncytiotrophoblast; VCT, villous cytotrophoblast; CCT, column cytotrophoblast; IS, intravillous stroma.

Enzyme	Location
3 β -HSD1	ST and CCT
17HSD2	IS
17HSD5	IS
17HSD7	ST, VCT, CCT and IS

5.4 Expression of 17HSD types 1, 2, 5, ER α , ER β , PR, Ki67 and c-*erbB2* in normal and cancerous breast tissues (IV)

In situ hybridization identified both 17HSD1 and 17HSD2 mRNAs in breast epithelial cells from normal premenopausal women, but not from normal postmenopausal women. However, 17HSD1 and 17HSD2 mRNAs were detected in the epithelial cells in malignant breast lesions, without any significant difference between the pre- and postmenopausal groups with regard to 17HSD1. On the other hand, the mean expression of 17HSD2 mRNA was higher in the premenopausal than the postmenopausal patients ($p < 0.01$).

In breast cancer specimens, the positive cases for the mRNA of 17HSD types 1, 2 and 5 were 16%, 25% and 65%, respectively. No difference in mean age between the patients positive and negative for 17HSD1 mRNA was observed. Significant differences in the patients' mean age between the positive and negative subjects were revealed by 17HSD2 and 17HSD5 mRNA expression. The mean ages of the 17HSD2-positive and negative cases were 54.5 and 58.3 years, respectively ($p = 0.004$), and those of the 17HSD5-positive and negative cases 58.5 and 55 years, respectively ($p = 0.0173$).

Grade III tumours showed more often 17HSD1 and 17HSD2 mRNA expression than the lower grades ($p = 0.04$ and 0.046 , respectively). Moreover, the 17HSD1-positive samples included more cases with larger tumours than the 17HSD2-positive ones. The expression of 17HSD5 was significantly higher in breast tumour specimens than in normal breast tissue ($p = 0.033$, Fisher's exact test). The strong expression of 17HSD5 was associated with positive nodal state ($p = 0.012$) but not with metastasis. A significant positive correlation was observed between ER α and ER β ($p = 0.02$), while significant negative correlations were found between ER α and 17HSD1 ($p = 0.04$) as well as between ER α and 17HSD5 ($p = 0.001$). Tumours with low ER β expression displayed significantly more metastatic growth ($p = 0.0006$).

Our study also revealed that patients with tumours expressing 17HSD1 mRNA had significantly worse survival ($p = 0.0010$, log rank) and a shorter disease-free interval ($p = 0.0134$) than all other cases. No such association was found with tumours expressing 17HSD2 mRNA. The group with 17HSD5 overexpression had a worse survival than the groups with lower or no expression ($p = 0.0146$). Patients with ER α positive breast cancer had better survival than those without ($p = 0.045$). Multivariate Cox analysis (forward stepwise regression) was used to determine the possible independent prognostic significance of the following parameters: tumour size, the presence of nodal and distant metastases, grade of the tumour, ER α , ER β , PR, 17HSD1, 17HSD2, 17HSD5 and c-*erbB2*. It showed that 17HSD1, tumour size and ER α had independent prognostic value.

6 Discussion

6.1 P450arom and 17HSD1 in the feto-maternal compartment in intrauterine and tubal pregnancy (II, III)

The human placenta is a unique organ specialized in supporting fetal development and maintaining maternal endocrine homeostasis. It has been established that the hormone production in the placenta takes place mostly in the ST cells of FV (Pepe & Albrecht 1999). The fallopian tube environment encountered by trophoblast cells is presumably different from the intrauterine environment. However, the present study demonstrated that P450arom and 17HSD1 are exclusively present in ST cells during both normal and tubal pregnancies. It can be suggested that the expression of P450arom and 17HSD1 and, therefore, E₂ biosynthesis are intrinsic features of placental ST cells. Furthermore, the location of the placenta is unlikely to affect the production of estrogens in it.

It has been demonstrated in several studies that villous CT cells express 17HSD1 (Beaudoin *et al.* 1995, Bonenfant *et al.* 2000). An interesting finding in the present study is that 17HSD1 was identified in a large portion of the EVCT cells invading into the fallopian stroma, but not in about 80% of CCT cells. The divergent expression of 17HSD1 in different CT cells suggests that the close contact of CT cells *in vivo* might play a role in silencing the HSD17B1 gene in CT cells. In accordance with a recent report showing that 17HSD1 is expressed in the invasive CT cells of the full-term placenta (Bonenfant *et al.* 2000), it indicates that the function of 17HSD1 is associated with invasive CT cells in both the early and the final stages of pregnancy.

It was discovered in this study that the fallopian tube expresses limited amounts of 17HSD1 during the menstrual cycle. However, its expression increases dramatically subsequent to the occurrence of the tubal pregnancy. This may be a way to ensure a sufficient E₂ supply for the maintenance of the pregnancy.

6.2 Influence of RAs on the expression of P450arom and 17HSD1 in placenta cells (I)

In choriocarcinoma cells, the protein kinase A (PKA) and protein kinase C (PKC) pathways play important roles in the induction of P450arom and 17HSD1 expression (Ritvos & Voutilainen 1992). However, the primary regulatory factors sustaining the high level of estrogenic enzymes in the placenta have been difficult to define because of the complexity of the placental milieu. It has been found that RAs stimulate 17HSD1 expression in JEG-3 choriocarcinoma cells in a dose- and time-dependent manner (Piao *et al.* 1997b). In the present study, it was shown that RAs induce aromatase activity and P450arom mRNA expression in JEG-3 cells. In addition, the mRNA levels of both P450arom and 17HSD1 are increased by RAs in cultured CBT cells isolated from a normal placenta, which further underlines the role of RAs in regulating estrogenic enzymes in the placenta. Moreover, the conversion of DHEA/A-dione to E_2 is significantly stimulated by RAs in cultured JEG-3 and CBT cells, as demonstrated in this study. Put together, our data suggested that RAs may act as regulatory factors for placental E_2 production.

It has been demonstrated that the effect of RAs on 17HSD1 expression is mediated by a RARE located in the 5'-flanking region of the HSD17B1 gene (Piao *et al.* 1995, 1997b). In human placenta, RAR α and RXR α are the major forms of retinoid receptors expressed in trophoblastic cells. RT-PCR performed in this study showed that RXR α and RAR α are the only retinoid receptors expressed in JEG-3 cells. In fact, the RXR α /RAR α heterodimer could bind to the RARE of the 17HSD1 gene (Piao *et al.* 1995). An interesting finding in this study was that Ro41, the selective inhibitor of RAR α , induces the mRNA expression and enzyme activity of 17HSD1 in JEG-3 cells and, moreover, potentiates the effects of RAs. On the other hand, Ro41 indeed acts as an inhibitory factor for P450arom expression in JEG-3 cells, as shown in this study, and for 17HSD1 expression in breast cancer T47D cells (Reed *et al.* 1994). The present study also showed that Ro41 blocks the activation of *at*-RA on the transcription of the HSD17B1 gene in T47D cells. These data imply that JEG-3 cells may possess a novel pathway for the action of retinoids on 17HSD1 expression. A further reporter gene analysis indicated that although Ro41 is able to increase the activity of the 17HSD1 enhancer containing the RARE in JEG-3 cells, the extent was remarkably less than its influence on the mRNA expression of 17HSD1. Moreover, the combined effect of Ro41 and *at*-RA on HSD17B1 enhancer in JEG-3 cells is only additive, and not so synergistic as shown by mRNA analysis. Therefore, the effect of Ro41 on the expression of 17HSD1 in JEG-3 cells could occur at both transcriptional and translational levels, especially the mRNA stabilization. Alternatively, Ro41 may activate a subtype of retinoid receptors, which is targeted to an unknown regulatory area in the HSD17B1 gene rather than the RARE located at the region from -512 to -479 in the enhancer. In addition, it was the first observation showing that Ro41 acts as agonist, broadening our knowledge of this compound in retinoid signaling.

The CYP19 gene contains a functional RARE in the distal promoter I.1 (Sun *et al.* 1998). However, this RARE does not seem to interact with the RXR α /RAR α heterodimer, the only form available in JEG-3 cells, as RAR α is not present in the protein

complex binding to this RARE (Sun *et al.* 1998). On the other hand, the present study showed that Ro40, a specific ligand for RAR α , could induce P450arom mRNA expression in JEG-3 cells and, further, that this induction can be blocked by Ro41. Collectively, it is indicated that the effect of RAs on P450arom expression is probably mediated by an unknown RARE, which interacts with the RXR α /RAR α heterodimer in the CYP19 gene.

The action of RAs on P450arom seems to occur at multiple levels. A short stimulation (6 h) with *at*-RA results in a maximal increase in aromatase activity in JEG-3 cells. However, the influence on P450arom mRNA expression appears to be time-dependent, being highest at 48 h, as examined. This is similar to the effect of *at*-RA on 17HSD1 mRNA expression (Piao *et al.* 1997b). Also, the extent of induction on enzyme activity is higher than that on mRNA expression. These findings indicate that RAs may also influence the translation or post-translational modification of aromatase in choriocarcinoma cells, in addition to increasing the transcription of the CYP19 gene or the stabilization of P450arom transcripts. Hence, inhibition of RNA synthesis in cultured cells with subsequent stimulation of RAs would provide more solid information about the effect on the post-transcriptional regulation of P450arom mRNA.

The placenta possesses multiple regulatory pathways in hormone production (Ritvos & Voutilainen 1992, Nestler 1993). In addition to the PKA and PKC pathways, the epidermal growth factor (EGF) and the calcium ion, either alone or in combination with RAs, promote the action of 17HSD1 in JEG-3 cells (Piao *et al.* 1997b). The present study further demonstrated that various regulatory factors, such as RAs, cAMP, TPA and IL-1 β can influence aromatase activity in JEG-3 cells. Therefore, the mechanisms controlling E₂ biosynthesis in the placenta include the regulation of P450arom and 17HSD1 by RAs in association with multiple regulators.

6.3 3 β -HSD1, P450arom and 17HSD1 in placental CCT cells (III)

It is well agreed that the biosynthesis of P and estrogens in the placenta predominantly occurs in ST cells, since all enzymes involved in the procedure, such as cholesterol side-chain cleavage cytochrome P450 (P450_{scc}), 3 β -HSD1, P450arom and 17HSD1, have been localized in ST cells (Fournet-Dulguerov *et al.* 1987, Beaudoin *et al.* 1997, Bonenfant *et al.* 2000). In this study, the expressions of 3 β -HSD1, P450arom and 17HSD1 mRNA were also detected in some CCT cells. These findings provide evidence that some CCT cells may undergo P and E₂ biosynthesis in early and mid-term intrauterine placentas. On the other hand, P450arom mRNA was not found in CCT cells during tubal pregnancy, indicating that the capacity of these cells in E₂ production differs from that of CCT cells during normal pregnancy.

To deepen the present study, a method to isolate CCT cells for in vitro culturing should be developed. This would enable a further investigation about the steroidogenesis in CCT cells under culture condition.

6.4 17HSD2, 5 and 7 in the placenta (III)

In the present study, 17HSD5 and 7 were localized, for the first time, in placental cells. The presence of 17HSD5 indicates that the placenta is capable of performing the biosynthesis of androgens from steroid precursors. The presence of 17HSD7 in various cell types in the placenta suggests that E₂ biosynthesis may also take place in the intravillous stroma. The expression of 17HSD5 and 7 could be detected from the 7th week of pregnancy. By 7 weeks of pregnancy, almost all circulating estrogens in the maternal body are, in fact, produced by the placenta (Simpson & Macdonald 1981). Around this time, too, the male fetal Leydig cells start to produce T, which can be introduced into the maternal circulation (Siiteri & Wilson 1974). Accordingly, 17HSD2 is already expressed in IS cells at week 7 of pregnancy, as shown in this study. In this way, the placenta could establish a timely defence against the action of E₂ on the fetus and the action of T produced by the placenta on a female fetus, and vice versa, against the unwanted influence of T synthesized by a male fetus on the maternal body. Furthermore, the increasing expression of 17HSD2 during the weeks 7-16 of gestation is concurrent with the increasing T production by male fetuses. In the previous report, 17HSD2 was localized in the intravillous endothelial cells in the placenta by immunohistochemistry (Moghrabi *et al.* 1997, Takeyama *et al.* 1998, Bonenfant *et al.* 2000). In the present study, we used in situ hybridization, a more reliable method, and found 17HSD2 was located in the intravillous stroma cells. The broader localization in the FV may ensure that 17HSD2 inactivates E₂ and T more efficiently. Meanwhile, 17HSD2 together with 17HSD5 and 7 also play a role in modulating the concentration of P. Put together, it can be suggested that from 7 weeks onwards, the human placenta contributes to maintaining the homeostasis of P, estrogens and androgens, which is necessary for both the maternal body and the fetus during the progression of pregnancy.

No quantitative measurement of 17HSD2 mRNA was made in the present study. Additional analysis, such as Northern blot or real time RT-PCR, may further support the finding of gestational increase of 17HSD2 mRNA in the placenta.

In further studies, steroid production in cultured placenta IS cells should be examined. It would provide direct evidence about the capability of IS cells in the biosynthesis and inactivation of sex steroid hormones.

6.5 Steroid metabolism in breast cancer: 17HSD type 1, an independent prognostic factor in breast cancer (IV)

Estrogens are essential for the growth and differentiation of the mammary gland. It has been shown that estrogens induce and promote mammary cancer in rodents (Nandi *et al.* 1995) and exert proliferative effects on cultured human breast cancer cells (Cullen & Lippman 1989), but the exact mechanisms of estrogen action in the development of breast cancer remain to be elucidated.

In the present study, we showed that mRNAs for the 17HSD1 and 17HSD2 enzymes are present in the epithelial cells of normal breast tissues of premenopausal women but

not in postmenopausal women, which is in agreement with the previous study (Miettinen *et al.* 1999) demonstrating constant expression of 17HSD1 and 17HSD2 mRNA in the breast throughout the menstrual cycle. In breast cancer tissues, the enzymes were expressed in both premenopausal and postmenopausal patients. In benign breast tissue and breast cancer, variable amounts of 17HSD1 and 17HSD2 enzymes have been reported (Poutanen *et al.* 1992, Sasano *et al.* 1996, Ariga *et al.* 2000, Suzuki *et al.* 2000, Gunnarsson *et al.* 2001). In this study we detected 17HSD1 mRNA in 16%, 17HSD2 mRNA in 25 % and 17HSD5 in 65% of breast cancer specimens.

Accumulation of estradiol in breast cancer tissue has been detected in postmenopausal women and may result from a higher aromatase, steroid sulfatase or reductive 17HSD activity or lower oxidative 17HSD activity. A recent study showed that the estradiol/estrone ratio and the expression of 17HSD1, but not that of aromatase or sulfatase, are higher in breast cancer tissues of postmenopausal compared to premenopausal patients (Miyoshi *et al.* 2001). This suggests that 17HSD1 is mainly responsible for the accumulation of estradiol in breast cancer tissue. 17HSD2 catalyzes the conversion of estradiol to estrone, and it may therefore have a protective role against estradiol influence in normal breast epithelium. The expression of 17HSD5 in breast tumour specimens was significantly higher than in normal breast tissue. The group with 17HSD5 overexpression had a worse survival than the groups with lower or no expression of 17HSD5. So far little is known about the role of 17HSD5 in breast tissue; however, the activities of 17HSD5 suggest that it might be involved in the production of progesterone and sex steroids in the breast.

Breast cancer is a multifactorial disease. In addition to the hereditary and acquired genetic alterations involved in the development of the disease, several environmental and hormonal factors interact with genetic alterations. A number of biological parameters appear to strongly influence tumour behaviour and have been tested as prognostic parameters in patients with breast cancer. Our study showed that patients with tumours expressing 17HSD1 mRNA had shorter disease-free and overall survival than the other cases in both ER-positive and negative patients, which indicates that 17HSD1 is an independent prognostic marker in breast cancer progression regardless of the ER status.

Estrogens exert their effect through two members of the nuclear receptor superfamily, ER α and ER β . It has been shown that ER α -positive breast cancer cells proliferate in response to estradiol (Cullen *et al.* 1989). Our study showed a significant correlation between ER α and ER β status. This is in agreement with the suggested co-expression of ER α and ER β in most breast cancers (Järvinen *et al.* 2000). Moreover, we found negative correlations between 17HSD1 and ER α , and 17HSD5 and ER α , respectively. It suggests the analysis of 17HSD1 and possibly 17HSD5 could be helpful in selection of the most suitable therapy for breast cancer patients.

In normal developing mammary gland, *c-erbB-2* has been suggested to have an important role in the regulation of cell growth and differentiation. Overexpression of *c-erbB-2* is characterized by a loss of estrogen sensitivity of tumour cells and aggressive tumour growth, leading to the development of resistance against a number of therapeutics (Jukkola *et al.* 2001, Gunnarsson *et al.* 2003). However, no correlation between 17HSDs and *c-erbB-2* expression was detected in our study.

6.6 Conclusions

In this study, the expressions of 3β -HSD1, P450arom, 17HSD types 1, 2, 5 and 7 as well as 20α -HSD in human placenta were investigated. With the exception of 20α -HSD, all the other enzymes were detected in various cells of intrauterine placentas during early and mid-gestation. Co-existence of these enzymes plays an important role in the maintenance of the homeostasis of P, estrogens and androgens, which is necessary for both the maternal body and the fetus during pregnancy.

Examination of 17HSD1 and P450arom expression at the fetomaternal interface during tubal pregnancy revealed that intrauterine and tubal pregnancies may possess similar mechanisms for estrogen biosynthesis in the placenta. The increasing expression of 17HSD1 in the fallopian epithelia during tubal pregnancy is thought to provide a sufficient local E_2 supply for the maintenance of the tubal pregnancy.

Using cultured JEG-3 and CBT cells, RAs were demonstrated to promote the action of P450arom and 17HSD1 on the biosynthesis of E_2 in the placenta.

The results of the study performed with breast cancer tissues suggest that 17HSD1 and 17HSD5 are associated with hormone independency of breast cancer. Since high 17HSD1 expression is correlated with a poor prognosis, it is suggested that inhibition of this enzyme could be a beneficial therapy for breast cancer patients.

The present study discovered that both the placenta and the breast possess the enzymes for androgen biosynthesis. Further studies need to be carried out to investigate whether these tissues can produce androgens from steroid precursors.

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