17-β HYDROXYSTEROID DEHYDROGENASE TYPES 1 AND 2
Expression and activities in various tissues and cell lines and effect of the type 1 enzyme on estrogen-dependent growth of breast cancer cells

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To my family
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Abstract

17β-Hydroxysteroid dehydrogenases (17HSDs) catalyze the reactions between 17-hydroxy and 17-keto steroids. In the present study, the enzyme activities and tissue distribution of 17HSD type 1, type 2 and type 4 were characterized. Furthermore, the role of 17HSD type 1 in estrogen-dependent growth was studied in MCF-7 breast cancer cells which were stably transfected with type 1 cDNA.

Endogenous oxidative 17HSD activity found in COS-m6 monkey kidney cells was first compared with that of human placental 17HSD. Cultured COS-m6 cells exclusively possessed oxidative 17HSD activity, converting estradiol (E2) to less active estrone (E1). When placental 17HSD was transfected into these cells, highly reductive activity appeared. The 17HSD enzyme in COS-m6 cells also catalyzed the conversion of testosterone to androstenedione, whereas the placental enzyme was estrogen-specific. These results further proved the existence of different 17HSD isoenzymes.

The enzymatic properties and cell- and tissue-specific expression of 17HSD type 1, type 2 and oxidative type 4 were further characterized. The data confirmed that in cultured cells the direction of 17HSD activity is determined by the expression of different isoenzymes and not by the intracellular environment. In addition, the 17HSD type 1 gene expresses two mRNA signals, 1.3 kb and 2.3 kb in size. The expression of 1.3 kb mRNA, but not 2.3 kb mRNA was related to enzyme concentration in all the cell types studied. The type 1 enzyme was expressed in the placenta, ovary and in some breast cancer specimens and in the cell lines originated from these tissues. 17HSD type 2 was more widely expressed in both steroidogenic and in target tissues of steroid action. 17HSD type 4 was expressed in almost all cell lines and in all tissues studied, but no correlation with 17HSD activity was detected. These results suggest that 17HSD type 1 is involved in E2 production in females and 17HSD type 2 is responsible for inactivation of sex steroids. However, the oxidation of 17β-hydroxysteroids seems not to be the primary activity of 17HSD type 4.

The mRNAs for 17HSD type 1, type 2 and type 4 were found to be expressed in human mammary epithelial cells. In breast tissue samples both 17HSD type 1 and type 2 were detected by in situ hybridization. Despite the presence of 17HSD type 1 mRNA in human mammary epithelial cells, only oxidative 17HSD activity was detected. The reason for the lack of reductive activity is not yet known.

Finally, MCF-7 breast cancer cells were stably transfected with 17HSD type 1 cDNA in order to study the effect of 17HSD type 1 on estrogen-dependent growth. In wild type MCF-7 cells, very low 17HSD activity was detected and E1 did not have any effect on cell growth. In the cells expressing 17HSD type 1, E1 was rapidly converted to E2. Hence in these cells E1 had a similar growth-promoting effect as E2 as a result of the action of 17HSD type 1. The presence of 17HSD type 1 in breast cancer cells may thus be an important factor regulating estrogen exposure and the estrogen-responsive growth of breast cancer tissue.

Keywords: steroid metabolism, estrogens
Acknowledgements

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Oulu, October 1999

Minna Miettinen
**Abbreviations**

### Hormones

- **3α-adiol**: 5α-androstane-3α,17β-diol
- **A-diol**: androstenediol, 5-androstene-3β,17β-diol
- **A-dione**: androstenedione, 4-androstene-3,17-dione
- **androsterone**: 3α-hydroxy-5α-androstan-17-one
- **cholesterol**: 5-cholesten-3β-ol
- **cortisol**: 11β,17,21-trihydroxy-4-pregnene-3,20-dione
- **cortisone**: 17,21-dihydroxy-4-pregnene-3,11,20-trione
- **DHEA**: dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one
- **DHEA-S**: dehydroepiandrosterone sulfate
- **DHT**: 5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one
- **E1**: estrone, 3-hydroxy-1,3,5(10)-estratriene-17-one
- **E1-S**: estrone sulfate
- **E2**: estradiol, 1,3,5(10)-estratriene-3,17β-diol
- **E3**: estriol, 1,3,5(10)-estratriene-3,16α,17β-triol
- **FSH**: follicle stimulating hormone
- **LH**: luteinizing hormone
- **P**: progesterone, 4-pregnene-3,20-dione
- **pregnenolone**: 3β-hydroxy-5-pregn-20-one
- **17α-hydroxyprogesterone**: 17α-hydroxy-4-pregnene-3,20-dione
- **17α-hydroxypregnenolone**: 3β,17α-dihydroxy-5-pregn-20-one
- **T**: testosterone, 17β-hydroxy-4-androsten-3-one

### Others

- **AKR**: aldoketoreductase
- **cAMP**: cyclic adenosine-3',5'-monophosphate
- **bp**: base pairs
- **Da**: dalton
- **cDNA**: complementary deoxyribonucleic acid
- **EGF**: epidermal growth factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERα</td>
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</tr>
<tr>
<td>ERβ</td>
<td>estrogen receptor β</td>
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<tr>
<td>ERE</td>
<td>estrogen response element</td>
</tr>
<tr>
<td>ERKO</td>
<td>ERα gene knock-out</td>
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<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HME cells</td>
<td>human mammary epithelial cells</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>20α-HSD</td>
<td>20α-hydroxysteroid dehydrogenase</td>
</tr>
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<td>3β-hydroxysteroid dehydrogenase/Δ5,Δ4-isomerase</td>
</tr>
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<tr>
<td>HSD17B2</td>
<td>17β-hydroxysteroid dehydrogenase type 2 gene</td>
</tr>
<tr>
<td>HSD17BP1</td>
<td>17β-hydroxysteroid dehydrogenase type 1 pseudogene</td>
</tr>
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<td>Hsp</td>
<td>heat shock protein</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
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<td>kilodalton</td>
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<tr>
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<td>multifunctional enzyme II</td>
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<tr>
<td>NAD</td>
<td>nicotinamide-adenine dinucleotide</td>
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<tr>
<td>NADP</td>
<td>nicotinamide-adenine dinucleotide phosphate</td>
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<tr>
<td>P450arom</td>
<td>cytochrome P450 aromatase</td>
</tr>
<tr>
<td>P450c17</td>
<td>17α-hydroxylase/C-17,20 lyase</td>
</tr>
<tr>
<td>P450ssc</td>
<td>type I mitochondrial cytochrome P450, cholesterol side-chain cleavage enzyme</td>
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<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RNA</td>
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</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>poly(A)+ RNA</td>
<td>polyadenylated RNA</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>SDR</td>
<td>short-chain dehydrogenase/reductase</td>
</tr>
<tr>
<td>TDLU</td>
<td>terminal ductal lobular units</td>
</tr>
<tr>
<td>X</td>
<td>any amino acid</td>
</tr>
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</table>
List of original publications

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


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Estrogens are steroid hormones, which belong to a group of lipophilic hormones synthesized from the common precursor cholesterol. Three endocrine organs are specialized in steroid hormone production in mammals: adrenal cortex, ovary and testis. During pregnancy the placenta develops as an additional major source of steroid hormones. The capability of steroidogenic tissues to secrete steroid hormones is regulated by specific extracellular hormones and factors that influence the rate of steroid hormone biosynthesis or catabolism.

In premenopausal females estrogens are predominantly synthesized in the ovaries as estradiol (E2), but estrogens are also made in some other tissues. Adipose tissue in particular, but also skin, skeletal muscle, hair follicles and bone have been indicated as estrogen-producing tissues. The major estrogen synthesized in these tissues is estrone (E1) and this peripheral production is regarded as an important source of estrogens in postmenopausal women and in men.

In addition to being endocrine factors, estrogens are autocrine regulators, which play a vital role at the site of their formation. They can also act as intracrine factors in the same cells in which they have been synthesized from circulating precursors. The intracrine function in peripheral tissues is especially dominant after menopause, and the steroid ligand supply is extensively modulated by it (Labrie 1991, Adams 1991).

1. Introduction

17β-hydroxysteroid dehydrogenases (17HSDs) catalyze the reactions between 17-oxosteroids and 17β-hydroxysteroids. Hence 17HSDs catalyze...
both oxidative and reductive reactions and control the last steps in the formation of E2 and testosterone (T). When this study was initiated only one 17HSD enzyme was cloned (Peltoketo et al. 1988, Luu-The et al. 1989a). Now, eight different enzymes named 17HSD have been characterized (Peltoketo et al. 1999 and refs. therein). The nomenclature of these enzymes has been variable. They have been called estradiol dehydrogenase, 17-ketosteroid reductase, 17β-hydroxysteroid oxidoreductase and 17β-hydroxysteroid dehydrogenases, for example. In this thesis the term 17β-hydroxysteroid dehydrogenase is used to describe these enzymes regardless of whether they catalyze oxidative, reductive or both reactions. These enzymes can regulate intracellular concentrations of active sex steroids. The important role of 17HSD probably explains the existence of such a large series of 17HSD isoenzymes with distinct cell-specific expression, substrate specificity, and regulatory mechanisms. In this study the expression and activities of 17HSD type 1, type 2 and type 4 have been characterized. In addition, the effect of 17HSD type 1 expression on estrogen-dependent growth was studied in MCF-7 breast cancer cells.
2. Review of the literature

2.1. Physiological effects of estrogens

E2 is the most potent female sex steroid and is mainly responsible for estrogen action in women. The two other major estrogens, E1 and estriol (E3), are far less active than E2. Estrogens are classical endocrine factors that induce and maintain female secondary characteristics and enable a large number of different functions outside the female reproductive system. During the reproductive years of women, the estrogens released from the ovary into the circulation exert a feedback action on the hypothalamic-pituitary unit to effect the synchronized preovulatory release of gonadotropins. Together with other hormones, estrogens therefore coordinate the menstrual cycle (Richards 1980). They are also involved in mammary gland development (Topper & Freeman 1980). The critical roles of estrogens, furthermore, include regulation of the maintenance of pregnancy, fetal maturation and neonatal self-sufficiency (Pepe & Albrecht 1995).

In addition to these effects in the reproductive system, estrogens are involved in a wide variety of other functions in the human body. Estrogens have cardioprotective effects, affecting cholesterol metabolism in the liver, and having direct effects on the arterial wall (Farhat et al. 1996). Estrogens also regulate bone metabolism. Diminished estrogen levels result in reduced new bone formation and thus estrogens appear to be the most important sex steroids as regards preventing osteoporosis in women (Oursler et al. 1993, Turner et al. 1994 and refs. therein, Kanis 1996). In addition, estrogens modulate brain function, for example mood and memory (Fink et al. 1996 and refs. therein).

2.2. Estrogen biosynthesis in human steroidogenic tissues

Three endocrine organs are specialized in steroid hormone production: the ovary is the main source of circulating estrogens, the adrenal cortex mainly produces dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S) and the testis synthesizes androgens. During pregnancy, the placenta develops as an additional major source of steroid hormones. Although each organ produces its own characteristic profile of steroid
hormones, the enzymes involved and the organization of the process are fundamentally similar in every case (Fig. 1).

All steroid hormones are synthesized from a common precursor, cholesterol. Endocrine organs are able to synthesize cholesterol from acetate (Rainey et al. 1986), but human steroidogenic cells derive most of their cholesterol from plasma low density lipoproteins (Faust et al. 1977, Kovanen et al. 1980). Conversion of cholesterol to pregnenolone in mitochondria is catalyzed by P450 side-chain-cleavage enzyme (P450scc, Lambeth et al. 1985, Zlotkin et al. 1986). This enzyme catalyzes three reactions: 20α-hydroxylation, 22-hydroxylation and cleavage of the cholesterol side chain at the bond between carbon atoms 20 and 22 (Jefcoate et al. 1970, 1992, Burstein & Gut 1976). Pregnenolone, after being transferred to the cytosol, is converted to progesterone (P). This conversion is modulated by 3β-hydroxysteroid dehydrogenase/Δ5-Δ7 isomerase (3β-HSD) type 2 in the adrenals, ovary and testis (Rhéaume et al. 1991). 3β-HSD type 1 catalyzes the same reactions as 3β-HSD type 2, but is expressed in the placenta and peripheral tissues, such as the skin and mammary gland (Luu The et al. 1989, Lachance et al. 1990, Lorence et al. 1990, Simard et al. 1993). Both pregnenolone and progesterone undergo 17α-hydroxylation to 17α-hydroxypregnenolone and 17α-hydroxyprogesterone and furthermore undergo cleavage at C-17,20 to yield DHEA and androstenedione (A-dione), respectively. These reactions are catalyzed by a single enzyme, 17α-hydroxylase/C-17,20 lyase (P450c17, Hedin et al. 1987, Sasano et al. 1989). DHEA is further converted to A-dione by 3β-HSDs. Then P450 aromatase (P450arom) aromatizes A-dione to E1 (Simpson et al. 1994 and refs. therein). 17HSD activity is needed to convert E1 to the more potent steroid, E2, as well as A-dione to T.

CHOLESTEROL

\[ \text{P450}_{\text{acc}} \]

PREGNENOLONE

\[ \text{P450}_{\text{17c17}} \] (17α-hydroxylase)↓

3HSDs

17α-HYDROXYPREGNENOLONE

\[ \text{P450}_{\text{17c17}} \] (17,20-lyase)↓

P450c17 (17α-hydroxylase)

DHEA

\[ \text{P450}_{\text{17c17}} \] (17,20-lyase)↓

P450c17 (17,20-lyase)

3HSDs↓

A-DIONE ← 17HSDs → T

\[ \text{P450}_{\text{arom}} \]↓

P450arom↓

E1 ← 17HSDs → E2

Fig. 1. Simplified catalytic pathway from cholesterol to estrogens

The main function of the ovary is to produce a mature fertilizable ovum (Krasnow 1991 and refs. therein). In the ovarian follicle androgen biosynthesis takes place in the theca-cells. The androgens are moved to the granulosa cells and are there aromatized and reduced by P450arom and 17HSD type 1. Steroid hormone production in the ovary is controlled by the two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). This co-work model is called the two cell-two gonadotropin theory,
where A-dione and testosterone, which are produced from cholesterol in LH-stimulated theca cells (Hedin et al. 1987), are used as substrates for estrogen biosynthesis in FSH-stimulated granulosa cells (Richards 1980 and refs. therein, Sasano et al. 1989, Tamura et al. 1992).

The most abundant products of the adrenals are DHEA and DHEA-S, which serve as substrates for steroid biosynthesis in peripheral tissues, such as breast tissue. The adrenals also synthesize mineralocorticoids, glucocorticoids, and small amounts of E2. During pregnancy the placenta and fetal adrenals produce a variety of steroid hormones. The human placenta can synthesize P from cholesterol, but it does not have the capacity to further convert C_{21}-steroids to C_{19}-steroids (Siiteri & MacDonald 1966). However, the human placenta is capable of catalyzing efficiently the conversion of maternal or fetal precursors, DHEA and DHEA-S, to androgens and further to estrogens (Pepe & Albrecht 1995 and refs. therein). Testicular steroidogenesis takes place in Leydig cells, which are the only male cell types that can produce T from cholesterol. The testes produce mainly androgens, but also small amounts of estrogens are synthesized (Huhtaniemi & Toppari 1995, Hall 1994).

2.3. Estrogen biosynthesis and metabolism in peripheral tissues

Human adrenals secrete large amounts of the inactive precursor steroids DHEA and DHEA-S, which are converted into potent androgens and estrogens in peripheral tissues (Longcope 1996). The rate at which each sex steroid is formed is dependent on the substrate supply and the expression of androgen- and estrogen-synthesizing enzymes in each tissue. Locally produced hormones exert their action inside the same cells, in which the final steps of their synthesis take place (Labrie 1997 and refs. therein). This is especially important in women after menopause, when most of the estrogens are formed in peripheral tissues.

Peripheral estrogen synthesis results from the activities of three main enzymes. The aromatase enzyme complex, which converts adrenal A-dione to E1, is widely distributed throughout the body (Folkerd & James 1983, Sasano & Harada 1998). Aromatase activity is also detected in normal breast tissue and in more than 50% of breast tumors (Tilson-Mallet et al. 1983, Lipton et al. 1987, Brodie et al. 1997). A great deal of the formed E1 is converted to its sulfate (E1-S) by estrone sulfotransferase. E1-S concentrations in blood and breast tissues are remarkably higher than those of unconjugated estrogens, and also the half-life of E1-S in blood is much longer than that of unconjugated estrogens. Hence E1-S can act as a reservoir for the formation of E1 after hydrolysis by estrone sulfatase (Santner et al. 1986, Pasqualini et al. 1992). In breast tumors E1 can be synthesized via sulfatase 10-fold more than via aromatase under conditions of limited substrate availability (Santner et al. 1984). E1 can then be converted to biologically active E2 by reductive 17HSD activity (Poutanen et al. 1995 and refs. therein).
Steroids are further metabolized mostly in the liver and excreted to the urine. E2 is rapidly converted to E1 by oxidative 17HSD activity (Wu et al. 1993, Adamski et al. 1995, Mustonen et al. 1997a, Biswas & Russel 1997, Fomitcheva et al. 1998). Some E1 reenters the circulation, but most is hydroxylated to 16α-hydroxyestrone and further to E3. In addition to these, a wide variety of other hydroxylated metabolites are formed (Bolt 1979, Zhu & Conney 1998, and refs. therein). After several reactions, inactivated metabolites are finally conjugated to glucuronic or sulfuric acid. Conjugation makes these lipids more water soluble and allows them to be eliminated through the kidney. Some of the metabolites of E2 are active and it has been suggested that several of these active molecules could have unique effects through their own, but still unknown, receptors (Zhu & Conney 1998).

**2.4. Estrogen receptors**

Estrogen action in target cells involves a pathway where E2 freely diffuses across the cell membrane and binds to its receptor. The unliganded estrogen receptor (ER) is associated with a large multiprotein complex, including heat shock protein 90 (Hsp90), Hsp56, Hsp70 and p23, which maintains the receptor in a conformation able to bind the ligand (Baulieu et al. 1987, Chambraud et al. 1990, Pratt 1990 and refs. therein, Segnitz & Gehring 1995). Following estrogen binding, the receptor dissociates from the complex and binds as a dimer to a specific estrogen response element (ERE, Kumar & Chambon 1988). The ERE is a palindromic sequence, the optimal sequence being 5'-C(A/G)GGTCAnnnTGACC(T/C)G-3' and any change from this diminishes ER-ERE interaction (Klein-Hitpaß et al. 1986, Driscoll et al. 1998). Once bound to an ERE in a target gene, the ER dimer interacts, directly or indirectly, with the basal transcription machinery to modulate transcription. The ER has been shown to interact directly with the basal transcription factors TFIIIB (Ing et al. 1992) and TATA-box binding protein (Sadovsky et al. 1995). Furthermore, the two individual transactivating functions in
different domains of the ER are dependent on cell and promoter type, suggesting that these different activities may be modulated by coactivators that are specific to one or other of the domains (Tasset et al. 1990). Several receptor interacting proteins have been identified, which can modulate transcriptional activity of ER in the presence of E2 but not in the presence of antiestrogens (Cavaillès et al. 1994, 1995, Halachmi et al. 1994, Horwitz et al. 1996, McKenna et al. 1999).

The ERs belongs to a family of nuclear proteins including receptors for the other classes of steroid hormones, thyroid hormone, vitamin D, and retinoids, in addition to a number of proteins with high sequence homology but as yet unidentified ligands (Evans 1988, Green & Chambon 1988, Parker 1993). The receptors act as ligand-inducible transcription factors whose binding sites act as enhancers (Evans 1988 and refs. therein). The receptor proteins can be divided into six regions (A-F) on the basis of sequence homology (Krust et al. 1986, Kumar et al. 1987), and shown to consist of a number of functionally independent domains.

The first ER, later named as ERα, has been cloned from the MCF-7 breast cancer cell line (Walter et al. 1985). Rat ERα is expressed in the uterus, testis, pituitary, ovary, kidney, epididymis and adrenal, as detected by using the reverse transcriptase polymerase chain reaction (RT-PCR, Kuiper et al. 1997). The distinct domain structure of ERα is well characterized. The N-terminal A/B region of ERα has a ligand-independent transcriptional activation function (Bocquel et al. 1989, Lees et al. 1989, Tora et al. 1989). The C-region, which is the most conserved among receptor family members, contains the DNA-binding region which enables the receptor to bind to its response element (Kumar et al. 1987, Kumar & Chambon 1988) and it also has weak dimerization activity (Kumar & Chambon 1988). Region D has a nuclear localization signal (Picard et al. 1990) and acts as a hinge region between domains C and E (Kumar et al. 1987). The conserved C-terminal region E/F contains the ligand-binding domain (Webster et al. 1988). In addition, the E/F region has a hormone-inducible activation function (Kumar et al. 1987, Webster et al. 1988, Bocquel et al. 1989, Tora 1989), and dimerization activity (Kumar & Chambon 1988, Fawell et al. 1990, White et al. 1991).

Recently, a novel estrogen receptor designated ERβ has been cloned from rat prostate (Kuiper et al. 1996), human testis (Mosselman et al. 1996) and mouse ovary (Tremblay et al. 1997, Pettersson et al. 1997). Although the two ERs are remarkably similar to one another and share many functional properties, they also have individual characteristics that suggest that both isoforms may perform specific biological functions (Kuiper et al. 1997). Human ERβ shows a high degree of conservation of the DNA-binding domain (96%), and of the ligand-binding domain (58%), compared with human ERα (Mosselman et al. 1996). In contrast, the A/B domain, the hinge region and the F domain are not conserved (Mosselman et al. 1996). Rat ERβ is expressed in the prostate, ovary, lung, bladder, brain, uterus and testis as detected by RT-PCR (Kuiper et al. 1997). The finding that ERβ mRNA is down-regulated by gonadotropins in granulosa cells, suggests that estrogen action in the rat ovary may be mediated primarily by ERβ (Byers et al. 1997). The ligand-binding affinity of the two receptors is similar overall for different physiological substrates (Kuiper et al. 1997). ERβ binds E2 with a slightly lower affinity than ERα. The dissociation constant for mouse ERβ is 0.5 nM and for mouse ERα 0.2 nM (Tremblay et al. 1997). ERα and ERβ form DNA-binding homodimers, but they can also form heterodimers in tissues where both receptors are expressed (Kumar &
ERα and ERβ heterodimers bind to DNA with an affinity similar to that of ERα homodimers and greater than that of ERβ homodimers (Cowley et al. 1997). Furthermore, the heterodimer is capable of stimulating transcription of a reporter gene in transfected cells (Cowley et al. 1997), which demonstrates its functionality.

To study further the details of estrogen action, mice lacking the functional ERα or ERβ gene have been generated (Lubahn et al. 1993 and Krege et al. 1998, respectively). ERα gene knock-out (ERKO) mice survive to adulthood with normal external phenotypes, but both females and males are infertile (Lubahn et al. 1993, Eddy et al. 1996). The female mouse has a hypoplastic uterus unresponsive to estrogen treatment, ovaries lacking corpora lutea and undeveloped mammary glands. The exact reason for the infertility in males is not known, although the development of the seminiferous tubules is affected together with alterations in spermatogenesis, sperm function and mating performance (Eddy et al. 1996). In addition, in both ERKO males and females, bone density is 20 to 25% lower than in wild-type mice (Korach 1994). The only reported case of ERα gene mutation in humans further confirms the importance of ERα in normal bone development. An adult man suffers from severe osteoporosis as a result of mutation in the ERα gene (Smith et al. 1994).

Mice lacking ERβ develop normally (Krege et al. 1998). Female mice are fertile and exhibit normal sexual behavior, but have fewer and smaller litters than wild-type mice. This reduction in fertility appears to be due to reduced ovarian efficiency. Furthermore, the mutant females have normal breast development and they lactate normally. Young male mice show no abnormalities and reproduce normally. However, older males display signs of prostate and bladder hyperplasia (Krege et al. 1998).

2.5. Estrogens and breast cancer

Female sex steroids are central to the control of proliferation of normal breast epithelial cells and of endocrine-responsive tumors derived from them. One hundred years ago Beatson (1896) first reported that ovariectomy brings about a response in premenopausal women with breast cancer. It is now known that this procedure removes the major source of estrogen, which supports the growth of hormone-dependent breast tumors.

The normal mammary gland contains a branching ductal system, ending in terminal ductal lobular units (TDLUs). Full development of the ductal system is completed during pregnancy. Breast cancers are thought to develop from the epithelial cells of the TDLUs (Russo et al. 1990). Growth and differentiation of the mammary gland is under hormonal control. In the normal breast E2 is required for mammary duct growth during adolescence as well as lobuloalveolar proliferation during pregnancy. In contrast, P is not required for ductal growth, but it is required for lobular development (Russo et al. 1990). Breast carcinogenesis is a multistage process, where cell sensitivities change during the progression from normal to hyperplastic, hormone-sensitive cancer and finally to the hormone-insensitive state (King 1991). According to the current hypothesis, initial carcinogenesis and progression of mammary epithelium to cancer probably requires both proliferative stimuli, such as estrogen and growth factors, and genetic damage.
Progression of hormone-dependent breast cancer to hormone independence probably involves oncogene activation, loss of the estrogen receptor or loss of hormone responsiveness of other gene products (Lippman & Dickson 1989).

The strongest evidence supporting the importance of estrogens in breast carcinogenesis comes from epidemiological studies. Early menarche, late menopause and lesser number of pregnancies correlates with an increased risk of breast cancer (Pike et al. 1981, Mansfield 1993, Lipworth 1995). This is due to the greater number of menstrual cycles and therefore greater amounts of circulating sex steroids during the lifetime. In addition, obesity, which correlates with the amount of circulating estrogens, is considered to be a risk factor (Cauley et al. 1989).

Peripheral tissues are important sources of estrogen, especially after menopause. In several studies estrogen concentrations in plasma have been compared with those in normal and malignant breast tissues. Concentrations of E1 and E2 in both normal and malignant breast tissues are significantly higher than the levels in plasma (Van Landeghem et al. 1985, Vermeulen et al. 1986). Furthermore, estrogen concentrations, in particular those of E2, are higher in malignant than in normal breast tissue (Bonney et al. 1986, McNeill et al. 1986, Vermeulen et al. 1986). The tumor/plasma E2 ratio is also much higher than that for E1 (Bonney et al. 1986, Van Landeghem et al. 1985). Finally, these studies have shown that while tissue E1 concentrations in postmenopausal women reflect the decrease in estrogen production that occurs at menopause, tumor E2 concentrations are independent of menopausal status (Thijissen et al. 1987).

Modern methods for assay of ER are based on immunological detection, mostly on immunohistochemistry, but previously ligand-binding assays were also used. In both methods estrogen receptors can be detected on average in 70% of breast cancer tissues (Hawkins et al. 1980 and refs. therein, Clarke et al. 1997). ERs and progesterone receptors (PRs) appear to be informative markers of prognosis in breast cancer. More than 50% of patients with ER- and PR-positive tumors respond to hormonal therapy (McGuire et al. 1991). The response to hormone treatment is decreased to less than 10% if the tumor is ER- and PR-negative. Most of the clinically used ER assays are based on antibodies to ERα. ERβ has also been detected in both normal and malignant breast tissue and cell lines (Dotzlaw et al. 1996, Enmark et al. 1997). The possible role of ERβ in normal breast development, and breast cancer development and therapy is still not known.

It has been suggested that changes in nuclear oncogenes, perhaps involving loss of function of tumor suppressor genes, may allow cells to enter the cell cycle. In addition, changes in growth factors, their receptors or intracellular second messenger systems may stimulate unregulated growth (Gullick 1990). Estrogens have been shown to regulate growth factors and their receptors in breast cancer cell lines (Dickson et al. 1986, Zajchowski et al. 1991, Dickson & Lippman 1995 and refs. therein). Estrogens also regulate the expression of protooncogenes such as c-fos, c-myc (van der Burg et al. 1989, Papa et al. 1991) and HER-2/neu (Read et al. 1990, Gullick 1990 and refs. therein). In addition, progesterone receptor expression is induced by estrogens in MCF-7 breast cancer cells (Nardulli et al. 1988).

Estrogen antagonists are used in breast cancer treatment to prevent estrogen action on cell proliferation. Tamoxifen is today the most widely used antiestrogen in the treatment of breast cancer. Tamoxifen is a non-steroidal compound that binds to ER and prevents
the effects of endogenous estrogens. Tamoxifen is also being used experimentally as a preventive agent where there is a high risk of breast cancer. After 5 years of treatment, tamoxifen has been shown to decrease the incidence of breast cancer (Fisher et al. 1998). However, tamoxifen and related compounds have also been shown to have partial agonist activity. Hence pure antiestrogens, such as ICI 164 384, have been developed and are under test. These pure antiestrogens could be particularly useful after the failure of tamoxifen or in the treatment of metastatic breast cancer.

Another way to reduce the estrogen effect on breast cancer is to directly decrease the amount of synthesized estrogens. This could be accomplished by inhibiting enzyme activities involved in estrogen synthesis. The most potent pathways for inhibition are the aromatase, estrogen sulfatase and 17HSD pathways. Several inhibitors of these enzymes have been generated and some are in the clinical trial phase (Labrie et al. 1992, Tremblay & Poirier 1998 and refs. therein).

Mutations in breast cancer genes (BRCAs) are thought to be responsible for the majority of inherited breast cancers. Although these mutations account for approximately only 5 % of all breast cancer cases, it has been calculated that these hereditary factors may be linked to 36 % of breast cancers in women under 30 years of age (Claus et al. 1991). Mutations in BRCA1, a gene identified by Miki et al. (1994) and localized to 17q21 (Hall et al. 1990), are thought to be responsible for half of all inherited breast cancers. Mutations in BRCA1 are also linked to the development of ovarian cancer. The function of BRCA1 is not known, but it is critical for normal embryonic development in mice (Gowen et al. 1996). It has been suggested that BRCA1 is involved in the proliferation and differentiation of multiple tissues especially in the mammary gland in response to ovarian hormones (Marquis et al. 1995). The results of recent studies show that BRCA1 protein may inhibit ERα mediated transcriptional pathways and therefore suppress estrogen-dependent proliferation of mammary epithelial cells (Fan et al. 1999).

On the other hand, estrogens have been shown to increase the expression of BRCA1 at both mRNA and protein levels (Gudas et al. 1995, Spillman & Bowcock 1996). The BRCA2 gene is located at chromosome region 13q12-q13 and mutations in this gene are probably responsible for a substantial number of cases of familial breast cancer not linked to BRCA1 (Tavtigian et al. 1996, Wooster et al. 1995). Like BRCA1, BRCA2 is also upregulated by E2 (Spillman & Bowcock 1996). In addition, breast cancer has been shown to occur more frequently in patients who have certain other rare hereditary diseases such as Li-Fraumeni syndrome, Ataxia telangiectasia and Cowden’s disease (Ellisen & Haber 1998 and refs. therein).

### 2.6. 17β-Hydroxysteroid dehydrogenases

17HSD enzymes have important roles in steroid hormone action since they catalyze the final steps in androgen and estrogen biosynthesis. 17HSDs primarily convert the relatively inactive sex steroids E1, A-dione and 5α-androstenedione to their more potent forms E2, T and 5α-dihydrotestosterone and vice versa. Reductive 17HSD activities are essential for E2 and T biosynthesis in the gonads as well as in certain extragonadal tissues of several species, including primates and rodents (Martel et al. 1992). In
contrast, the widely expressed oxidative 17HSD activity decreases the potency of estrogens and androgens and therefore may protect tissues from excessive hormone action (Moghrabi et al. 1997, Mustonen et al. 1998b).

Six different human 17HSDs (types 1-5 and 8, Peltoketo et al. 1988, Luu The et al. 1989a, Qin et al. 1993, Wu et al. 1993, Geissler et al. 1994, Adami et al. 1995, Ando et al. 1996, Lin et al. 1997) and eight 17HSDs from other species (types 1-8, see refs. in Table I) have been cloned to date. Each 17HSD isoenzyme has a preferred substrate and reaction direction, and often a unique tissue distribution (Labrie 1997, Peltoketo et al. 1999 and refs. therein). All the 17HSDs, except 17HSD type 5, belong to the short chain dehydrogenase/reductase (SDR) family. The type 5 enzyme, however, belongs to the aldo-keto reductase family (AKR).

17HSDs differ in their substrate and cofactor specificity. 17HSD types 1, 3, 5 and 7 mainly catalyze reductive reactions of estrogens and androgens (Poutanen et al. 1995, Geissler et al. 1994, Deyashiki et al. 1995, Nokelainen et al. 1998, respectively), while types 2, 4, 6 and 8 are oxidative (Wu et al. 1993, Adami et al. 1995, Biswas and Russel 1997, Formitcheva et al. 1998, respectively). Furthermore, some of the enzymes named 17HSD also catalyze several other reactions, such as β-oxidation of fatty acids in the case of 17HSD type 4 (Qin et al. 1997).

The expression patterns of 17HSDs are different, although overlapping in some cases. The expression of 17HSD type 1 correlates with E2 concentrations in human ovarian granulosa cells (Ghersevitch et al. 1994) and placental trophoplasts (Mäentausta et al. 1991a), and 17HSD type 1 is also expressed in certain target tissues of estrogen action. 17HSD type 3 is almost exclusively expressed in the testes (Geissler et al. 1994, Sha et al. 1997), the type 5 enzyme is most abundantly expressed in the liver and kidney (Deyashiki et al. 1995), and type 6 in liver and prostate (Biswas & Russel 1997). 17HSD type 7 is most abundantly expressed in the ovaries of pregnant animals and in the placenta, and small signals have also been found in the ovaries of adult nonpregnant mice, and in some peripheral tissues (Nokelainen et al. 1998). 17HSD type 8 is expressed in the spleen, liver and gonads (Formitcheva et al. 1998). On the other hand, the type 2 and type 4 enzymes are widely expressed in both steroidogenic and peripheral tissues (Casey et al. 1994, Moghrabi et al. 1997, Mustonen et al. 1997a, 1998ab, Adami et al. 1995). For more details see Table 1.
<table>
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<th>Type/protein family</th>
<th>Species cloned</th>
<th>References for cloning</th>
<th>Subcellular localization</th>
<th>Abundant expression</th>
<th>Substrate specificity</th>
<th>Main Activity</th>
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<td></td>
<td>Rat</td>
<td>Ghersevich et al. 1994c, Akinola et al. 1996</td>
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<td>Placenta, liver, gastrointestinal tract</td>
<td>Estrogens, androgens (progestins)</td>
<td>oxidative activity</td>
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<td>Fatty acyl-CoA, estrogens</td>
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<td>Prostate, liver</td>
<td>Androgens</td>
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<tr>
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<td>Ovary</td>
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<td></td>
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<tr>
<td>8/SDR</td>
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<td>Liver, kidney</td>
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<td>Aziz et al. 1993</td>
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2.6.1. 17HSD type 1

2.6.1.1. Structure and function of 17HSD type 1

17HSD type 1 is needed for E2 biosynthesis in human ovarian granulosa cells (Ghersevich et al. 1994a) and in placental syncytiotrophoblasts (Dupont et al. 1991, Mäentausta et al. 1991a). Human 17HSD type 1 mainly catalyzes reduction of E1 to E2, being able to use both nicotinamide-adenine dinucleotide (NADH) and its phosphate (NADPH) as cofactors. However, structural data suggest that NADP⁺ is the preferred cofactor over NAD⁺ (Mazza et al. 1998). The human type 1 enzyme also reduces androgens in cultured cells to some extent, but it clearly prefers phenolic substrates over neutral ones (Nokelainen et al. 1996, Puranen et al. 1997a). In contrast, rodent type 1 enzymes effectively catalyze both androgenic and estrogenic substrates (Akinola et al. 1996, Nokelainen et al. 1996, Puranen et al. 1997a). 17HSD type 1 enzymes can also catalyze oxidative reactions, but only in vitro in the presence of an excess of the cofactors NAD⁺ or NADP⁺ (Karavolas & Engel 1971, Puranen et al. 1994).

The open reading frame of human 17HSD type 1 cDNA (Peltoketo et al. 1988, Gast et al. 1989, Luu-The et al. 1989a) encodes a polypeptide of 327 amino acids with a calculated molecular mass of 34 853 Da (Peltoketo et al. 1988). The 17HSD type 1 protein exists as a homodimer (Burns et al. 1972, Nicholas and Harris et al. 1973, Lin et al. 1992) consisting of non-covalently bound, but strongly associated subunits (Puranen et al. 1997a). The firm association ensures stable dimerization of the enzyme, which is evidently necessary for its proper function. Asymmetry in substrate binding has been suggested: ligand binding at one subunit might result in reduced ligand binding affinity in the other subunit (Sawicki et al. 1999). The X-ray structure of the enzyme dimer suggests the presence of two dimerization helices in each monomer, forming a four-helix bundle (Ghosh et al. 1995). If formation of the helices is compromised by substitutions at the dimeric interface, the enzyme does not fold properly and an aggregated and inactive protein of more than 300 kDa in size is formed (Puranen et al. 1997a).

17HSD type 1, as are most of the other 17HSD enzymes, belongs to the SDR protein family. Despite their low overall similarity, the members of this family contain conserved regions which have been suggested to be involved in the binding of cofactors or the conformation of the proteins (Krozowski 1992). The conserved region between amino acids 148 and 268 is mainly responsible for the differences in substrate specificity of human and rat enzymes (Puranen et al. 1997b). E2 binds close to the catalytically important residues Ser¹⁴², Tyr¹⁵⁵, Lys¹⁵⁹, His²²¹ and Glu²⁸² (Azzi et al. 1996). A highly conserved peptide, Tyr-X-X-X-Lys (X represents any amino acid), corresponding to Tyr¹⁵⁵ to Lys¹⁵⁹ in human 17HSD type 1, is located in the vicinity of the modeled catalytic site comprising a cofactor-binding cleft and a steroid-binding cleft (Ghosh et al. 1995). Mutation of Tyr¹⁵⁵ of 17HSD type 1 results in almost complete inactivation of the enzyme (Puranen et al. 1994). Substitution of Lys¹⁵⁹ also abolishes the activity of 17HSD type 1, which further confirms the importance of the residue for the function of the enzyme (Puranen et al. 1997a). According to the current model for the catalytic mechanism of 17HSD type 1, the tyrosine residue is a proton donor, while Lys¹⁵⁹ appears to stabilize cofactor binding of the enzyme (Ghosh et al. 1995, Azzi et al. 1996, Breton et
Mutation of a third well conserved residue, Ser^{142}, also results in an inactive enzyme (Puranen et al. 1994). This is in agreement with a suggestion that the serine residue lowers the pKa of Tyr^{155} by forming a hydrogen bond with its side chain (Ghosh et al. 1995, Labrie et al. 1997).

2.6.1.2. The gene encoding human 17HSD type 1

The gene encoding human 17HSD type 1, hHSD17B1, is localized in the chromosome 17 region q12-21 (Luu-The et al. 1989a, Winqvist et al. 1990). Another gene, hHSD17BP1, is located in the same region (Luu-The et al. 1990, Peltoketo et al. 1992). These two genes share 89% overall identity, but hHSD17BP1 has a premature stop codon (Luu-The et al. 1990, Peltoketo et al. 1992). The function of hHSD17BP1, if any, is unknown. Human HSD17B1 has two transcription starting points leading to two messenger RNA (mRNA) transcripts, 1.3 kb and 2.3 kb in size, which differ only in the length of their 5'-noncoding regions (Luu-The et al. 1989a, 1990). The longer mRNA is constitutively expressed in most tissues and cell lines studied (Tremblay et al. 1989, Luu-The et al. 1990), whereas the 1.3 kb mRNA is expressed in cells producing 17HSD type 1 protein (Tremblay et al. 1989, Poutanen et al. 1992a). It is also the 1.3 kb mRNA whose transcription is subject to regulation (Tremblay & Beaudoin 1993, Poutanen et al. 1992a, Ritvos & Voutilainen 1992, Tremblay et al. 1989, Jantus Lewintre et al. 1994ab, Piao et al. 1995, 1997a).

The region from -78 to +9 nucleotides upstream from the transcription starting point of the 1.3 kb mRNA for 17HSD type 1 contains certain characteristic elements of a gene promoter. The promoter fragment contains a sequence typical of a TATA box, a GC-rich area and an inverted CAAT element, indicating that this region might be responsible for transcription of the 1.3 kb mRNA (Luu-The et al. 1990, Peltoketo et al. 1992). The GC-rich area of the promoter contains binding sites for Sp1 and AP-2 transcription factors (Piao et al. 1997b). Mutation of the Sp1 motif decreases the promoter activity to 30% in JEG-3 cells and to 60% in JAR choriocarcinoma cells, suggesting that binding to the Sp1 motif has a substantial role in the function of the hHSD17B1 promoter (Piao et al. 1997b). Mutation of the AP-2 element increases promoter activity 260% in JEG-3 cells. Moreover, binding of AP-2 to its motif leads to reduced binding of Sp1 and Sp3 to the Sp1 motif. Thus the data imply that AP-2 can repress the function of the hHSD17B1 promoter by preventing binding to the Sp1 motif (Piao et al. 1997b).

An enhancer of the hHSD17B1 gene is localized between -661 and -393 nucleotides from the start codon of 1.3 kb 17HSD type 1 mRNA (Piao et al. 1995). The enhancer increases transcription efficacy in both orientations, regardless of the promoter type or the distance between the promoter and the enhancer. The action of the enhancer is also cell type-dependent, being most effective in choriocarcinoma cell lines. A retinoid acid responsive element has been found in hHSD17B1 enhancer, between -503 and -487 (Piao et al. 1995). Retinoid acids (RAs) have been shown to increase 17HSD type 1 expression in the T47D breast cancer cell line and in the JEG-3 choriocarcinoma cell line (Piao et al. 1995). The enhancer regions of hHSD17B1 and hHSD17BP1 genes have 98% identity, but the enhancer for hHSD17BP1 has only 10% of the activity of the hHSD17B1
enhancer. This is due to just two different nucleotides in hHSD17B1 and hHSD17BP1 (Leivonen et al. 1999). One silencer element has also been localized between the promoter and the enhancer (Piao et al. 1995). GATA factors have been demonstrated to bind their cognate sequence in the silencer region, and mutations in the GATA-binding site increase promoter activity (Piao et al. 1997b). These results suggest that regulation of 17HSD type 1 expression is balanced between the function of the enhancer and silencer.

2.6.1.3. Expression and regulation of 17HSD type 1 in different tissues

17HSD type 1 is expressed in both classical steroidogenic tissue, namely the ovaries, and in the placenta during pregnancy (Luu-The et al. 1990), as well as in some steroid target tissues including benign and malignant breast tissue (Poutanen et al. 1992b) and endometrium (Mäentausta et al. 1991a). In addition, in immunochemical studies minor signals have been detected in the glial cells of the brain (Pelletier et al. 1995, Mensah-Nyagan et al. 1996).

The 17HSD type 1 enzyme is expressed in the granulosa cells in human ovaries (Ghersevich et al. 1994a, Sawetawan et al. 1994) and in granulosa-lutein cells in the corpus luteum (Sawetawan et al. 1994). In cultured rat granulosa cells the expression of 17HSD type 1, and 17HSD activity, is detected in parallel, indicating that 17HSD type 1 is responsible for E2 biosynthesis in the ovary (Ghersevich et al. 1994b). The expression of 17HSD type 1 in granulosa cells is related to the differentiation stage of the follicles (Ghersevich et al. 1994a). Expression is low in rat antral follicles, up-regulated during follicular maturation, and highest in Graafian follicles. Thereafter, the expression of 17HSD type 1 in the rat ovary decreases during luteinization (Ghersevich et al. 1994a).

The expression of 17HSD type 1 in rat granulosa cells of developing follicles is under multi-hormonal regulation (Ghersevich et al. 1994acd). Expression of the enzyme is regulated by FSH, which acts via cyclic adenosine-3’,5’-monophosphate (cAMP), and the effects of FSH are modulated by at least estrogen, androgens and epidermal growth factor (EGF) (Ghersevich et al. 1994d). In cultured human granulosa-lutein cells, enzyme expression is decreased by Br-cAMP (Tremblay et al. 1989), while it has been shown to be increased by cAMP analogues in cultured rat granulosa cells (Ghersevich et al. 1994d), normal human trophoblasts and choriocarcinoma cells (Tremblay et al. 1989, 1993, Ritvos & Voutilainen 1992). Hence, it is likely that the effect of cAMP is dependent on the cell type and, in the case of granulosa cells, also on the differentiation stage of the cells.

In humans, the placenta is the major source of estradiol during pregnancy, and as in the ovaries, 17HSD type 1 catalyzes the final step of estradiol biosynthesis. 17HSD type 1 is highly expressed in the human placenta in syncytiotrophoblasts (Fournet-Dulguerov et al. 1987, Dupont et al. 1991, Mäentausta et al. 1991a). In addition, in three choriocarcinoma cell lines, JAR, JEG-3 and BeWo, 17HSD type 1 expression has been detected, and it correlates with their 17HSD activity (Jantus-Lewintre et al. 1994a). Several growth factors, including fibroblast growth factor, EGF and transforming growth factor α, stimulate the expression of 17HSD type 1 in choriocarcinoma cells, and these
growth factors may hence have a stimulatory role in estradiol production in the placenta (Jantus-Lewintre et al. 1994a,b). In addition, RAs increase 17HSD type 1 expression and reductive activity in JEG-3 cells (Piao et al. 1995).

Highly variable amounts of 17HSD type 1 have been detected in about 70% and 50% of benign and malignant breast specimens, respectively (Poutanen et al. 1992b, 1995, Sasano et al. 1996), and the expression of 17HSD type 1 has also been detected in a number of breast cancer cell lines (Poutanen et al. 1992a). 17HSD type 1 expression is induced by progestins in the T-47D human breast cancer cell line (Poutanen et al. 1990). On the other hand, considerable 17HSD type 1 protein expression has also been detected in breast cancer specimens lacking progestin receptors (Poutanen 1992b), indicating that other factors are also involved in regulation of the enzyme in breast tissue. Several factors have been found to regulate reductive 17HSD activity in breast cancer cells: interleukin 6 (IL-6, Adams et al. 1991), IL-1β (Duncan et al. 1994), tumor necrosis factor α (Duncan et al. 1994) and insulin-like growth factors type I and type II (Singh & Reed 1991). In addition, RAs increase 17HSD type 1 expression and reductive 17HSD activity in the T47D human breast cancer cell line (Reed et al. 1994, Piao et al. 1997).

Using immunohistochemical staining, 17HSD type 1 has been localized in glandular and epithelial cells of normal and malignant endometrium (Mäentausta et al. 1992). In the normal menstrual cycle type 1 expression is regulated by a progestin-dependent mechanism. During the proliferative phase, no immunohistochemical staining for 17HSD type 1 has been seen (Mäentausta et al. 1991a). In the early secretory phase of the menstrual cycle 17HSD type 1 is expressed in gland and surface epithelial cells. The staining is maximal by the mid-secretory phase, coinciding with an increased serum progesterone concentration, and then it disappears towards the late secretory phase (Mäentausta et al. 1991a). Regulation by progestins has been further confirmed by the finding that an antiprogestin, mifepristone, blocked the progestin-induced expression of 17HSD type 1 in the endometrium (Mäentausta et al. 1993). On the other hand, about 40% of endometrial adenocarcinoma specimens that are positive for the enzyme, have been detected to be negative as regards ER and PR (Mäentausta et al. 1992). In addition, the correlation between serum P concentrations and 17HSD type 1 staining is negative (Mäentausta et al. 1992). These facts indicate that 17HSD type 1 protein expression in the endometrium is also regulated by endocrine or paracrine factors other than progestins, at least in malignant endometrium.

2.6.2. 17HSD type 2

The second 17HSD isoenzyme was cloned in 1993 by Ling Wu and co-workers. Before that several investigators had presented indirect evidence of the existence of more than one 17HSD isoenzyme (Blomquist et al. 1985, Tait et al. 1989,). The 17HSD type 2 isoenzyme was cloned from a human prostate cDNA library using an expression cloning method (Wu et al. 1993). A 1.4 kb cDNA clone was identified, and DNA sequence analysis indicated that 17HSD type 2 is a protein of 387 amino acids with a calculated molecular weight of 42 782 Da. 17HSD type 2 is a membrane-associated protein that contains an amino-terminal type II signal-anchor motif and a carboxyl-terminal
endoplasmic reticulum retention motif (Wu et al. 1993, Akinola et al. 1996). The type 2 enzyme is an oxidative enzyme that catalyzes the interconversion of T and A-dione, as well as E2 and E1. The enzyme also possesses 20α-HSD activity toward 20α-dihydroprogesterone (Wu et al. 1993). The human 17HSD type 2 gene, hHSD17B2, contains 7 exons and is localized in chromosome 16, at q24 (Casey et al. 1994, Durocher et al. 1995, Labrie et al. 1995).

In human placenta, 17HSD type 2 is alternatively spliced to two species of mRNA (Labrie et al. 1995). 17HSD type 2A mRNA is 1.5 kb in size and encodes an active protein, whereas 17HSD type 2B mRNA encodes an inactive protein of 291 amino acid residues (Labrie et al. 1995). The 1.5 kb mRNA for 17HSD type 2 is highly expressed in human placenta, liver and small intestine, and is expressed in much smaller amounts in the prostate, kidney, pancreas and colon (Casey et al. 1994). According to Northern blot analysis 17HSD type 2 mRNA is also expressed in endometrial tissues obtained during the mid- to late secretory phase of the menstrual cycle, correlating with the plasma P concentration (Casey et al. 1994). This has also been shown using in situ hybridization (Mustonen et al. 1998a). In human placenta the 17HSD type 2 protein has been detected in endothelial cells of fetal capillaries (Moghrabi et al. 1997). Furthermore, dichotomous immunostaining has been observed among pairs of placental cotyledonary and chorionic vessels. In human fetal liver, staining has been detected in the hepatocytes, but not in the cells lining blood vessels (Moghrabi et al. 1997).

Mouse and rat cDNAs for 17HSD type 2 share 82% sequence identity (Mustonen et al. 1997a). The rat 17HSD type 2 enzyme, 42 kDa in size, shows a hydropathy profile, tissue distribution and catalytic properties similar to those of the human enzyme, though the two proteins share only 62% sequence homology (Akinola et al. 1996). In the mouse, 17HSD type 2 is expressed highly in the liver, placenta, kidney and brain. Lower levels of mouse 17HSD type 2 have been detected in the adrenals, spleen, uterus, mammary gland and prostate (Mustonen et al. 1997a).

Using in situ hybridization techniques, Mustonen and co-workers (1998b) have studied 17HSD type 2 expression in more detail in mouse tissues. In both male and female mice strong expression of 17HSD type 2 was detected in epithelial cells of the gastrointestinal and urinary tracts. The 17HSD type 2 enzyme was localized in surface epithelial cells of the stomach, small intestine and colon. Strong expression was also detected in hepatocytes of the liver and the thick limbs of the loops of Henle in the kidneys, as well as in the epithelium of the urinary bladder (Mustonen et al. 1998b). These results further suggest a role for 17HSD type 2 in steroid inactivation in a range of tissues and cell types not considered as classical sex steroid target tissues. Furthermore, the expression of 17HSD type 2 mRNA has been analyzed during mouse embryogenesis, and the results showed that the expression is strongly increased in parallel with the fetal development of gastrointestinal organs (Mustonen et al. 1998b). This expression in the placenta and in fetal liver is in line with the proposed physiological role of 17HSD type 2, namely to protect tissues from active estrogen and androgen (Moghrabi et al. 1997, Mustonen et al. 1998b).
2.6.3. 17HSD type 4

17HSD type 4 has been cloned from several species (see Table I for references). The cDNA for human 17HSD type 4 encodes a protein of 736 amino acids long, with a predicted molecular mass of 79,595 Da (Adamski et al. 1995). The human gene encoding the type 4 enzyme has been located to chromosome region 5q2 (Leenders et al. 1996a). The type 4 enzyme is peroxisomal (Markus et al. 1995) and is expressed nearly ubiquitously in different adult tissues and cell lines (Adamski et al. 1995, Mustonen et al. 1997a). The type 4 enzyme is also expressed throughout mouse fetal life (Mustonen et al. 1997a).

The 17HSD type 4 enzyme is a multifunctional protein, known also as multifunctional enzyme II, MFE-II. The 80 kDa protein is post-translationally cleaved to three parts with distinct substrate and reaction specificities (Dieuaide-Noubhani et al. 1996, Leenders et al. 1996b, Qin et al. 1997). The N-terminally cleaved 32 kDa fragment catalyzes dehydrogenase reactions, not only with steroids at the C17 position, but also with 3-hydroxyacyl-CoA. The central part of the protein catalyzes the 2-enoyl-acyl-CoA hydratase reaction. The C-terminal part of the protein is similar to sterol carrier protein 2 and facilitates the transfer of 7-dehydrocholesterol and phosphatidylcholine between membranes in vitro (Leenders et al. 1996b).

The human, porcine, mouse and rat 17HSD type 4 proteins (Leenders et al. 1994ab, Adamski et al. 1995, Normand et al. 1995, Corton et al. 1996, Dieuaide-Noubhani et al. 1996, et al. Qin 1997) show a close relationship, revealing 85% amino acid similarity, the same multi-domain structure and identical kinetic parameters, suggesting an essential physiological function for the enzyme. Both rat and porcine enzymes show very low activity as regards the oxidation of E2 compared with dehydrogenation of 3-hydroxyacyl-CoA (Leenders et al. 1996b, Qin et al. 1997). Thus, the role of 17HSD type 4 in estrogen metabolism needs to be clarified. Its ubiquitous expression in different tissues (Adamski et al. 1995, Dieuaide-Noubhani et al. 1996) might suggest a housekeeping role in inactivating steroids. 17HSD type 4 deficiency has been linked to a disease called the Zellweger syndrome (van Grunsven et al. 1998). Patients with this disease lack morphologically distinguishable peroxisomes, leading to the loss of virtually all peroxisomal functions.

2.6.4. Other 17HSD isoenzymes

17HSD type 3 has been isolated from a human testicular cDNA library (Geissler et al. 1994). The cDNA encodes a microsomal protein of 310 amino acids with a molecular weight of 35 kDa. The human gene for 17HSD type 3 contains 11 exons and spans more than 60 kb of genomic DNA at chromosome region 9q22. 17HSD type 3 predominantly catalyzes the reduction of A to T, and at lower conversion rates, E1 to E2, and DHEA to androstenediol (Geissler et al. 1994). 17HSD type 3 is highly expressed in the testes (Geissler et al. 1994). The physiological importance of the 17HSD type 3 isoenzyme is reflected in its necessity as regards the normal development of male genitalia. Deficiency of the type 3 enzyme results in male pseudohermaphroditism (Geissler et al. 1994). The
disease is characterized by a 46XY karyotype with normal Wolffian duct structures but female external genitalia (Andersson et al. 1996 and refs. therein). 17HSD type 3 is also expressed in adipose tissue in women, where it may play an important role in peripheral production of T and E2 (Corbould et al. 1998).

The cDNA for human 17HSD type 5 encodes a protein of 323 amino acids (Lin et al. 1997), which belongs to the aldoketoreductase family (Deyashiki 1995). 17HSD type 5 was previously characterized as type 2 3α-HSD (Khamma et al. 1995, Lin et al. 1997). The 17HSD activity of the human type 5 enzyme is highly labile and it was not detected in early studies involving cell homogenates or purified enzyme (Dufort et al. 1999). In intact cells, both human and mouse 17HSD type 5 efficiently catalyze the conversion of A to T, and to a lesser extent the transformation of dihydrotestosterone (DHT) to 3α-adiol (Dufort et al. 1999). Human 17HSD type 5 also effectively converts P to 20α-dihydroprogesterone (Dufort et al. 1999). Human 17HSD type 5 is expressed in the liver, testis, adrenal, prostate, ovary, mammary gland, endometrium, DU-145 and LNCaP prostatic cancer cell lines and MG-63 bone carcinoma cells (Dufort et al. 1999, Pelletier et al. 1999). 17HSD type 5 may be involved in the formation of active androgens in the testis and other tissues.

17HSD type 6 has been cloned from rat ventral prostate (Biswas & Russel 1997). It catalyzes the oxidation of androgen and estrogen substrates, particularly that of 3α-adiol to androsterone, 3α-adiol being catabolic product of DHT. The mRNA of 17HSD type 6 is expressed in rat liver and prostate. These properties indicate that the possible role of the 17HSD type 6 enzyme is to inactivate androgens and estrogens in these tissues.

17HSD type 7 has recently been cloned from the HC11 cell line, derived from mouse mammary gland tissues (Nokelainen et al. 1998). The rat analogue was previously characterized as prolactin receptor-associated protein (Duan et al. 1996). The cloned mouse cDNA encodes a protein of 334 amino acids with a calculated mass of 37 317 Da (Nokelainen et al. 1998). 17HSD type 7 efficiently catalyzes conversion of E1 to E2. 17HSD type 7 is most abundantly expressed in corpora lutea of the ovaries of pregnant animals. It is expressed in the middle and second half of pregnancy, correlating with E2 secretion from the corpus luteum. The mRNA of 17HSD type 7 is also apparent in the placenta, and a slight signal is found in the ovaries of adult nonpregnant mice, in the mammary gland, liver, kidney and testis (Nokelainen et al. 1998).

The gene for 17HSD type 8 was earlier characterized as the Ke 6 gene, which has been linked to the development of recessive polycystic kidney disease in mice (Aziz et al. 1993). In in vitro conditions 17HSD type 8 is preferentially an oxidative enzyme efficiently catalyzing conversion of E2 to E1, and to the same extent the oxidation of T and DHT (Fomiticheva et al. 1998). 17HSD type 8 also has some reductive activity, converting E1 to E2. The enzyme is expressed in the ovary, testis, kidney and spleen. In the ovary 17HSD type 8 is expressed in cumulus cells surrounding the oocyte, which suggests that it may have a role in controlling the level of steroids to which the egg is exposed.
3. Outlines of the present study

17HSDs catalyze the interconversion of low activity 17-ketosteroids and highly active 17β-hydroxysteroids and thereby regulate the biological activity of sex steroids. This study was aimed at characterization of 17HSD type 1, type 2 and type 4 in different cell lines and tissues. In addition, the effect of 17HSD type 1 on the estrogen-dependent growth of breast cancer cells was evaluated.

The specific topics of the study were:

1. to compare the endogenous enzyme found in COS-m6 cells with placental 17HSD,
2. to characterize in more detail the activity and expression of 17HSD type 1, type 2 and type 4 enzymes in different cell lines and tissues,
3. to evaluate the role of 17HSD type 1 in the estrogen-dependent growth of MCF-7 human breast cancer cells,
4. to analyze 17HSDs in normal breast cells and breast tissue.
4. Materials and methods

Detailed descriptions of the materials and methods are presented in the original articles, I-IV.

4.1. Cell culture (I-IV)

All the carcinoma cell lines used (breast cancer cell lines: BT-20, MCF-7, MDA-MB-231, MDA-MB-361 and T-47D, choriocarcinoma cell lines: BeWo, JAR and JEG-3, endometrial cancer cell lines: HEC-1-A, HEC-1-B and RL95-2, ovarian cancer cell line: OVCAR-3, prostate cancer cell lines: DU145, LNCaP and PC-3, liver carcinoma cell lines: A-431, Hep G2 and SK-HEP-1, embryonal kidney cell line: 293 and monkey kidney cell lines: COS-7 and CV-1) were obtained from the American Type Culture Collection and were cultured according to the instructions of the supplier. COS-m6 cells were kindly provided by Dr. K. von Figura (Georg-August-University, Gottingen, Germany). Stock cultures were maintained in almost continuous exponential growth by weekly passage of the appropriate number of cells following treatment with 0.05% trypsin and 0.02% EDTA in PBS without Mg2+ and Ca2+.

Normal human mammary epithelial cells (HME cells, donor age 21 years, strain 4144 and donor age 51 years, strain 4224), as well as the media, buffers and supplements for the cells were obtained from Clonetics. The cells were maintained in serum-free medium, supplemented with growth factors and antimicrobial agents, according to the instructions of the supplier.

4.2. Tissue specimens (II, IV)

Tissue specimens for isolation of RNA were obtained surgically from patients with breast lesions or patients undergoing hysterectomy. All tissue specimens were stored in liquid nitrogen until processed. Normal breast tissue samples for in situ hybridization were collected during breast reduction operations.
4.3. Expression of 17HSD type 1 and type 2 in different cell lines

4.3.1. Construction of the expression vectors (I-IV)

An EcoRI-EcoRI fragment (1.3 kb) of 17HSD type 1 cDNA, containing the entire coding region, was first inserted into a pSG5 expression vector (Stratagene). pSG5 and pSG5-17HSD vectors were amplified in E. coli AG1 cells. These plasmids, as well as plasmids described later, were purified using Qiagen columns, according to the instructions of the manufacturer (I).

Full-length 17HSD type 1 cDNA (1.3 kb) was then inserted into pRC/CMV vectors, which also contain resistance to the selection drug geneticin (G-418 sulfate). Recombinant plasmids and intact plasmids were amplified in E. coli JM109 cells. Full-length cDNA encoding 17HSD type 2, cloned into the pCMV6 eukaryotic expression vector, was provided by Dr. S. Andersson. Recombinant plasmids and intact plasmids were amplified in DH5α cells (II, III, IV).

4.3.2. Transient transfection of 17HSDs (I, II, IV)

Tranfections were carried out using a lipofection-based DOTAP method, according the manufacturer’s instructions (Boehringer), with minor modifications in cell number and in the amount of DNA as described in detail in the original articles. Briefly, HEC-1-B, LNCaP and MCF-7 cells (II) were plated in 10 cm dishes (1.5 x 10⁶ cells) and were allowed to attach overnight in the culture medium. For each dish 10 μg of plasmid DNA was incubated for 10 min with 150 μg/ml DOTAP in 350 μl of 20mM HEPES/150 mM NaCl pH 7.4. After incubation this reaction mixture was added to 10 ml of the culture medium, containing 5% fetal calf serum, and this medium was applied to the cells. Transfection was carried out for 20 h, the transfection mixture was replaced with fresh medium and the cells were further cultured for 48 h before activity measurements or collection of the cells.

4.3.3. Stable transfection of 17HSD type 1 (III)

MCF-7 cells were plated for stable transfection in 96-well plates at 1 000 cells/well and allowed to attach overnight in the culture medium. Transfection was carried out, using 10 μg pRC/CMV or pRC/CMV-17HSD type 1 plasmids per plate, as described above. After 20 h transfection, the transfection mixture was replaced with fresh medium containing the selection drug G-418 sulfate (1.6 mg/ml). The cells were grown for 2-3 weeks in the selection medium and thereafter resistant cell clones were further expanded into separate cell lines and grown without G-418 sulfate.
4.4. Measurement of 17HSD type 1 concentration (II, III)

Breast lesion specimens or breast cancer cells were homogenized in buffer A (10 mM phosphate buffer, pH 7.5, containing 1 mM EDTA, 0.5 mM phenyl-methylsulfonyl fluoride, 0.02% NaN₃ and 20% glycerol). Thereafter, the concentrations of 17HSD type 1 in cytosol fractions of breast cancer specimens or in supernatants (800 × g) of cells were measured by time-resolved immunofluorometric assay (Mäentausta et al. 1991b).

4.5. 17HSD activity measurements (I-IV)

Both reductive (E₁ → E₂) and oxidative (E₂ → E₁) 17HSD activities were measured in cultured cells. Modifications in cell number, substrate concentrations and in incubation times are described in detail in the original papers (I-IV). Briefly, cells were applied to plates and were allowed to attach overnight prior to activity measurements. The culture media were aspirated from the wells and medium containing E₁ or E₂ was added. The metabolism of the steroids was followed by adding the corresponding [³H]-labeled substrate (200 000 cpm/ml). The cells were then incubated for indicated times at 37 °C in cell culture conditions. After incubation the media were removed, frozen in dry ice and kept at -20 °C until the steroids were extracted into ether. The specimens were then evaporated to dryness, dissolved in acetonitrile:water and further separated using Lipidex column chromatography (I), or on a Sephasil C₁₈ reverse phase chromatography column connected to a SMART™ System (II, III, Pharmacia LKB Biotechnology), or alternatively on a reverse phase chromatography column connected to a high performance liquid chromatography (HPLC) system (IV, Waters™). The radioactivities were measured in a β-counter and the 17HSD activities were calculated as percentages of the substrates used.

4.6. RNA isolation and Northern blot analysis (I, II, IV)

RNA was isolated from different cell lines and tissues using various methods described in detail in the original articles. In articles I and II total RNA in cultured cells was prepared by washing the plates with PBS and lysing the cells in 4 M guanidine thiocyanate with 25 mM sodium acetate. The homogenates were centrifuged (21 h, 179 000 × g) through a cushion of 5.7 M CsCl, followed by ethanol precipitation of RNA. Total RNA from breast cancer specimens and normal endometrium was prepared similarly, except that the tissues were homogenized in 4 M guanidine thiocyanate with 25 mM sodium acetate, using an Ultra-Turrax homogenizer. In the study described in article IV, total RNA from cell lines was isolated by using TRIZOL Reagent (Life Technologies Inc.) according to the manufacturer’s instructions. Poly(A)*RNA was isolated by oligo-dT cellulose chromatography. From normal human mammary epithelial cells (IV), poly(A)*RNA was isolated by using an Oligotex Direct mRNA kit (Qiagen).
For each sample, 10 µg of poly(A)^+RNA was resolved by 1% agarose gel electrophoresis, transferred to nylon membrane by capillarity and cross-linked by UV radiation. Tissue distribution of the 17HSD type 1 and type 2 enzymes was analyzed using commercially available Northern blots containing 2 µg of poly(A)^+RNA from various human tissues (Clontech). The membranes were prehybridized for 2 h at 42 °C in 5 x SSPE (1 x SSPE = 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, 0.1 mM EDTA) containing 50% formamide, 0.1% BSA, 0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 0.5% SDS and salmon sperm DNA (20 mg/ml). The membranes were then hybridized overnight at 42 °C using appropriate [^32P]-labeled probes, as described in the original reports. After hybridization the membranes were washed twice in 2 x SSPE, containing 0.1% SDS, for 15 min each and once in 1 x SSPE, containing 0.1% SDS, for 30 min. The membranes were then exposed to Kodak XAR films. To quantify the amount of mRNA applied to the gel, the membranes were also hybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or γ-actin cDNA probes.

4.7. Immunoblot analysis of 17HSD type 1 (I, III)

For immunoblot analysis, the cytosolic proteins from cell homogenates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or native gel electrophoresis (native PAGE). Thereafter, the proteins were electrophoretically transferred to nitrocellulose membranes (Towbin et al. 1979). The membranes were immunostained as previously described (Poutanen 1992ab), using rabbit polyclonal antibodies raised against human 17HSD type 1 (Mäentausta et al. 1990).

4.8. Measurement of estrogen receptor concentrations (III)

Cultured cells were harvested from the plates by scraping. The cells were disrupted by sonicating twice for 15 s and the homogenates were then centrifuged at 800 x g for 10 min. Estrogen receptor concentrations in the supernatants were measured in triplicate using an Abbot ER-RIA Monoclonal kit, according to the manufacturer’s instructions. Total protein concentrations were measured by using Bio-Rad protein assays.

4.9. Measurement of estrogen-dependent growth (III)

Control cells or 17HSD type 1-expressing cells were plated in 96-well plates, 1000 cells/well, in phenol red-free DMEM containing 5% dextran-coated charcoal-treated FCS and antibiotics. The cells were allowed to attach overnight, after which they were incubated for 7 days with a range of concentrations of E1 or E2. Cell proliferation was measured using the method described by Mosmann (1983), which is based on the use of a tetrazolium salt, MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.
Briefly, 20 µl of MTT solution (5 mg/ml in PBS) were added to each well, containing 200 µl of cell culture medium. The cells were further incubated for 4 h at 37 °C, after which the medium was removed and the cells were disrupted by adding 100 µl of DMSO. The concentration of the solubilized dye was then measured at a wavelength of 570 nm.

4.10. In Situ hybridization (IV)

Probes for in situ hybridization were prepared from a 376 bp fragment (nucleotides 1-376) of human 17HSD type 1 cDNA (Peltoketo et al. 1988), and a 380 bp fragment (nucleotides 191-570) of human 17HSD type 2 cDNA (Wu et al. 1993), cloned in pGEM-4Z plasmids (Promega). Sense and antisense RNA probes were transcribed from linearized plasmids by using T7 or SP6 RNA polymerases (Promega). In vitro transcription reactions with the polymerases were performed according to the manufacturer’s specifications, using [α-35S]dCTP (1300 Ci/mmol, DupontNEN).

Twenty tissue specimens of normal breast tissue obtained at different stages of the menstrual cycle and three specimens from postmenopausal women were collected during breast reduction operations. The specimens were examined microscopically to ensure that they did not contain any carcinoma tissue. All specimens were briefly washed twice with PBS, fixed overnight at 4 °C in 4% paraformaldehyde-PBS, dehydrated and embedded in paraffin. Thereafter, 7 µm sections were collected on glass slides and stored at 4 °C.

In situ hybridization was performed as previously described by Mustonen et al. (1997b). Hybridization was detected after 15-day exposure to autoradiographic NTB2-emulsion (Eastman Kodak) at 4 °C. Finally, the slides were stained with Hoechst 33258 intercalating DNA dye (Sigma Chemical Co.) and mounted in glycergel (DAKO A/S).
5. Results

5.1. Tissue distribution of human 17HSD types 1, 2 and 4 and expression of the 17HSDs in certain cell lines (II, IV)

The tissue distribution of human 17HSD type 1 and type 2 was characterized. Abundant 17HSD type 1 1.3 kb mRNA expression was detected in the placenta and ovary, and low expression was detected in the endometrium. No expression was detected in heart, brain, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, small intestine, colon or leukocytes. The 2.3 kb mRNA for 17HSD type 1 was detected in almost all tissues analyzed. 17HSD type 2 was seen to be strongly expressed in the endometrium, liver, placenta and small intestine. In addition, low expression of 17HSD type 2 mRNA was detected in the colon, kidney, pancreas and prostate. In breast cancer specimens the 1.3 kb mRNA for 17HSD type 1 was detected in some specimens and the 2.3 kb mRNA in all of the specimens studied. In contrast, the expression of 17HSD type 2 was not present in these breast cancer specimens.

Several cell lines were used to further characterize the expression of 17HSD isoenzymes in different tissues (Table 2). The 1.3 kb mRNA for 17HSD type 1 was detected in some of the breast cancer cell lines (BT-20, T-47D and MDA-MB-361) and in choriocarcinoma cell lines (BeWo, JAR and JEG-3). The 2.3 kb mRNA for 17HSD type 1 was expressed in all the human cell lines studied except the BT-20 breast cancer cell line. The 1.5 kb mRNA for 17HSD type 2 was expressed in breast cancer cell lines (BT-20 and MDA-MB-361), endometrial cancer cell lines (RL95-2 and HEC-1A), liver carcinoma cell lines (A-431 and Hep G2), PC-3 prostate cancer cells and CV-1 monkey kidney cells. Northern blot analysis showed moderate constitutive expression of the 2.9 kb 17HSD type 4 mRNA in all the cell lines studied except the BT-20 breast cancer cell line. Southern blot analysis of the BT-20 cell line indicated that the 17HSD type 4 gene is not deleted from BT-20 cells (data not shown) and hence the reason for the lack of the 2.9 kb 17HSD type 4 mRNA in these cells remains to be elucidated.
### Table 2. 17HSD activities and expression of 17HSD type 1, type 2 and type 4 in different cell lines. 17HSD activity was measured in triplicate and the results are presented as the percentage of substrate used ± SD in 20 h. Expression of 17HSD enzymes is shown as – (no expression) or + (mRNA signal present).

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>substrate converted</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E1→E2</td>
<td>E2→E1</td>
<td>17HSD type 1</td>
<td>17HSD type 2</td>
<td>17HSD type 4</td>
</tr>
<tr>
<td>Breast cancer cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BT-20</td>
<td>70.2±0.9</td>
<td>26.7±2.4</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MCF-7</td>
<td>4.5±1.1</td>
<td>1.7±0.5</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>1.6±0.1</td>
<td>1.8±0.1</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>23.3±1.0</td>
<td>73.0±1.0</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T-47D</td>
<td>89.0±1.9</td>
<td>7.1±0.2</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Choriocarcinoma cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>BeWo</td>
<td>78.0±0.6</td>
<td>19.9±0.6</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>JAR</td>
<td>92.2±0.3</td>
<td>4.9±0.1</td>
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<td>+</td>
<td>-</td>
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<tr>
<td>JEG-3</td>
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<td>6.0±0.1</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Endometrial cancer cells</td>
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<tr>
<td>HEC-1-A</td>
<td>4.7±0.9</td>
<td>3.5±0.9</td>
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<tr>
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<td>2.8±0.4</td>
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<tr>
<td>RL95-2</td>
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<td>95.0±0.4</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Ovarian cancer cells</td>
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<td></td>
</tr>
<tr>
<td>OVCAR-3</td>
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<td>8.0±6.3</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Prostate cancer cells</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DU145</td>
<td>2.4±1.2</td>
<td>0.7±0.2</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>LNCaP</td>
<td>5.8±0.5</td>
<td>8.9±0.9</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PC-3</td>
<td>1.5±0.2</td>
<td>59.5±0.2</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Human skin fibroblasts</td>
<td>2.8±0.2</td>
<td>1.2±0.2</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Monkey kidney cells</td>
<td></td>
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</tr>
<tr>
<td>CV-1</td>
<td>7.7±0.2</td>
<td>77.2±1.5</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

#### 5.2. Enzymatic properties of 17HSD types 1, 2 and 4 (I, II, IV)

The opposing oxidative and reductive 17HSD activities were characterized in COS-m6 cells. At the time only 17HSD type 1 had been cloned and characterized. These cells have endogenous oxidative activity, which did not correlate with the expression of the type 1 enzyme. Transfection of 17HSD type 1 in COS-m6 cells resulted in reductive activity. Furthermore, a 10-fold excess of A-dione slightly inhibited the activity of the recombinant enzyme. The endogenous enzyme converted T to A-dione at about the same rate as measured with E2.

The activity of 17HSD was measured in several different cell lines and was compared with the expression of various 17HSD isoenzymes (Table 2). In the cell lines where
reductive activity was measured, both 17HSD type 1 protein and the 1.3 kb mRNA for 17HSD type 1 were detected. In contrast to the 1.3 kb mRNA, the 2.3 kb mRNA for 17HSD type 1 was detected in all the human cell lines analyzed, even in cell lines without detectable 17HSD type 1 protein or major reductive activity. The expression of 17HSD type 2 correlated with the oxidative activity measured in the cell lines. In those cells where both reductive and oxidative activities were measured, both 17HSD type 1 and type 2 enzymes were detected. In these cell lines the relative activity was dependent on the amounts of isoenzymes expressed. In contrast to 17HSD type 1 and type 2, the 2.9 kb mRNA for 17HSD type 4 was detected in all cell lines analyzed apart from the BT-20 cell line, and furthermore it did not correlate with the 17HSD activities measured. In contrast, 17HSD activity in the analyzed cells always appeared to be associated with the presence of 17HSD type 1 and type 2 mRNAs. The expression of 17HSD type 4 presented in Table 2 is not published in the original article.

To investigate if the intracellular environment could modulate enzyme activities in different cell types, 17HSD type 1 and type 2 were transfected into cell lines where no significant 17HSD activity was present. The data showed that the activities of the transiently expressed enzymes were identical to those found in the cells endogenously expressing the enzymes. In addition, cells endogenously expressing 17HSD type 1 were transfected with 17HSD type 2 and vice versa. The results again showed opposite activities of the enzymes. Furthermore, the results showed that in cultured cells 17HSD type 1 has a higher catalytic efficacy than the type 2 enzyme, since 17HSD type 1, when transfected into PC-3 prostate cancer cells endogenously expressing the type 2 enzyme, reversed the activity to the reductive direction, whereas 17HSD type 2 did not have a significant effect when expressed in 17HSD type 1-producing T-47D breast cancer cells.

5.3. Properties of 17HSDs in normal breast tissue and cell lines (IV)

The activity and expression of 17HSD isoenzymes in normal breast cells were characterized using human mammary epithelial (HME) cells obtained from two donors. In both of the cell lines (4144 and 4224), oxidative 17HSD activity prevailed. Fifty to sixty percent of 100 nM E2 was converted to E1 in 10 h, whereas the opposite reductive activity was not detected. Northern blot analyses were used to characterize the expression of 17HSD enzymes in these cells. In line with the predominant oxidative activity measured in cultured cells, a 1.5 kb signal for 17HSD type 2 was detected in both cell lines. However, both 1.3 kb and 2.3 kb mRNA signals for 17HSD type 1 were also present. This was surprising, as no significant reductive activity typical of the type 1 enzyme was detected in these cells. In addition, the 2.9 kb mRNA signal for 17HSD type 4 was detected in both of the HME cell lines.

The expression of 17HSD type 1 and type 2 was further analyzed by using in situ hybridization in sections of normal breast tissues obtained at different stages of the menstrual cycle. Both 17HSD type 1 and type 2 mRNAs were more or less evenly expressed in glandular epithelium regardless of the stage of the menstrual cycle. Furthermore, the mRNAs of the type 1 and type 2 enzymes were expressed pari passu in all specimens analyzed.
Since type 1 17HSD activity was not detected in the HME cells even though the mRNA of the enzyme was expressed, the presence of 17HSD type 1 protein was evaluated using Western blot analyses. In intact cells a 35 kDa signal was detected, corresponding to recombinant human 17HSD type 1 used as a control. We next investigated whether or not the intracellular environment could modulate 17HSD type 1 activity in these cells. For this, human 17HSD type 1 cDNA was transiently transfected into the 4224 cells. In the transfected cells, a protein with the correct molecular weight was detected (data not shown) and the reductive 17HSD activity (E1→E2) measured increased with the amount of 17HSD type 1 cDNA transfected.

5.4 Characterization of estrogen-dependent growth of cultured MCF-7 breast cancer cells expressing 17HSD type 1 (III)

In order to analyze the role of 17HSD type 1 in estrogen metabolism and estrogen-dependent growth of human breast cancer cells, MCF-7 cells were stably transfected with a full length cDNA coding for the enzyme.

After stable transfection, 35 different G-418 sulfate-resistant MCF-7 cell lines were analyzed as regards 17HSD type 1 expression. Three control cell lines and six 17HSD type 1-expressing cell lines were then further characterized. The enzyme concentrations in the different cell clones expressing the enzyme were between 295-2894 ng/mg protein as measured by immunofluorometric assay. In the control clones, enzyme expression was under the detection limit of the assay and it is therefore evident that nearly all of the 17HSD activity detected in the constructed cells was a result of transfected 17HSD type 1. The correct molecular weight (35 kDa) and dimerization of the monomers were detected using Western blot analysis. ER concentrations were analyzed in both the 17HSD type 1-expressing and control cells. The data showed that high expression of 17HSD type 1 did not affect the basal ER concentrations in the constructed cell lines. Both the reduction of E1 to E2 and oxidation of E2 to E1 were then studied in the different cell clones. The results showed that in the 17HSD type 1-expressing cells most of the E1 was converted to E2 in 2 h with both 1 nM and 1 μM concentrations of E1, whereas only 8% of E2 was converted to E1 in 20 h. The reductive reaction in the cultured cells was related to the increased 17HSD type 1 enzyme protein concentration, while the oxidative activity (E2→E1) was not affected in the same manner.

Estrogen-dependent growth of the cultured MCF-7 cells expressing 17HSD type 1 was then characterized and compared with that of the control cells. Both the control cells and 17HSD type 1-expressing cells showed dose-dependent increases in cell proliferation in the presence of E2. However, identical concentrations of E1 did not affect the proliferation of control cells, whereas the increases in cell proliferation of 17HSD type 1-expressing cells were practically identical to those observed with E2. It is therefore evident that in these cells the conversion of E1 to E2 catalyzed by 17HSD type 1 results in increased biological activity. With the E1 concentrations used, the rate of E2 production was identical with all the 17HSD type 1-expressing cells, thereby suggesting that other factors such as substrate and cofactor availability also limit the reaction rate in
cultured cells. This is backed up by the fact that similar dose-response curves for E1 were observed with all the stable cell clones characterized.
6. Discussion

At the time this study was started, only one 17HSD enzyme had been cloned and characterized (Peltoketo *et al.* 1988, Luu-The *et al.* 1989). There was, however, increasing evidence of the existence of multiple 17HSD enzymes. Both oxidative and reductive 17HSD activities with various substrate specificities had been detected in human and rat tissues (Martel *et al.* 1992). Several purification and fractionation experiments had also demonstrated cytosolic and microsomal 17HSDs with different enzymatic characteristics and substrate specificities (Blomquist *et al.* 1985, Tait *et al.* 1988, Mann *et al.* 1991). The opposite 17HSD activities found in different tissues and cell lines could not be explained by different cofactor pools in different cell types, for instance. We could unquestionably demonstrate that COS monkey kidney cells contain a 17HSD, which is not placental 17HSD (17HSD type 1) and which has opposite activity to 17HSD type 1. This confirmed the presence of at least two different 17HSDs. The 17HSD activity detected in COS cells was later characterized as 17HSD type 2 (Wu *et al.* 1993). At present eight distinct enzymes having 17HSD activity have been characterized and cloned (Peltoketo *et al.* 1999 and refs. therein).

Previously it had been shown that 17HSD type 1 can catalyze both reductive and oxidative reactions equally in *in vitro* conditions (Karavolas & Engel 1971, Puranen *et al.* 1994). Our results suggest that *in vivo*, 17HSD type 1 predominantly catalyzes reductive reactions. Our data also suggest that the direction of the 17HSD reaction in each cell is dependent on the 17HSD forms expressed. In the cell lines we used, the direction of conversion between E1 and E2 was determined by the expression of 17HSD type 1 and/or 17HSD type 2. In cell culture conditions, the cells stably or transiently transfected with 17HSD type 1 showed predominantly reductive activity, converting E1 to E2. In contrast, in 17HSD type 2-expressing cells oxidative activity was present. Hence, the intracellular environment could not modulate the direction of enzyme activity in the transfection experiments.

Intracellular cofactor equilibrium and membrane association of 17HSD type 1 have been suggested to affect the different behavior of the enzyme *in vivo* and *in vitro*. 17HSD type 1 and type 3, which catalyze the reductive reaction *in vivo* prefer NADPH as cofactor, while oxidative 17HSD type 2 uses NAD⁺ (Wu *et al.* 1993, Geissler *et al.* 1994, Jin & Lin 1999). Similar results have been observed with 11β-hydroxysteroid dehydrogenases (Agarwal *et al.* 1990, 1994). 11HSD type 1 catalyzes reduction of
cortisone to cortisol, using NADPH as cofactor, whereas oxidative 11HSD type 2 uses NAD⁺. The most abundant intracellular concentrations of nicotinamide cofactors are those of NADPH and NAD⁺ rather than NADH and NADP⁺. Furthermore, *in vitro* and *in vivo* conditions may modulate the enzymatic characteristics of 17HSD type 1 protein. The X-ray structure of 17HSD type 1, determined from the solubilized enzyme *in vitro*, suggests that *His*²²¹ is involved in substrate recognition (Ghosh *et al.* 1995). *In vitro* the substitution of *His*²²¹ with another amino acid reduced the activity of the enzyme, but this was not so *in vivo*, indicating that *His*²²¹ is not essential in substrate binding (Puranen *et al.* 1994, 1997a). It is suggested that membrane association near the active site of 17HSD type 1 may modulate its activity *in vivo*.

The existence of several 17HSD isoenzymes with opposite activities can complicate the interpretation of earlier studies based on enzymatic activities only. For instance, in several studies conversion of E1 and E2 has been measured in normal and malignant breast tissue homogenates (Bonney *et al.* 1983, Vermeulen *et al.* 1986). These *in vitro* activity measurements can be misleading, since 17HSD type 1 activity can be driven to either an oxidative or reductive direction depending on the cofactor and substrate used in the assay (Karavolas & Engel 1971, Puranen *et al.* 1994). In such conditions the measured oxidative activity can result from both 17HSD type 1 and oxidative isoenzymes together. Hence it is not possible to conclude which isoenzyme causes the 17HSD activity if the isoenzymes have not been otherwise identified.

Expression of the 1.3 kb mRNA for 17HSD type 1 but not the 2.3 kb mRNA was shown to result in 17HSD protein and reductive 17HSD activity in the cells. The human 17HSD type 1 gene, *hHSD17B1*, has two transcription starting points, which lead to the two mRNA transcripts of 1.3 kb and 2.3 kb in size. The only difference between the 1.3 kb and the 2.3 kb mRNA is that the 2.3 kb mRNA has an additional 971-nucleotide-long 5’ non-coding region (Luu-The *et al.* 1989, 1990). The longer 2.3 kb mRNA is evidently not transcribed to active protein. Several studies have shown that only expression of the 1.3 kb mRNA is regulated (Tremblay *et al.* 1989, Poutanen *et al.* 1992a, Ritvos & Voutilainen 1992, Reed *et al.* 1994, Jantus-Lewintre *et al.* 1994a,b, Piao *et al.* 1995, 1997a), which further indicates the importance of the 1.3 kb mRNA over the 2.3 kb mRNA.

We found 17HSD type 1 expression in the ovary, placenta and in some breast cancer specimens. In ovarian granulosa cells 17HSD type 1 has an important role in E2 biosynthesis (Ghersevich *et al.* 1994a, Sawetawan *et al.* 1994). The expression of 17HSD type 1 is extensively regulated during follicular maturation, being highest in Graafian follicles (Ghersevich *et al.* 1994a, Sawetawan *et al.* 1994). 17HSD type 1 expression has been detected in the placenta (Fournet-Dulguerov *et al.* 1987, Dupont *et al.* 1991, Mäentausta *et al.* 1991) as well as in JAR, JEG-3 and BeWo choriocarcinoma cell lines (Jantus-Lewintre *et al.* 1994a) and in some target tissues of estrogen action such as normal and malignant breast tissue, and breast cancer cell lines also express 17HSD type 1 (Poutanen *et al.* 1992ab, 1995). 17HSD type 2 is a less estrogen-specific enzyme than 17HSD type 1. The type 2 enzyme also converts T to A-dione and in addition it has 20α-HSD activity, converting P to 20α-dihydroprogesterone (Wu *et al.* 1993). Human 17HSD type 2 is expressed in several tissues including the placenta and liver. In addition, mouse 17HSD type 2 is expressed throughout the gastrointestinal and urinary tracts, which suggests that 17HSD type 2 could have a role in inactivation of sex steroids and
steroid-like compounds (Mustonen et al. 1997b, 1998b). Thus 17HSD type 1 and type 2 have opposite activities, 17HSD type 1 being reductive and producing the active estrogen E2, whereas 17HSD type 2 inactivates E2 to E1. The physiological role of 17HSD type 1 is to act in the biosynthesis of E2 both in steroidogenic and peripheral tissues. The oxidative activity of 17HSD type 2 could protect tissues from excess E2.

The expression of 17HSD type 4 mRNA was detected in all tissues and almost all cell lines characterized. In our studies a correlation between the presence of 17HSD type 4 mRNA and oxidative 17HSD activity in cultured cells was not detected. The data, therefore, suggest that compared with the type 4 enzyme, 17HSD type 2 is far more potent in inactivating E2 to E1. These data strongly suggest that oxidation of 17β-hydroxy steroids is not the primary activity of the 17HSD type 4/MFE-II enzyme.

We also characterized the 17HSD isoenzymes and their activities in normal mammary cells in more detail. In two human mammary epithelial (HME) cell lines only oxidative activity was detected. The mRNA signals for all studied isoenzymes, 17HSD types 1, 2 and 4, were present in these cell lines. In addition, in normal breast specimens both 17HSD type 1 and type 2 were expressed. In our experiments with several cancer cell lines, as well as in other studies, expression of the 1.3 kb mRNA for 17HSD type 1 is usually associated with detectable reductive activity. The reason for the lack of reductive activity is not known. Possibly the oxidative activity might be strong enough to hide the reductive activity. In addition to 17HSD type 2, the oxidative activity may result from cooperation with other oxidative 17HSD enzymes.

The presence of 17HSD type 1 in malignant breast tissue might explain the higher E2 concentrations in breast cancer specimens compared with normal breast tissue or plasma concentrations (Bonney et al. 1986, McNeill et al. 1986, Vermeulen et al. 1986, Blankenstein et al. 1999). Using immunohistochemistry, 17HSD type 1 has been detected in 71% of benign breast lesions and in 47% of cancer specimens (Poutanen et al. 1992b, Sasano et al. 1996). Most of the known risk factors for breast cancer, such as early menarche, late menopause, late childbirth and obesity, increase the influence of estrogen (Pike et al. 1981, Mansfield 1993, Lipworth 1995). We used MCF-7 breast cancer cells to study further the role of 17HSD type 1 in estrogen metabolism and in estrogen-dependent growth of breast cancer cells. Transfected reductive activity led to enhanced biological activity of E1. In these cells both E1 and E2 resulted in similar responses in cell growth, whereas in wild type cells E1 at these concentrations (1-100 pM) did not have any significant effect on cell growth. These results further suggest that 17HSD type 1 may be an important regulatory factor in the estrogen-responsive growth of breast cancer tissue. 17HSD type 2 was also expressed in two breast cancer cell lines, BT-20 and MDA-MB-361. In previous studies microsomal oxidative activity has been found in normal, benign and malignant breast tissue (Pollow et al. 1977, Bonney et al. 1983, 1986, Tait et al. 1989, Mann et al. 1991). The possible role of 17HSD type 2 in breast cancer needs further characterization. A suggestion is that 17HSD type 2 protects the tissue from excessive estrogen exposure.

An interesting possibility is to use 17HSD type 1 inhibitors in the prevention and treatment of estrogen-dependent breast cancer. Several potential inhibitors have already been generated (Penning 1996 and refs. therein). A problem with 17HSD type 1 inhibitors might be that they result in increased E1 concentrations, and since E1 has some affinity towards ER, at higher concentrations E1 could have an effect on cell growth
similar to that of E2. However, use of 17HSD type 1 inhibitors together with other treatments, such as aromatase or sulfatase inhibitors, might provide useful tools to lower E2 concentrations.

The properties of most 17HSD isoenzymes are only superficially known. Clarification of the enzymatic properties, tissue-specific expression and regulation of the 17HSD enzymes requires further investigations. More studies are also needed to specify the role of each 17HSD enzyme in steroid hormone action. Additional 17HSD enzymes may exist. Two of the 17HSD isoenzymes have been linked to inherited disease. A defect in the function of 17HSD type 3 causes male pseudohermaphroditism (Geissler et al. 1994) and mutations in 17HSD type 4 leads to Zellweger syndrome (van Grunsven et al. 1998). The generation of knock-out mice might create a useful tool to study the physiological function of 17HSD isoenzymes.

Modern techniques in molecular biology have made it possible to investigate the structure and function of separate enzymes and their genes. Characterization of 17HSD isoenzymes has made it possible to study the effects of single isoenzymes rather than 17HSD activity. In addition to steroidogenic tissues many peripheral tissues possess the enzymatic activity needed for formation of active steroids from adrenal precursors (Labrie 1991). Hence, the effect of estrogens can be regulated by enzymes expressed in the target tissues of estrogen action. The biological activity of estrogens in different cell types can locally be modulated by regulating the expression and the activity of different 17HSD isoenzymes. When the regulation of local steroid metabolism is better understood, it may be possible develop more specific drugs to control the steroid content of specific tissues.
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