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The Effects of Estradiol, Androgens and the Selective Estrogen Modulators: Ospemifene, Raloxifene and Tamoxifen on Explant Cultures of Human Breast Tissue

by

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"I know that I know nothing ..."

Socrates (469 BC – 399 BC)

ABSTRACT

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The Effects of Estradiol, Androgens and the Selective Estrogen Modulators: Ospemifene, Raloxifene and Tamoxifen on Explant Cultures of Human Breast Tissue

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The impact of menopausal hormone therapy (MHT) on increasing the risk for breast cancer (BC) remains controversial. To understand MHT-elicited cellular breast effects and the potential risks, included with using this therapy, a further investigation into this controversy is the subject of this thesis.

In this thesis, to study the effects of estrogen, progestin, androgens and selective estrogen receptor modulators (SERMs), a modified tissue explant culture system was used. The different types of human breast tissues (HBTs) used in this study were normal HBTs, obtained from reduction mammoplasties of premenopausal women (prem-HBTs) or postmenopausal (postm-HBTs) women and peritumoral HBTs (peritum-HBTs) which were obtained from surgeries on postmenopausal BC patients. The explants were cultured up to three weeks in the presence or absence of estradiol (E_2), medroxyprogesterone acetate (MPA), testosterone (T), dihydrotestosterone (DHT) and SERMs - ospemifene (OSP), raloxifene (RAL) and tamoxifen (TAM).

The cultured HBTs maintained morphological integrity and responded to hormonal treatment *in vitro*. E_2, MPA or E_2/MPA increased proliferative activity and was associated with increased cyclin-D_1 and caused changes in the cell cycle inhibitors p21 and p27, whereas the androgens T and DHT inhibited proliferation and increased apoptosis in HBT epithelia and opposed E_2-stimulated proliferation and cell survival. The postm-HBTs were more sensitive to E_2 than prem-HBTs. The effects of OSP, RAL and TAM on HBT epithelium were antiproliferative. E_2, androgens and SERMs were associated with marked changes in the proportions of epithelial cells expressing steroid hormone receptors: E_2 increased ERα expressing cells and decreased androgen receptor (AR) positive cells, whereas T and DHT had opposite effects. The OSP, RAL and TAM, also decreased a proportion of ERα positive cells in HBT epithelium. At 100 nM, these compounds maintained the relative number of AR positive cells, present at control level, which may partly explain proliferative inhibition.

In conclusion, the proliferative activity of E_2, in the epithelium of postm-HBTs, is opposed by T and DHT, which suggests that the inclusion of androgens in MHT may decrease the risk for developing BC.

KEY WORDS: human breast tissue, menopausal hormone therapy, explant culture, estrogen, androgens, testosterone, dihydrotestosterone, SERMs, ospemifene, raloxifene, tamoxifen

TIIVISTELMÄ

Natalija Eigeliene

Estradiolin, androgeenin ja selektiivisten estrogeenireseptorin muuntelijoiden (SERM), ospemifeenin, raloksifeenin ja tamoksifeenin vaikutus ihmisen rintakudokseen

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Vaihdevuosien hormonihoito (VHH) sisältää tyypillisesti estrogeenia, usein yhdistettynä keltarauhashormoniin tai androgeeniin. Hoidoilla on myönteinen vaikutus vaihdevuosioireisiin, luun tiheyteen ja rasva-aineenvaihduntaan, mutta VHH:n mahdollinen rintasyöpäriskiä lisäävä vaikutus on edelleen kiistanalainen ja aihetta koskevat tutkimusraportit ovat tuottaneet ristiriitaista tietoa.

Tämän tutkimuksen tarkoituksena oli selvittää vaihdevuosien hormonihoidossa (VHH:ssa) käytettyjen hormonien ja muiden yhdisteiden vaikutusta ihmisen normaaliin maitorauhaskudokseen. Tutkimuksessa käytettiin kudosviljelymallia, jossa tutkittiin estrogeenin, keltarauhashormonin (medroksiprogesteroni-asetaatti, MPA), androgeenin (testosteroni, T ja dihydrotestosteroni, DHT) ja kolmen selektiivisen estrogeenireseptorin muuntelijan (SERM), ospemifeenin (OSP), raloksifeenin (RAL) ja tamoksifeenin (TAM) rintakudosvaikutuksia ihmisten rmaitorauhaskudokseen.

Maitorauhaskudosta viljeltiin hormonien ja SERM- yhdisteiden kanssa 14-21 päivää. Tulokset osoittavat, että rintakudos säilyttää viljelyssä hormonivasteensa kuten E_2:n aiheuttaman ER-spesifisten kohdegeenien (amfireguliini ja TFF1) sekä androgeenien aiheuttaman kohdegeenien (apolipoproteiini-D ja prostataspesifinen antigeeni, PSA) induktion. E_2, MPA tai E_2/MPA lisäsivät rintaepiteelisolujen proliferaatiota, kun taas androgeenit T ja DHT estivät sitä ja lisäsivät solukuolemaa. Ne myös vähensivät E_2:n stimuloivaa vaikutusta proliferaatioon. Postmenopausaalisten naisten rintakudos oli yleisesti herkempi hormonihoidoille kuin premenopausaalisten naisten maitorauhasrintakudos. SERM-yhdisteet OSP, RAL ja TAM vähensivät maitorauhasen epiteelisolujen proliferaatiota pitoisuudesta riippuvalla tavalla. E_2-, androgeeni- ja SERM-käsittelyihin liittyi voimakkaita muutoksia rintaepiteelien ER- ja AR-reseptorien tasoissa ja vaikutusta välittävissä tekijöissä, mitkä selittänevät osaltaan yhdisteiden vaikutuksia.

Yhteenvetona voi todeta, että androgeenit estävät maitorauhasen epiteelisolujen proliferaatiota sekä estrogeenin aiheuttamaa stimulaatiota ja siihen liittyvää rintasyöpäriskin lisääntymistä. Postmenopausaalisten naisten hormonihoitoihin suunnitellut SERM-yhdisteet OSP ja RAL estivät normaalin rintaepiteelin proliferaatiota lähes rintasyöpälääkkeenä käytettävään tamoksifeeniin verrattavalla tavalla. Tuloksilla on merkitystä vaihdevuosien hormonihoitojen kehitetyksessä.

AVAINSANAT: ihmisen normaali rintakudos, kudosviljely, vaihdevuosien hormonihoito estradioli, , selektiivinen estrogeenireseptorinmuuntelija (SERM), ospemifeeni, raloksifeeni, tamoksifeeni, androgeeni, testosteroni, dihydrotestosteroni

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ABBREVATIONS

Apo-D	Apolipoprotein D
AR	Androgen receptor
AREG	Amphiregulin
BC	Breast cancer
Bclt	Bicalutamide
CE	Conjugated estrogens
CK	Cytokeratin
CPP32	Caspase-3
DHT	Dihydrotestosterone
E_2	17β-Estradiol
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERT	Estrogen replacement therapy
FOXA1	forkhead box family member
Fulv	Fulvestrant (ICI 182,780)
H&E	Hematoxylin & eosin
HBTs	Human breast tissues
postm-HBT	postmenopausal HBT
prem-HBT	premenopausal HBT
peritum-HBT	peritumoral HBT
Lob	Lobule
LEC	Luminal epithelial cells
K	Keratin
MC	Menstrual cycle
MHT	Menopausal hormone therapy
MEC	Myoepithelial cells
MPA	Medroxyprogesterone acetate
OSP	Ospemifene
P	Progesterone
PI	Proliferation index (-es)
PHH3	Phospho-histone3
PR	Progesterone receptor
PSA	Prostate specific antigen
SERM	Selective estrogen receptor modulator
SMA	Smooth muscle actin
SSH	Sex steroid hormone
TAM	Tamoxifen citrate
TDLU	Terminal duct lobular unit
T	Testosterone
TFF1	Trefoil factor 1

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original articles, which are referred in the text by the Roman numerals (I-IV)

I Natalija Eigeliene, Pirkko Härkönen, Risto Erkkola. Effects of estradiol and medroxyprogesterone acetate on morphology, proliferation and apoptosis of human breast tissue in organ cultures. *BMC Cancer* 2006, 6:246

II Natalija Eigeliene, Pirkko Härkönen, Risto Erkkola. Effects of Estradiol and Medroxyprogesterone Acetate on Expression of the Cell Cycle Proteins Cyclin D1, p21 and p27 in Cultured Human Breast Tissues. *Cell Cycle* 2008; 7(1):71-80

III Natalija Eigeliene, Teresa Elo, Mari Linhala, Saija Hurme, Risto Erkkola, Pirkko Härkönen. Androgens Inhibit the Stimulatory Action of 17β-Estradiol on Normal Human Breast Tissue in Explant Cultures. *J Clin Endocrinol Metab*, July 2012, 97:E1116-1127

IV Natalija Eigeliene, Lauri Kangas, Christina Hellmer, Risto Erkkola, Pirkko Härkönen. Effects of Selective Estrogen Modulator Ospemifene on Normal Human Breast Tissue *in vitro* in comparison with 17β-Estradiol, Raloxifene and Tamoxifen. *(Manuscript)*

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1 INTRODUCTION

Women, in western countries, spend approximately one-third of their life beyond menopause and the vast majority of these women have severe menopausal symptoms (vasomotor symptoms, mood and sexual dysfunction) and morbidities (osteoporosis and bone fractures). These are caused by the cessation of ovarian sex steroid hormones, in particular estrogens. The ovarian steroid hormones: estrogen (E_2) and progesterone (P) have essential roles in the development and maintenance in the proper function of human mammary gland *(von Schoultz et al. 1996, Howard et al. 2000)*. However, E_2 and progestins associate with breast carcinogenesis, which is a common cancer and is a leading cause of mortality among females in western countries *(Hulka et al. 1995, Lopez-Otin et al. 1998, Rossouw et al. 2002, Beral 2003, Folkerd et al. 2010, Santen et al. 2010, Stevenson et al. 2011, Taylor et al. 2011, Gompel et al. 2012)*. Breast cancer (BC) incidence strongly correlates with age and during menopause, peaks.

Estrogen-alone, or in combination with progestins, are widely used for menopausal hormone therapy (MHT) for prevention, amelioration and treatment of unwanted conditions that occur during menopause. Despite the beneficial effects of MHT, numerous studies show that long-term MHT use increases the risk of BC [reviewed in *(Justenhoven et al. 2012)*].

Contradictory results exist, from *in vitro* and *in vivo* studies, concerning the role of sex steroid hormones (SSH), estrogens, androgens and progestins in breast epithelial cell proliferation and in breast carcinogenesis *(Bergkvist et al. 1996)*. BC is described as a complex disease caused by the progressive accumulation of mutations of certain genes in combination with epigenetic deregulation and distortion of signalling pathways *(Giovannelli et al. 2012)*. SSH are major regulators of breast tumorigenesis and they affect transcriptional machinery *(Giovannelli et al. 2012)*. One of the mechanisms facilitating breast carcinogenesis is increased cell proliferation caused by endogenous estrogens, and P as well as their synthetic and/or exogenously administered derivates and metabolites in breast glandular tissue *(Preston-Martin et al. 1990, Henderson et al. 2000, Giovannelli et al. 2012)*. Intensive proliferative responses predispose epithelial cells to somatic mutations and eventually to malignant changes *(Feigelson et al. 1998)*, especially during long-term MHT *(CGHFBC 1997, Rossouw et al. 2002, Chlebowski et al. 2010)*. Further studies that could clarify the effects of MHT on normal human mammary gland are needed *(Zhuang et al. 2003)*.

Evaluations, of MHT on normal breast tissues (HBTs), are performed by using large-scale clinical studies. These studies demand many years to reach completion and need great numbers of healthy participants. Experimental studies using cell lines and experimental animals (*e.g*. rodents), although useful, have their limitations due to lack of epithelial-stromal interaction in cell cultures *(Burdall et al. 2003)* or in the essential biological differences between the human and rodent mammary gland *(Zhuang et al.*

2003). There are also studies on non-human primates' mammary gland, which have well-documented similarities to the anatomy of the human mammary gland, reproductive physiology and peripheral steroid hormone metabolism *(Cline et al. 1998, Isaksson et al. 2003, Stute et al. 2004)*. These studies yield highly relevant information, but they are extensive, expensive and require animal sacrifice.

Many experimental models show that the epithelial-stromal interaction is essential for proliferation and differentiation of epithelial cells both *in vivo* and *in vitro* *(Levine et al. 1985, Ronnov-Jessen et al. 1996, Parmar et al. 2004)*. The advantage of tissue explant culture compared to cell lines is the maintenance of the three-dimensional structure of the tissues and in the dynamics of epithelial-stromal interactions. Previous studies show that tissue-specific hormonal effects are maintained more properly in tissue cultures when compared to primary culture models *(Levine et al. 1985, Ronnov-Jessen et al. 1996, Parmar et al. 2004)*.

A difficulty in assessing direct hormonal effects, *in vivo,* are in verification, controlling hormonal interactions, and that the actual hormone concentrations at the level of target organ are not known *(Martikainen 1986)*. Culture systems possess some evident advantages, in the studies of these complicated variables, such as lack of systemic effects, easy manipulation of the environment and the possibility to study the direct effects of active compounds.

In the present thesis, by using tissue explant cultures of HBTs, the effects of estrogen (E_2), progestin (MPA), androgens and the SERMs ospemifene (OSP), raloxifene (RAL) and tamoxifen (TAM) were assessed.

2 REVIEW OF THE LITERATURE

2.1 The breast of an adult female

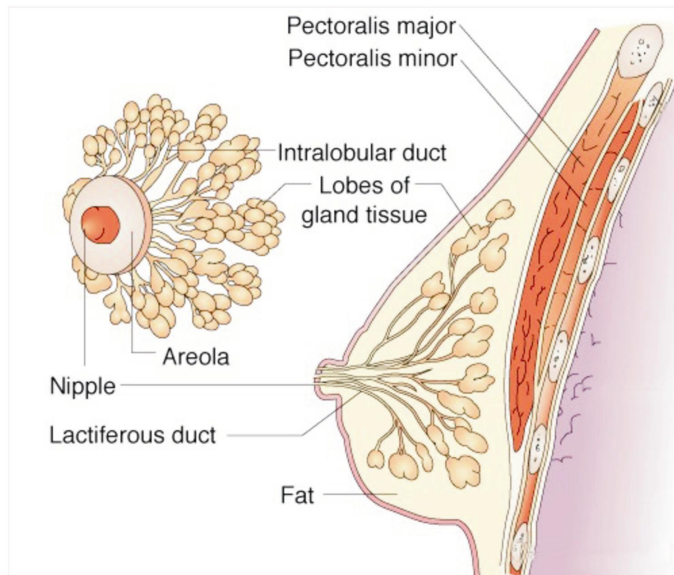
Mammals have a unique paired organ, the mammary gland or breast, which allow breastfeeding and produces a milk, which provides nourishment for their offspring *(Kumar Vinay 2010)*. In humans, breasts are a pair of mammary glands located on the upper chest wall *(Kumar Vinay 2010)*. The development of mammary glands begins in fetal life and it is an organ that is not completely developed at birth.

The normal human breast is a modified sweat gland that undergoes morphological and functional changes during a woman's lifespan (Fig.1). No other organ undergoes through such distinct changes (in size, shape and morphology) that occur during puberty, pregnancy and lactation *(Russo et al. 1997)*. HBTs undergo phases of proliferation and differentiation and, finally, regression that are regulated by the changes of the levels of circulating sex hormones along with the stage of the menstrual cycle (MC) or age.

The glandular part of the breast is formed from six to ten major lactiferous ducts which begin inside the nipple, branching and ending up in small grapelike structures referred to as alveoli or acini. The alveoli (acini) arise from one terminal duct and is embedded in the intralobular stroma and is collectively called the terminal ductal lobular unit (TDLU) which is considered as the functional unit of the breast *(Parmar et al. 2004)*.

Normal HBTs of adult women contain stromal compartments. In the HBTs, the interstitial stroma accounts for >80% of its volume, which can change up to 20% during the MC *(Ronnov-Jessen et al. 1996)*. The stroma are divided into two types – the interlobular and intralobular *(Kumar Vinay 2010)*. The interlobular stroma is composed of a dense, fibrous connective and adipose tissue. The intralobular stroma surrounds the alveoli (acini) of the lobules and contains the specific hormonally responsive fibroblast-like cells mixed together with scattered lymphocytes. The crosstalk between breast epithelium and stroma promotes the maintenance of the normal breast structure and function *(Kumar Vinay 2010)*.

Breasts of young premenopausal women consist of approximately 15% epithelial cells. With age, progressively, breast epithelium is replaced with adipose tissue and HBTs of postmenopausal woman contain less than 5% epithelium *(Russo et al. 1997)*.



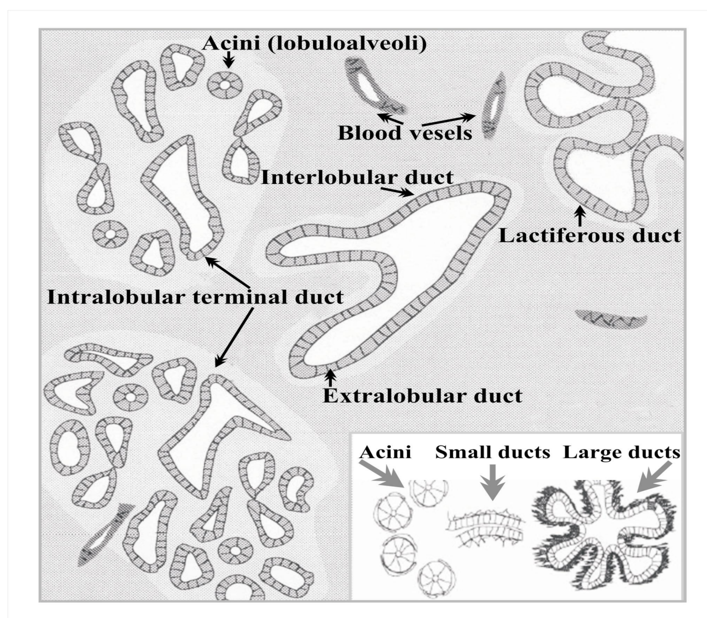


Figure 1. **The structure and a schematic presentation of resting human breast.**

Upper panel: Lactiferous ducts start inside the nipple, branch and end up in terminal ductal lobular units (TDLU). Lactiferous ducts start inside the nipple, branch and end up in terminal ductal lobular units (TDLU) (Adapted from *Lippincott Wlliams&Wilkins, Concepts of Altered Health States, 7th Edition*);

Lower panel: Duct and alveoli (acini) localization in the adult human breast. Each of intralobular terminal and extralobular terminal ducts are drained into interlobular ducts (Adapted and modified from *Rønnov-Jessen et al, 1996*).

2.1.1 Breast morphology

Each duct and acinus of an adult mammary gland consist of three different cell lineages: *epithelial cells* that surround a lumen are considered to be luminal epithelial cells *(Richert et al. 2000, Parmar et al. 2004)*, and the underlying cells, are called *myoepithelial cells (Richert et al. 2000)* forming a basal layer that rests on a laminin-containing basement membrane separating the parenchymal and stromal compartments *(Parmar et al. 2004)* and *alveolar epithelial cell*s that synthesize milk proteins *(Rudland et al. 1998, Daniel et al. 1999, Richert et al. 2000)*. As in other organs of the mammals, the epithelial cells of the mammary gland can be generated by a committed stem cell in the terminal duct that can give rise to luminal or myoepithelial cells *(Kumar Vinay 2010)*.

The complete differentiation of mammary glands occurs only during pregnancy and lactation and this differentiation is influenced by specific hormones *(Russo et al. 1998, Russo et al. 2004)*. There are differences in the histological structure of TDLU at various ages of a woman's lifespan, which represent different stages of mammary gland development *(Russo et al. 2004)*.

Four lobule types (Lob) exist in HBTs *(Russo et al. 2004)*. In Lob1 (referred as virginal lobule), alveolar buds surround a terminal duct. Both these structures - alveolar buds and terminal ducts consists of a two-layered epithelium, whereas four layers of epithelial cells is found in the terminal end buds *(Russo et al. 2004)*. The transition from Lob1 to Lob4 is a progressive process that involves sprouting of new alveolar buds. In Lob2 and Lob3, ductules increase in number from about 11 per Lob1 to 47 and 80 ductules per Lob2 and Lob3 respectively *(Russo et al. 2004)* (Fig.2).

Lob1 is mainly located in the breast of nulliparous young women, whereas Lob2 and Lob3 are more often found in the gland of parous women (Fig.3). Lob4 is considered to be completely developed and differentiated in the adult gland and presented in the late pregnancy and during lactation when glands are secreting milk and have undergone complete functional differentiation. After menopause, the breast tissue possess changes both in nulliparous and parous women which are manifested as an increase in the number of Lob1, and a concomitant decline in the number of Lob2 and Lob3 *(Russo et al. 2004)*. By the end of the women’s 60’s, HBTs of both nulliparous and parous women mainly contain Lob1 which is associated with the highest level of cell proliferation, decreasing progressively in Lob 2, 3 and 4 *(Russo et al. 1992, Russo et al. 2004)*. Nevertheless, the proliferative activity of Lob1 of nulliparous women at menopause is 2-fold greater than that of the Lob1 of parous women’s breast. The proliferative activity of the mammary epithelium varies as a function of the degree of lobular differentiation, which, in turn, is driven by E_2, P and by pregnancy hormones.

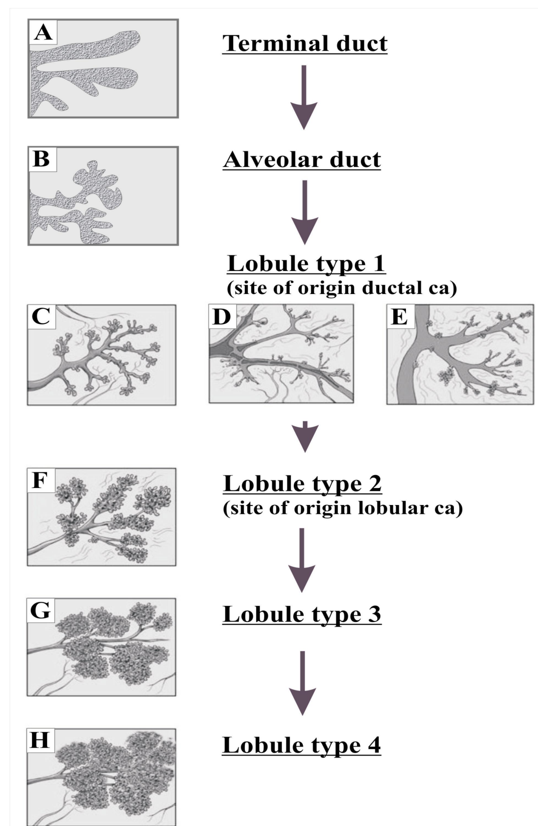


Figure 2. **Formation and involution of differentiated breast lobules during woman's lifespan** (Adapted *from Russo et al, 1998; Parmer and Cuhna 2004*).

The histological structure of human breast tissue (HBT) is dependent on its developmental phase. The adult female HBT undergoes recurrent cycles of growth, differentiation, and regression, whereas ovarian sex steroid hormones, in particular estrogen and progesterone, play a major role. The essential stages that occur in epithelium of HBT can be divided into: puberty, pregnancy, lactation, and involution. Each has specific morphological features in the main structure of HBTs – TDLU or lobule (Lob). A-B - At birth, the mammary gland is formed by several ducts, ending in terminal ducts with elongation and further divisions of the alveolar buds. Before the onset of puberty, the ducts and alveolar buds grow and divide in "a dichotomous basis" *(Russo et al. 2004)*, forming Lob1; C – Lob1 is typical in teenage HBTs;
D – Lob1 in postmenopausal woman HBTs, where ducts are dilated, and TDLUs are atrophied; E – Lob1 of an old postmenopausal woman, where marked atrophy of TDLUs is seen; F - TDLU from a young nulliparous woman breast having well developed lobules and acini; G – lobule structure of a pregnant woman with well developed TDLUs; H – Lob4 is found during lactation.

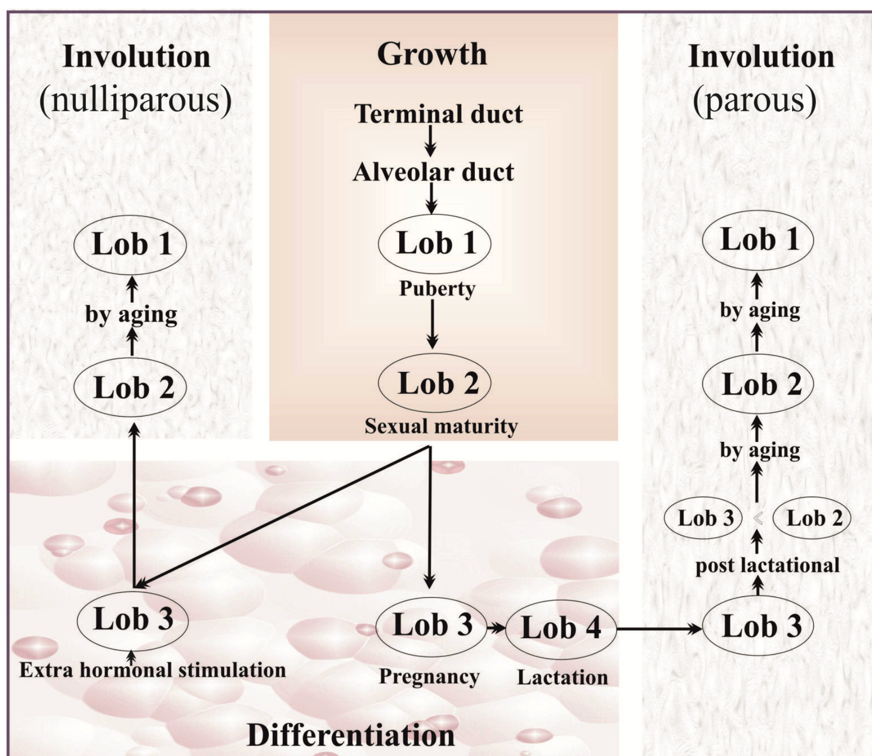


Figure 3. Morphological changes occurring during breast development in the nulliparous and the parous woman.

The lobule 1 (Lob1) is the most undifferentiated structure, which eventually progresses to Lob2. During the pregnancy, Lob1 and Lob2 rapidly evolve to Lob3 and Lob4, which is present only in the last trimester of pregnancy and lactation. The breast of the parous women is composed mainly of Lob3 (80-100%) at reproductive age with sequential regression to Lob2 and Lob1 during menopause *(Russo et al. 2004)*. Breasts of the nulliparous women at all ages is primarily composed of Lob1 (50-60% of structure), with some progression to Lob2 (30-35%), and minimal formation of Lob3 (10-15%) during sexual maturity with gradual regression to Lob1 during menopause *(Russo et al. 2004)*. An extra hormonal stimulation (hormonal contraceptives and MHT) increase the number of Lob3 in the breast of the nulliparous woman *(Russo et al. 2004)*. Lob1 and Lob2 exhibit higher cell proliferative activity than more differentiated Lob3. Thus, Lob1 and Lob2, in nulliparous women's breasts have a higher proliferation ratio than parous woman's breasts which are comprised of Lob3.

2.1.2 Hormonal regulation of the breast

Mammary gland growth, differentiation and involution consist of many ordered events involving cross-talk between distinct cell types, and this process is regulated by the interaction among various SSH and growth factors *(Hennighausen et al. 1998, McCave et*

al. 2010). The key players are E_2 and P, which are essential for mammary epithelial cell turnover *(Parmar et al. 2004)*.

E_2 is the most important circulating estrogen in women, whereas P is the most important progestagen *(Anderson et al. 2004, McCave et al. 2010)*. From the advent of menarche until menopause, both E_2 and P are synthesized and secreted in a cyclical manner by the ovaries under the control of the pituitary gonadotropins *(Anderson et al. 2004)*. Studies in rodents suggest that P promotes side-branching and normal secretory alveolar development (alveologenesis), whereas E_2 stimulates ductal elongation *(Giovannelli et al. 2012)*. P has a similar role in HBTs, demonstrated in rodents, and it stimulates TDLU formation and expansion during puberty and pregnancy *(Anderson et al. 2004)*. However, this was not demonstrated in HBTs, probably because it is almost impossible to study human mammary epithelium at these developmental stages *(Anderson et al. 2004)*.

SSH action occurs through their cognate intracellular receptors such as estrogen receptor (ER) and progesterone receptor (PR). *In vivo* studies, using mouse models with these receptors genetically deleted, show that the loss of ERα results in impaired ductal branching and development and PR depletion results in impaired alveologenesis [reviewed in *(McCave et al. 2010)*].

Breast epithelial proliferation ratios vary during the MC: epithelial cell division is slow during the follicular phase, when E_2 and P levels are low, and is increased during the luteal phase of MC due to high levels of these ovarian SSH *(Pike et al. 1993, Bernstein 2002)*. The epithelial cell proliferation in HBTs is very low during the menopause, when estrogen levels are low and P is absent *(Bernstein 2002, Walker et al. 2007b)*.

The epithelial-stromal interactions are essential for the initiation and response of E_2 and/or P maintenance in mammary epithelial cells *(Haslam et al. 2002)*. This epithelial-stromal interaction appears to be important for the coordinated regulation of growth and differentiation of the normal mammary gland *(Woodward et al. 1998, Parmar et al. 2004)*. Stromal cells can influence epithelial cell behavior by producing of soluble growth factors and thereby modify the composition of the extracellular matrix *(Woodward et al. 1998)*. SSH are lipophilic and could be stored in adipose tissue. Stroma could affect the diffusion of SSH by slowing or blocking it *(Gompel et al. 2004)*. Additionally, the mammary gland could have an active SSH metabolism, where ovarian and adrenal androgens provide an important source of estrogens through their aromatization. Results from various studies indicate that aromatase activity increases in pre- and malignant HBTs *(Labrie et al. 2003, Bulun et al. 2005, Singer et al. 2006, Shibuya et al. 2008, Suzuki et al. 2010, Lonning et al. 2011)*.

"Programmed cell death or apoptosis, also deprives the proliferating cellular pool and allows the elimination of genetically damaged cells after their division" *(Gompel et al. 2000, Gompel et al. 2004)*. Some of the molecules involved in the

regulation of apoptosis are influenced by SSH and antihormones are particularly susceptible to E_2 and P action *(Gompel et al. 2004)*. In normal breast epithelial cells, apoptosis occurs in response to changes in hormonal levels *(Gompel et al. 2004)*. As proliferation, apoptosis also shows cyclic variations in normal mammary gland reaching the peak at the end of luteal phase of MC. This peak could either be due the decrease of E_2 and/or P levels occurring at the end of MC. The effects of E_2 on cell survival in E_2-dependent BC cell lines, in which apoptosis can be induced by E_2 deprivation or antiestrogen treatment *(Somai et al. 2003)*, is known. This antiapoptotic effect of E_2 is reinforced with the mitogenic effect in breast epithelial cells and may contribute to the development of BC. The results obtained in normal breast epithelial cells show that progestins and antiestrogens are able to modulate the expression of certain molecules in the apoptotic cascade, *e.g.* p53 (increasing the level) and caspase-3, while E_2 has an opposite effect on the level of these proteins *(Gompel et al. 2004)*.

The effects of E_2 and P on the mammary gland are mediated by the nuclear receptors – ER, PR and androgen (AR) receptors. These receptors are members of the nuclear hormone receptor superfamily and consist of classic DNA binding, hormone-binding, and activation domains. The SSH receptors are nuclear proteins which function as transcription factors to regulate the expression of specific target genes *(Lofgren et al. 2006)*.

Normal epithelial cells of resting human mammary gland express very low levels of ER and PR, which fluctuate in accordance with the cyclic changes in E_2 and P during MC or age.

2.1.2.1 Estrogen receptors

Two forms of ER, encoded by different genes, are known. The first estrogen receptor, ER alpha (ERα) was sequenced in 1986 and the second isoform called ER beta (ERß) was discovered and sequenced ten years later *(Green et al. 1986)*. Both ERs have similar DNA binding domains, but lack similarities (about 58%) in the ligand binding domain *(Kuiper et al. 1996, Lofgren et al. 2006)*. ERα and ERß elicit a different response to various hormonally active compounds, estrogen agonist and/or antagonist characteristics *(Gustafsson et al. 2000)*. In addition, ER isoforms have different distribution in human breast *(Speirs et al. 2002)*.

Estrogen receptor alpha (ERα). In the premenopausal HBTs, low percentages of ER*α*-positive cells (~10–20%) can be identified only in luminar and ductal epithelial cells *(Petersen et al. 1987, Ricketts et al. 1991, Shoker et al. 1999a)*. Population of ERα positive cells are heterogeneous within and between lobules and in ducts, where lower expression of ERα is found *(Walker et al. 1992)*.

ER*α* levels in human mammary gland are higher in the luteal phase and lower in the follicular phase of MC and during pregnancy *(Shaw et al. 2002, Walker et al. 2007b, Li et al. 2010)*.

The numbers of ERα positive cells in premenopausal HBTs (prem-HBTs) vary during the MC or with the use of oral contraceptives *(Battersby et al. 1992, Boyd et al. 1996)*. During lactation, approximately 80% of the luminal cells express ERα *(Speirs et al. 2002)*. Postmenopausal HBTs (postm-HBTs) express more ERα than premenopausal HBTs and many studies *(Battersby et al. 1992, Walker et al. 1992, Boyd et al. 1996, Clarke et al. 1997c, Shoker et al. 1999b, Walker et al. 2007b)* report increased ERα positivity with age. Unlike normal prem-HBTs, where minimal ERα-positive cells are found, in postm-HBTs, there is a higher frequency of contiguous ERα-positive cells in both lobules and ducts *(Shoker et al. 1999b)*. A small proportion of lobules showing involutional change had ERα–positive in more than 90% of cells *(Shoker et al. 1999b)*. Moreover, there is an almost mutual exclusion of ER*α* and Ki-67 positivity in the prem-HBTs, as assessed by dual immunohistocheminal stainings *(Clarke et al. 1997c)*. In postm-HBTs, even though the percentage of proliferating cells is low, the incidence of ER*α*- and Ki-67-positive cell coexpression increases with age *(Shoker et al. 1999b)*. However, there are also reports indicating that ER levels in HBTs decrease after menopause [reviewed in *(Li et al. 2010)*].

Estrogen receptor beta (ERβ). This is more abundantly expressed in normal HBTs than ERα and there are indications that ERβ may modulate and even counteract the effects of ERα *(Roger et al. 2001, Heldring et al. 2007, Li et al. 2010)*. Accordingly, the current opinion is that ERα and ERβ have opposite actions on breast–growth stimulatory and inhibitory effects, respectively [reviewed in *(Maehle et al. 2009)*]. Data suggest that low estrogen concentration poorly activate the expression of ERβ, but once ERβ is activated, it acts as an inhibitor of ERα *(Hall et al. 1999)*. ERβ is expressed not only in luminal epithelial, but also in myoepithelial and stromal cells *(Speirs et al. 2002)*. ERβ expression is high in resting breast epithelium (~70-85%) *(Speirs et al. 2002, Li et al. 2010)*, while lower levels of ERβ exist in proliferating breast tissue *(Lofgren et al. 2006)*.

Changes of the mRNA and protein levels of ERβ/ERα occur during breast carcinogenesis *(Leygue et al. 1998, Roger et al. 2001)*. ERβ may be a tumor suppressor and it is down-regulated during tumor progression *(Omoto et al. 2002, Nakopoulou et al. 2004)*. There are some reports suggesting that ERβ may be a favorable prognostic factor in BC, but the role of ERβ needs to be further evaluated *(Speirs et al. 2002, Maehle et al. 2009)*.

2.1.1.2 Progesterone receptors

The ovarian SSH, progesterone (P), acts by binding and activating its own receptor – progesterone receptor (PR). Progesterone receptor also has three isoforms – PR-A, PR-B and PR-C, encoded by a single gene [reviewed in *(Conneely et al. 2000a, Hagan et al. 2012)*].

The full-length PR-B (116kDa), contains a unique N-terminal segment, named B-upstream segment (BUS), that is not presented in PR-A (94kDa) and PR-C (60kDa) [reviewed in *(Hagan et al. 2012)*]. PR-C lacks the BUS and a portion of DNA-binding domain, thus it lacks transcriptional activity. PR-A and PR-B are transcriptionally active forms, which contain a flexible hinge region, a ligand binding domain and multiple activating function domains. Studies from knock-out mice show that PR-B is required for alveologenesis of normal mammary gland development, whereas PR-A is necessary for uterine development. PR-C inhibits PR-B action in the uterus and potentiates the transcriptional activity of two other PR isoforms in the mammary gland *(Hagan et al. 2012)*.

Experimental *in vivo* and *in vitro* studies indicates that PR-A may have repressive functions on PR-B action *(Conneely et al. 2000b)*. The ratio between different PR isoforms varies depending on cell type, which may contribute to appropriate P responsiveness *(Tsai et al. 1994)*. In the endometrium, the expression of PR showed cyclic variations depending on the phase of the menstrual cycle *(Mangal et al. 1997)*. Both PR-A and PR-B are expressed constantly throughout the phases of menstrual cycle *(Mote et al. 2002)*. Rodent studies show that PR-A is necessary for the action of P in the genital tract, whereas stimulation of PR-B induces proliferative effects in breast tissue *(Conneely et al. 2002)*. In the breast, PR expression is induced by E_2 through ER activation, whereas P suppresses the levels of ER *(Alexander et al. 1990)*. The changes in the expression pattern of PR isoforms in HBTs during hormonal treatment are currently unclear. In the non-human primates, conjugated estrogens (CE) and medroxyprogesterone acetate (MPA) co-exposure reduced PR-A levels and PR-A/PR-B ratio with proliferation-inducing properties as compared to treatment with CE alone *(Isaksson et al. 2003)*.

PR is located, like ERα, only in luminal epithelial cells (15–30%) *(Clarke et al. 1997c)*. Using dual-label immunofluorescent techniques, almost complete coexpression between ERα and PR (96% of the cells expressing PR also contain ERα) was found *(Clarke et al. 1997c)*. In normal HBTs, only differentiated, non-proliferating cells express ERα and PR *(Russo et al. 1999)*. Only undifferentiated cells (the stem cell/early transit cell populations) are capable to proliferate *(Anderson et al. 1998)*. Almost every proliferating cell is adjacent to a cell expressing ERα and PR *(Anderson et al. 1998)*. Thus, it is possible that ERα- and PR-positive cells control the proliferative activity of

undifferentiated cells *via* juxtacrine and/or paracrine signalling depending on the prevailing hormonal milieu *(Anderson 2002)*.

2.1.1.3 Androgen receptors

The androgens, in particular 5α-dihydrotestosterone (DHT) and testosterone (T), exert their effects through a specific receptor – androgen receptor (AR). The AR encoding gene, is located on the X chromosome and does not have any corresponding allele on the Y chromosome. Hence, AR functions as a single-copy gene and mutations in the AR results in complete loss of androgen effect in XY individuals *(Chang et al. 1995, Avila et al. 2001, Dimitrakakis et al. 2002)*. There are AR isoforms – AR-A and AR-B [reviewed in *(Li et al. 2009)*], but no information is available about the expression of these different isoforms in HBTs. AR is abundantly expressed in normal HBTs and in the vast majority of BC specimens and cell lines *(Haapasalo et al. 1993, Birrell et al. 1995, Hall et al. 1996, Zhou et al. 2000)*. AR is co-expressed with ERα and PR in epithelial cells and according to some studies, it is not detected in mammary stroma or myoepithelium *(Ruizeveld de Winter et al. 1991)*. On the other hand, there are also reports showing that AR is expressed in myoepithelial and stromal cells [*(Zhou et al. 2001)*, reviewed in *(Li et al. 2010)*]. The different AR expression pattern may be explained by different primary antibodies used.

The coexpression of ERα and AR in mammary epithelial cells suggests that the effects of E_2 and androgens on mammary epithelial cell proliferation are integrated. Preliminary results indicate that AR is not co-expressed with Ki-67 in normal human breast epithelium *(Hickey et al. 2012)*.

2.1.1.4 Signalling by sex steroid receptors in mammary tissue

Mammary gland adipose tissue is an important location. Here, signalling factors and epithelial-stromal cross-talk occurs and this is essential for the initiation and maintenance of E_2 and/or P responsiveness in mammary epithelial cells *(Haslam 1991, Haslam et al. 1991, Woodward et al. 1998, Parmar et al. 2004, Brisken et al. 2010, McCave et al. 2010)*. This epithelial-stromal interaction is vital for the coordinated regulation of cellular functions such as growth, proliferation and differentiation in the normal mammary gland *(Woodward et al. 1998, Parmar et al. 2004)*.

A number of essential stromal-derived growth factors are autocrine and paracrine mediators of epithelial–mesenchymal cross-talk in HBTs. These stromal growth factors include *epidermal growth factor* (EGF), *insulin-like growth factor* (IGF), *hepatocyte growth factor* (HGF), *fibroblast growth factor* (FGF) and

transforming growth factor (TGF) and they mediate signals influencing mammary epithelial growth *(Parmar et al. 2004, McCave et al. 2010)*.

The EGF family and their receptors (EGFRs) are important molecules that function through autocrine, paracrine and juxtacrine mechanisms *(McCave et al. 2010)*. EGFRs are critical downstream mediators of SSH action in the mammary gland by locally regulating growth and development *via* stromal–epithelial interaction *(Parmar et al. 2004, Hynes et al. 2010)*.

The EGF family members amphiregulin (AREG), EGF, betacellulin, heparin-binding EGF (HB-EGF), epiregulin, neuroregulin, TGFα and TGFß are all expressed in postnatal mammary gland and their expression changes throughout the mammary gland development, pregnancy, and lactation *(Schroeder et al. 1998, Sebastian et al. 1998, Hansen et al. 2000, McCave et al. 2010)*.

The EGF family member, AREG, is the only EGF family member induced by E_2 and is most highly expressed during the pubertal growth of mammary glands *(Brisken et al. 2010)*. AREG is required for ERα-mediated ductal elongation and branching. AREG binds and activates the epidermal factor receptor (EGFR), which is expressed in the stroma *(Brisken et al. 2010)*.

The expression of EGF is low during puberty, pregnancy and involution, but it increases dramatically at the end of pregnancy, reaching a peak during lactation. Within the first weeks after the birth, high levels of EGF, an important factor for the development of the gastrointestinal tract of newborns, are found in colostrum and breast milk *(McCave et al. 2010)*. This milk is a complex fluid that has multifunctional roles, such as providing immunoprotection *(Dvorak 2010)*. HB-EGF is also detected in breast milk, but its concentration is a few times lower than EGF. In pathophysiological conditions, EGF and HB-EGF contribute to intestinal epithelium protection and mucosal repair *(Dvorak 2010)*.

TGFα is another stromal factor inducing cell proliferation. It is expressed throughout mammary development *(McCave et al. 2010)*. TGF describes two classes of polypeptide growth factors, namely TGF-α and TGF-β *(Albert et al. 2002)*. TGF-α is unregulated in some human cancers. It is produced in macrophages, brain cells, and keratinocytes, and induces epithelial development *(Albert et al. 2002)*. TGF-β exists in three known subtypes in humans, TGF-β1, TGF-β2, and TGF-β3 *(Albert et al. 2002)*. All three isoforms are expressed in the mammalian mammary gland *(Parmar et al. 2004, McCave et al. 2010)*. There are high levels of TGF-β subtypes in mammary epithelial tissues and lower levels of transcripts in fat cells and fibrous connective tissue stroma *(Daniel et al. 1992, McCave et al. 2010)*.

FGFs are one of the largest families of growth and differentiation factors for stromal cells *(Albert et al. 2002, Pond et al. 2013)*. Critical roles for FGFs and their receptors are well-established and occur during embryonic development *(Schwertfeger 2009)*. FGFs are potent mitogens for mammary epithelial cells and fibroblasts in culture

(Woodward et al. 1998). However, nearly all FGFs promote angiogenesis, which is critical for normal mammary growth/differentiation, as well as, tumor growth *(Woodward et al. 1998)*. In addition, aberrant activation of the FGF pathway associates with tumor progression in both breast and prostate cancer. Recent studies have linked FGFR1 expression and single nucleotide polymorphisms in FGFR2 to BC *(Schwertfeger 2009)*.

FOXA1 (forkhead box family member) is an essential transcription factor for ERα expression and subsequent stimulation of mammary gland development *(Bernardo et al. 2010)*. In breast and prostate cancer models, FOXA1 is a critical factor that facilitates ER and AR binding and activity *(Augello et al. 2011)*. In the mammary gland, FOXA1 is required for ER activity and ER expression. FOXA1 and ER are co-expressed in mammary luminal epithelial cells during development *(Bernardo et al. 2010)*. In mouse models, loss of FOXA1 completely blocks epithelial growth and inhibits ductal elongation *(Bernardo et al. 2010)*.

One key factor mediating the effects of P on the mammary gland is NF-κB (RANK) and its ligand (RANKL). RANK and RANKL are expressed in epithelial cells of mammary gland under the control of SSH and other hormones. RANK expression is induced by P, prolactin and by the parathyroid hormone protein-related peptide [reviewed in *(Schramek et al. 2011)*]. The mammary epithelium from mice, lacking either RANK or RANKL, did not proliferate nor from milk secreting acini during the pregnancy which leads to failure to lactate. In rodent models, P induces two waves of mammary epithelium proliferation, where the first occurs within the 24 hours and affects PR-positive cells and its dependent on cyclin-D_1 *(Beleut et al. 2010)*. The second, is triggered by RANKL, which drives proliferation of PR-negative mammary epithelial cells in a paracrine fashion *(Beleut et al. 2010)*. RANKL is critical for P induced expansion of the mammary stem cell population *(Fernandez-Valdivia et al. 2012)* by induction of proliferation of daughter cells followed by the asymmetric division of stem cells *(McCave et al. 2010)*. Recent data suggests that the RANK-RANKL system controls the metastasis of BC to the bones and drives SSH primary mammary cancer *(Schramek et al. 2011)*.

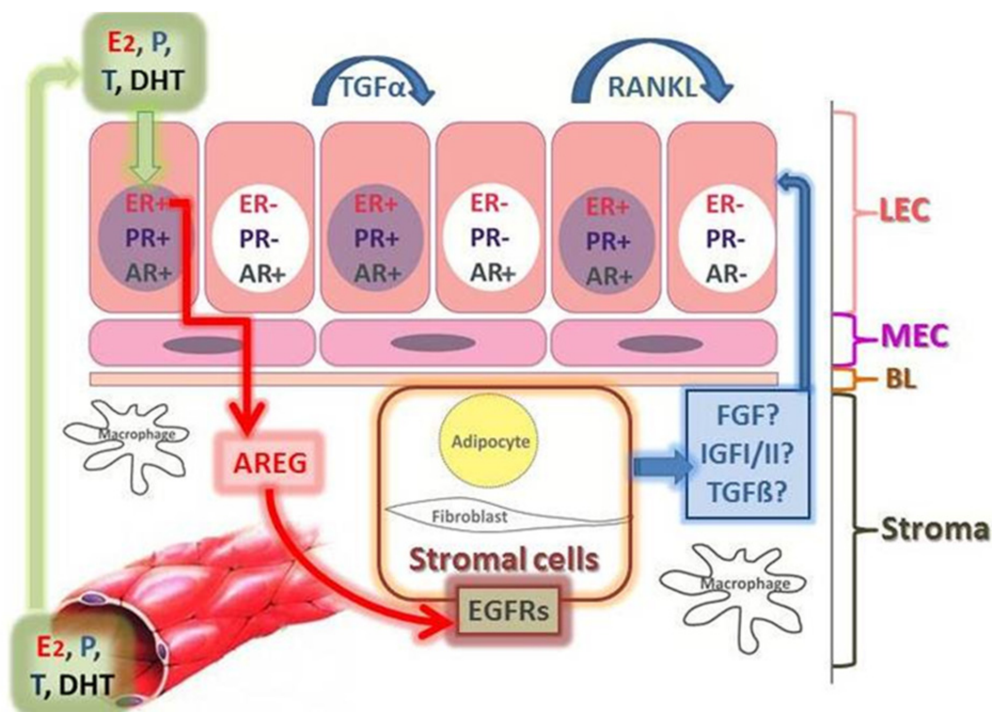


Figure 4. Schematic illustration of the hormone regulation of normal breast epithelium. The mechanisms of epithelial and stromal cell interaction are tightly regulated. E_2, P, T and DHT achieve their effects through their cognate receptors located in the luminal epithelial cell nuclei. In the epithelium of HBTs, AR expression is high (up to 70% AR-positive cells) *(Hickey et al. 2012)*, whereas ERα 10-20% *(Li et al. 2010)* and PR 15-30% *(Clarke et al. 1997c)* are expressed at lower frequency. Coexpression of AR, ERα and PR is reported, *e.g* ERα is almost completely coexpressed with PR *(Clarke et al. 1997c)*. Some ERα-positive cells coexpress AR-positive cells, but the proportion of AR-positive cell numbers is higher than that of ERα-positive cells, thus many breast epithelial cells are ERα-negative and AR-positive *(Hickey et al. 2012)*. The receptor-positive cells are surrounded by other luminal epithelial cells that are negative for one or several of these receptor types. As a result of receptor-ligand action, paracrine/autocrine factors are produced and secreted and they interact with luminal cells that are negative for SSH receptors or with stromal cells which express receptors for these factors. AREG is presented as an example of a factor mediating E_2-effects on proliferation and it is produced by epithelial cells, binds to stromal cells expressing EGFR and possibly helps produce other factors (such as FGFs, HGF, IGFs, TGFß), which bind to epithelial cells expressing appropriate receptors and may stimulate their proliferation. *Abbrevations:* AREG-amphiregulin, BL-basal lamina, EGFR-epidermal growth factor receptor, FGF – fibroblast growth factor; HGF- hepatocyte growth factor; IGF- insulin growth factor; TGFα- transforming growth factor alpha, TGFß- transforming growth factor beta; LEC-luminal epithelial cells, MEC-myoepithelial cells.

2.1.3 The morphological changes of the breast during the menstrual cycle

The cyclical changes in HBTs are most dynamic and prominent during the reproductive years *(Kumar Vinay 2010)*. The cyclic changes in the endometrium have been well documented, but the occurrence of similar changes in the breast still remain controversial issue *(Ramakrishnan et al. 2002)*. It was proposed that the normal breast undergoes similar changes as endometrium during the phases of the MC, depending on the levels of circulating SSH and affect breast morphology, protein expression, breast epithelial cell proliferation and apoptosis *(Ramakrishnan et al. 2002)*.

The distinct and typical changes in the morphology of the TDLUs and the intralobular stroma of HBTs with some degree of overlap were identified in accordance to the phase of the MC *(Ramakrishnan et al. 2002)*. Interlobular stroma was reported to be relatively unaltered in all these phases. In the first half of the MC, the lobules are relatively quiescent or resting *(Kumar Vinay 2010)*. Very rare mitotic figures were presented in the epithelial cells, most likely persistent from the previous cycle, observed in the early follicular phase *(Ramakrishnan et al. 2002)*. After ovulation, under the influence of E_2 and increasing levels of P, cell proliferation ratio and the number of acini per lobule increases *(Kumar Vinay 2010)*. Both cell turnover events - proliferation and apoptosis in HBTs were found to be higher in the second half than in the first half of the MC with the peak of apoptosis occurring approximately two days after the peak of mitotic activity *(Ramakrishnan et al. 2002)*. This could be explained as a result of the cumulative effect of high levels of E_2 and P that occurs in the late luteal phase *(Ramakrishnan et al. 2002)*. The intralobular stroma also becomes markedly edematous in luteal phase *(Kumar Vinay 2010)*. Upon menstruation, the decrease in E_2 and P levels causes the regression of the lobules and the disappearance of the stromal edema *(Kumar Vinay 2010)*.

There are established criteria to define four separate phases of the MC in the breast ("breast cycle") as the cumulative response to E_2 and P. Each of these phases have typical features in breast morphology based on type of the lobules, appearances of the luminal cells, myoepithelial vacuolisation, dual lining, intraluminal secretion, stromal edema, and events of cell turnover (mitosis and apoptosis) (Tab.1) *(Ramakrishnan et al. 2002)*. When planning breast surgeries, it would be beneficial to pay attention to the phase in MC *(Zurrida et al. 2001)*. Some studies demonstrate that BC surgeries, performed during the luteal phase of the MC, led to better outcomes (on survival) and this information is used in some centers as part of surgery protocol, especially when the operating node is positive and hormone receptor positive BC cases *(Zurrida et al. 2001, Chaudhry et al. 2006b)*. This observation is explained on the biological-endocrinological basis. First, in the follicular phase, E_2 reduces immune and phagocytic activity and circulating levels of interleukin-2 which all may raise the metastatic potential of BC cells *(Hrushesky et al. 1988)*. At the same time, they stimulate

the activity of IGF, which induces proliferation in breast cells *(Clarke et al. 1997b, Dabrosin 2003, Chaudhry et al. 2006a)*. Furthermore, angiogenesis, which promotes tumor growth and facilitates metastasis, is favored by high circulating levels of E_2 which induces vascular endothelial growth factor (VEGF) *(Coradini et al. 2003, Wood et al. 2005, Chaudhry et al. 2006a)*. During the luteal phase, circulating levels of endogenous P increase. Thus, the proliferation of normal and neoplastic HBTs may be affected and increases in intercellular cohesion may reduce metastatic potential *(Soderqvist et al. 1997, Navarrete et al. 2005, Chaudhry et al. 2006a)*. P also regulates the balance between IGF1 (pro-mitotic effect)/TGFb (anti-mitotic effect) and thereby reduces the number of ER positive breast epithelial cell that limits estrogenic stimulation and promotes apoptosis in breast epithelial cells *(Ramakrishnan et al. 2002)*.

Table 1. **The modified morphological criteria which correlates to the stage of the cycle in surgically obtained breast tissue samples** (adapted from *(Ramakrishnan et al. 2002)*). Epithelial myoepithelial distinction – epithelial and myoepithelial layers distinction; Myoepithelial vacuolation in the acini proportion of myoepithelial cells (MEC) exhibiting vacuolation.

PHASES / CRITERIA	I	II	III	IV
	early follicular MC days 0-5	**late follicular** MC days 6-15	**early luteal** MC days 16-24	**late luteal** MC days 25-28
Epithelial myoepithelial distinction	not seen	< 30% of the lobules	31-74% of the lobules	> 75% of the lobules
Myoepithelial vacuolization in the acini	absent	< 30% of the cells	31-74% of the cells	> 75% of the cells
Stromal edema	absent	mild	moderate	marked
Stromal infiltration	absent	mild	moderate	marked
Mitosis (mitotic figures)	absent	1-2	frequent	frequent
Apoptosis (apoptotic bodies)	absent	1-2	frequent	increased

2.1.4 The normal breast after menopause ("Aged breast")

In women, well before menopause, approximately in the fourth decade of life, breast tissue begins an involutionary process - lobules and their specialized stroma start to regress and involutionary changes occur in both epithelium and in the stroma *(Kumar Vinay 2010)*. Morphometric analysis of HBTs show that the proportion of the epithelial tissue in HBTs declines steadily during aging, with a corresponding decline in the volume of lobules, with no correlation between the amount of epithelium and the number of previous pregnancies *(Walker et al. 2007b)*. The prominent involutionary changes (the complete atrophy of lobules) exist in elderly females. The interlobular

stroma also changes, which can be examined by x-ray: the radio opaque fibrous stroma of the young women of reproductive age is progressively replaced by radiolucent fat tissue *(Kumar Vinay 2010)*.

The main hormonal changes that occur during and after menopause are a decline in plasma SSH, in particular - E_2, P and androgen levels *(Labrie 2006)*. Subsequently, the TDLU - the intralobular stroma becomes more collagenous. In addition, the basement membrane of acini become thicker and the size of the acini is reduced *(Walker et al. 2007b)*. These involutionary changes are scattered throughout HBTs. In many women, over 65–70 years, lobules are atrophied and ducts of varying sizes with a prominent myoepithelial layer are seen *(Walker et al. 2007b)*. The amount of fat and stroma present may vary, but basically, the relative amount of adipose tissue has increased.

The proliferative activity of the normal HBTs declines after the menopause *(Walker et al. 2007b)*. *In vivo* studies show that proliferation indices for both acinar and ductal epithelium in premenopausal BC patients were higher than in postmenopausal BC patients *(Christov et al. 1991, Hassan et al. 1998, Shoker et al. 1999a, Walker et al. 2007b)*. The decrease of the proliferative indices in the acini also continues during postmenopause and in women older than 60 years and they are lower than in women of 50-59 years *(Hassan et al. 1998)*.

There is a clear association between the development of BC and age, with 80% of BC occurring in women more than 50 years of age and one-third of BC occurring in women over 70 years old. In postmen-HBTs, though the percentage of proliferating cells is lower, the incidence of dual expression of ERα- and PR positive and the proliferation markers Ki-67-positive increases and is associated with BC risk *(Shoker et al. 1999a, Obr et al. 2012)*. Increased percentages of proliferating ERα/PR positive cells were observed in HBTs adjacent in the tumor area of BC patients, as well in hyperplasias and ductal carcinoma *in situ (Shoker et al. 1999a, Shoker et al. 1999b, Lawson et al. 2002)*. The data indicates that a switch from a paracrine to an autocrine regulation of proliferation leads to pre-neoplastic progeression to BC *(Obr et al. 2012)*.

2.2 Effects of menopausal hormone therapy on human breast

Menopausal hormone therapy (MHT) - is a generic term that refers to any type of hormone therapy used during menopause to alleviate the symptoms caused by cessation of ovarian SSH *(Santen et al. 2010)*. This term is used when studies do not specifically stipulate estrogen alone or combined therapy*, e.g.* estrogen plus progestin or estrogen plus androgen. The term “MHT” includes **HRT** (hormone replacement therapy) and **HT** (hormone therapy) *(Santen et al. 2010)*. Estrogen, alone (estrogen therapy, ET) or in combination with progestins (estrogen-progestin therapy, EPT), has

been widely used for MHT for reducing climacteric symptoms, degenerative changes and improving quality of life *(Grady et al. 1992, Kenemans 1999, Kagan 2012)*.

MHT may consist of a wide variety of both natural and synthetic hormonally active compounds, with many different routes of administration *(Kenemans et al. 2003)* (Table 2). There are three principle regimens for MHT – estrogen only or in cyclic or continuous combination with progesterone. These regimens have marked differences with regard to their effects on the endometrium. Whether such differences apply also to other target organs*, e.g.* the breast, is incompletely understood and controversial *(Conner 2004)*.

Despite the benefits of MHT, MHT may increase the risk of BC. One explanation for this could be that MHT activates and increases epithelial cell proliferation that leads to the neoplastic transformation *(Pike et al. 1993, von Schoultz 2007)*. Results from animal models indicate that even a short term application of MHT increases breast cell proliferation *(Pike et al. 1993, Conner et al. 2003, Conner et al. 2004, von Schoultz 2007)*.

The risk for BC might differ depending on the regimen used, the route of administration applied, and the type of SSH administered. In addition to the classical MHT compounds used, in particular estrogens and progestins, other SSH, such as androgens, are increasingly being used and the risk for BC that will differ from that seen with the classical estrogens and progestins *(Kenemans et al. 2003)*.

2.2.1 Estrogens

ET, including a variety of natural and synthetic estrogens, has been used for more than 60 years. There are different types of estrogens with different characteristics and biological properties. CE and E_2 are regarded as moderately potent, whereas the synthetic preparation ethinylestradiol has high biological potency *(Conner 2007)*. Today, CEs and the native hormone E_2 are the most common substances used for systemic treatment in postmenopausal women.

In addition to the type and dosage of estrogens, the route of administration is also important for metabolism of SSH. After oral administration, the vast majority of the given dose of E_2 is metabolized and excreted *via* the liver. Simultaneously, the organism reacts to it with increased levels of plasma proteins, *e.g.* binding proteins, sex hormone-binding globulin (SHBG), insulin-like growth factor-binding proteins, coagulation factors lipoproteins and triglycerides. After parenteral administration of estrogens, the effects on proteins, lipid synthesis, gall secretion and glucose metabolism are substantially reduced *(Holst et al. 1983, Hemelaar et al. 2003, Scarabin et al. 2003)*.

2.2.2 Progestins

In the mid 1970s, unopposed estrogens (estrogen-alone) use resulted in an increased risk for endometrial cancer. Thus, progestagenic compounds were added to the estrogen combinations, commonly for 10–14 days of each artificial cycle, and a dramatic decline in the incidence of endometrial cancer occurred *(Ziel et al. 1998, Kenemans et al. 2003)*.

Treatment with unopposed estrogens causes a stimulatory effect on the endometrium and, simultaneously, increases the risk of malignant transformation during long-term use. Thus, all postmenopausal women with an intact uterus, have to receive the addition of a progesteronic compound to counteract the estrogenic effect on the uterus. In addition, progestins may also exert an additional agonistic effect to estrogens with regard to relief of hot flushes and sweating as well as an effect on bone metabolism *(Utian et al. 2005)*. Despite these positive effects, progestins cause many of unwanted side effects in women receiving MHT, such as irregular bleeding, breast symptoms, mood disturbances and hair loss *(Bjorn et al. 2000)*. In addition to the natural P, produced and secreted by the ovaries, there are various synthetic progestins, which are generally classified according to whether they are structurally related to testosterone (19-nortestosterone derivatives) or to naturally occurring P. In addition to a wide variation in their progestogenic potency, every progestin differs in its ability to exert either estrogenic, antiestrogenic or androgen-like effects, and in absorption, metabolism, binding to serum proteins and biological effects mediated through binding to the progesterone receptors PR-A and PR-B *(Schindler et al. 2003)*. All progestins have one effect in common – secretory transformation of the E_2-primed endometrium. There are controversial results regarding the action of P on the E_2-induced cellular mitotic activity – some studies show synergistic effects *(Spicer et al. 1992, Pike et al. 1993)*, but others antagonistic *(Pasqualini et al. 1998, Kandouz et al. 1999)*. The antagonistic effects could be explained by downregulation of the ER, activation of metabolic pathways *(Pasqualini et al. 1998)* or stimulation of apoptosis *(Kandouz et al. 1999)*. However, discrepancies exist among experimental studies about the precise role of progestins in breast epithelial cell proliferation and in breast carcinogenesis *(Bergkvist et al. 1996)*. *In vitro* studies in breast cell lines show that E_2 increases cell proliferation, but addition of P has an opposite effect on breast epithelial cells *(Kramer et al. 2005)*. In contrast, *in vivo* studies of the human normal breast show that the proliferative activity of breast epithelial cells is the highest during the luteal phase of MC coinciding with high serum estrogens and P levels *(Meyer 1977, Going et al. 1988, Potten et al. 1988, Olsson et al. 1996, Soderqvist et al. 1997)*.

2.2.3 Androgens

The role that androgens play in BC development, growth and progression is not fully understood *(Kotsopoulos et al. 2012)*. Numerous studies show controversial results regarding this issue. There are two major theories regarding the mechanism underlying the effect of androgens on breast epithelial cell proliferation and BC development [reviewed in *(Shufelt et al. 2008, Dimitrakakis et al. 2009, Kotsopoulos et al. 2012)*]. Androgens could increase BC risk either directly by increasing epithelial cell proliferation *via* the AR or indirectly through their aromatization to estrogens *(Liao et al. 2002)*. In the late 1960's, *Grattarola* proposed "hyperandrogenic theory" based on his observation that BC patients often had elevated blood androgen levels, increased urinary androgen excretion, atypical endometrial hyperplasia, indicative of anovulation and a reduction of luteal P production [reviewed in *(Kotsopoulos et al. 2012)*]. He assumed that ovarian interstitial cell hyperplasia, as a result of increased ovarian androgen production, was associated with a high BC risk [reviewed in *(Kotsopoulos et al. 2012)*]. Secreto et al. later suggested the "androgen excess theory" which states that high circulating levels of androgens are associated with an increased risk of BC in healthy women, in particular for ER-positive cancers [reviewed in *(Kotsopoulos et al. 2012)*].

According to another theory, androgens protect mammary glands from estrogen induced stimulation, followed by increased proliferation and BC development by competitive blockade. There are *in vivo (Zhou et al. 2000)*, *in vitro (Somboonporn et al. 2004b)* and clinical evidence from multiple studies that androgens have an inhibitory effect on the mammary epithelium *(Dimitrakakis et al. 2002, Dimitrakakis et al. 2003, Labrie et al. 2003, Dimitrakakis et al. 2004, Dimitrakakis et al. 2009, Peters et al. 2011)*. *In vitro* studies show the dichotomous effects of androgens, such as antiproliferative and proapoptotic effects of androgens as well as stimulation of cell proliferation [reviewed in *(Somboonporn et al. 2004b)*]. The differences in the results may be explained by different cell lines, dosages and type of androgen used [reviewed in *(Kotsopoulos et al. 2012)*]. *In vivo* data from rodents and non-human primates suggest that T limits the mitogenic and cancer-promoting effects of E_2 on the mammary epithelium [reviewed in *(Somboonporn et al. 2004b)*]. Women taking MHT standard formulations (estrogen-alone or estrogen plus progestin) often experience loss of libido, most likely due to the decline in androgen levels [reviewed in *(Kotsopoulos et al. 2012)*]. The addition of T to estrogen plus progestin, improves the quality of their sexual life and alleviates the menopausal vasomotor symptoms *(Braunstein 2002)*. There is also some evidence from small clinical studies that E_2 in combination with T may restore bone mineral density and improve cognitive function in menopausal women [reviewed in *(Kotsopoulos et al. 2012)*]. The potential side effects of long term androgen use have not been elucidated, but these may include adverse effects on cardiovascular system and lipid profiles, as

well as increased in BC risk. Data regarding androgen effects on the breast and epidemiological data relating to BC risk with E_2/T treatment have led to conflicting conclusions *(Santen et al. 2010)*. However, in one clinical study, T administration significantly counteracted breast cell proliferation induced by E_2/P therapy in postmenopausal women, suggesting that the addition of T to estrogen or combination of E_2/P therapy could attenuate the risk of BC *(Hofling et al. 2007)*.

2.2.4 Selective estrogen receptor modulators (SERMs)

Selective estrogen modulators (SERMs) interact with ER as an antagonist and/or agonist depending on the target tissue *(Pickar et al. 2010)*. Currently available SERMs are used to prevent and/or treat diseases and conditions such as BC, osteoporosis, ovulatory dysfuntions and even used in contraception *(Pickar et al. 2010)* (Tab.4). The ideal SERM characteristics would be ER agonistic activity in tissue, where estrogen action is desirable (*e.g.* bone) and ER neutral or antagonistic activity – where estrogen effects are unwanted (*e.g.* endometrium, breast, *etc.)* (Table 3).

Tamoxifen (TAM) was initially discovered as a nonsteroidal contraceptive *(Pickar et al. 2010)* and later used for BC treatment and is considered to be a highly efficient drug for ER-positive BC treatment *(Taylor 2009)*. TAM provides beneficial effects on skeletal and cardiovascular system, but has a negative impact on endometrium, causing hyperplastic changes.

Raloxifene (RAL) is considered to be the prototypical second-generation SERM *(Taylor 2009)*. Previously, RAL was found to be effective in reducing the incidence of vertebral fractures and BC and did not increase the risk for endometrial cancer. However, RAL is linked to an increased risk for deep venous thromboembolisms and fatal strokes *(Taylor 2009)*.

2.2.4.1 Mechanism of SERM action

SERMs achieve their biological effects through specific, high-affinity interactions with ERs resulting in ligand-ER conformational changes specific for each SERM *(Riggs et al. 2003, Pickar et al. 2010)*. This ligand-receptor complex binds to one of the DNA response elements, specifically to estrogen response element (EREs), located in the promoter regions of E_2 target genes, triggering the mRNA and synthesis process. Depending on SERMs, cell type, gene regulatory regions as well the ratio between co-activator and co-repressor proteins, the ligand-ER complex may activate or repress transcription of the target gene. Co-activators bind non-uniformly to the dimerized ligand-ER complexes depending on their unique structure *(Riggs et al. 2003, Pickar et al. 2010)*. The relative concentrations of members of the co-activator family such as (SRC-1, -2 or -3) or co-repressors may regulate the response of a tissue to ERα *(Jordan 2004)*.

SERMs regulate the levels of SRC-1 and SRC-3 and they can affect the transcriptional activity of other genes than ER members of nuclear receptor family *(Lonard et al. 2004)*. Recent gene profiling studies demonstrate that various SERMs differentially regulate many ER-regulated genes *(Berrodin et al. 2009, Chang et al. 2010)*. The gene regulation by individual SERMs ultimately contributes to the different cell- and tissue-specific activities of SERMs *(Riggs et al. 2003, Pickar et al. 2010)*.

2.2.4.2 SERMs as MHT alternative

Unfortunately, SERMs have not fully met the goals set for an "ideal MHT" - a compound that prevents fractures, reduces vasomotor symptoms, and treats vaginal atrophy and its symptoms, while being neutral or protective for normal breast, uterus, or cardiovascular system (Table 4) *(Pickar et al. 2010)*. The current clinical utility of the TAM and RAL is limited to postmenopausal women, because both increase the risk of developing ovarian cysts in premenopausal women. In addition, these compounds may have adverse effects on pregnancy and fetal development *(Buelke-Sam et al. 1998, Inal et al. 2005, Premkumar et al. 2007, Peano et al. 2009)*. In regard to MHT, SERMs have potential to treat most of the symptoms of menopause caused by reduced levels of estrogens, but currently marketed SERMs as well as the ones in late stage of development are lacking a key feature which is that they fail to reduce vasomotor symptoms (*i.e.* hot flushes and night sweats), and they can occasionally exacerbate them. Because vasomotor symptoms are the primary reason that women seek treatment at menopause, there is still a need to develop better therapies for symptomatic women *(Peano et al. 2009)*. Even though some studies indicate that a few SERMs are currently being studied for possible future use as MHT, their potential to be an optimal SERM for the postmenopausal woman has not been demonstrated.

One of the SERMs, in development, is ospemifene (OSP) for the treatment of vulvar-vaginal atrophy occurring in menopause. Results from phase 2 studies indicate that OSP has significant estrogenic effects on the vaginal epithelium. OSP also dose dependently decreases markers of bone turnover *(Komi et al. 2004)*, without endometrial hyperplasia or exacerbation of hot flushes and had an overall neutral cardiovascular impact *(Ylikorkala et al. 2003, Komi et al. 2005)*. Experimental *in vivo* studies have showed that OSP does not increase BC risk in animals, in contrast, it blunted cancer development and progression *(Wurz et al. 2005, Burich et al. 2012)*. Direct effects on human breast of OSP are unclear.

2.2.4.4 Tissue Selective Estrogen Complex (TSEC)

Recently, SERMs, in combination with estrogen(s) for use in postmenopausal women as MHT, are being investigated. This combination of a SERM with estrogen(s) is called: "a tissue selective estrogen complex (TSEC) which was designed to achieve a more favorable clinical profile based on the blended tissue selective activity profiles of the components" *(Pickar et al. 2010)*. The idea of TSEC is to exploit the capacity of low levels of estrogens to inhibit vasomotor symptoms and the capacity of selected SERMs to have beneficial tissue specific effects and oppose adverse effects of estrogens. "An optimal or ideal TSEC would diminish hot flushes, treat vaginal atrophy and its symptoms, and prevent loss of bone mass, without stimulating the breast or endometrium" *(Pickar et al. 2010)*. TSECs show tissue-specific activities as the result of ligand binding, specific conformational changes in ligand-ER complex, various cofactor recruitments, and subsequent changes in gene expression *(Pickar et al. 2010)*. In addition to differential responses for individual SERMs, the RAL, lasofoxifene, and bazedoxifene have unique effects on gene expression profiles in combination with CE, suggesting that different pairings will have different effects in the clinic *(Berrodin et al. 2009, Chang et al. 2010, Pickar et al. 2010)*. Microarray analyses and MCF-7 cell proliferation assays show that certain SERMs, in particular raloxifene, lazoxifene and bazedoxifen, have significant antagonistic effect against CE-stimulated cell proliferation and gene expression, with the order of antagonist activity being bazedoxifene > RAL > lasofoxifene. These results indicate that SERMs, combined with CE, exhibit differential pharmacological profiles *(Chang et al. 2010)*.

Table 2. MHT compounds, formulations and administration routes.

MHT formulations	Route of administration	Active compounds
ESTROGEN	Oral (Tablets)	CE A CE B CE Estradiol Estradiol micronized Esterified estrogens Estropipate
	Transdermal (Gel - Patch – Spray)	Estradiol
	Vaginal (Cream – Ring – Tablets)	CE Estradiol
PROGESTERONE	Oral	Norethindrone acetate Progesterone acetate Medroxyprogesterone acetate
ESTROGEN/ PROGESTERONE	Oral	Estradiol+ norethindrone acetate Estradiol + drospirenone Ethinyl estradiol + norethindrone acetate CE + norethindrone acetate
	Transdermal (Patch)	Estradiol + levonorgestrel Estradiol+ norethindrone acetate
ANDROGEN	Oral	Methyltestosterone (Android-10) Fluoxymesterone (Halotestin, Fluoxymesterone)
	Parenteral	Testosterone enanthate (Delasteryl) Testosterone cypionate (Depo Testosterone)
ESTROGEN/ANDROGEN	Oral	Esterified estrogens + methyltestosterone (Estratest)
OTHERS		
Livial	Oral	Tibolon
Gynodian-depot	Parenteral	Estradiol valerate + prasterone enanthate
Abbr.: **CE – conjugated estrogen**		

Table 3. Various SERMs, indications and current status of investigation or clinical use.

SERMs	Indications	Clinical Use
I. Benzopyrans 1. Levormeloxifene 2. Ormeloxifen 3. Acolbifene	1. The osteoporosis treatment 2. An oral contraceptive 3. Prevention and treatment of established mammary tumors in animal models	1. Discountinued 2. In use 3. Phase 2 and 3
II. Benzothiophenes 1. Raloxifene 2. Arzoxifene	1. Prevention of osteoporosis and invasive BC and treatment of osteoporosis in postmenopausal women 2. The prevention and treatment of BC	1. Phase 2 2. Phase 3
III. Chloroethylene 1. Clomiphene	1. The treatment of ovulatory dysfunction in women desiring pregnancy	1. In use
IV. Indols 1. Pipendoxifene 2. Bazedoxifene	1. The treatment of TAM resistant metastatic BC. 2. The prevention and treatment of postmenopausal osteoporosis	1. Phase 2 2. Phase 3
V. Naphtalene 1. Lasoxifene	1. The prevention of osteoporosis and the treatment of osteoporosis and VVA in postmenopausal women.	1. Discountinued
VI. Steroidals 1. Fulvestrant 2. HMR3339	1. The treatment of ER-positive metastatic BC in postmenopausal women 2. Preclinical studies suggest a beneficial effect on vasomotor symptoms and the cardiovascular system, on bone and a good safety profile on the breast and uterus.	1.In use 2. Awaiting long clinical trials
VII. Triphenylethylenes 1. Tamoxifen 2. Toremifene 3. Droloxifene 4. Idoxifene 5. Ospemifene	1. Treatment of metastatic BC; adjuvant treatment of BC; reduction of invasive BC risk in women with DCIS after surgery and radiation; reduction of BC incidence in high-risk women 2.The treatment of metastatic BC in postmenopausal women with ER positive or unknown tumors 3. The treatment of the BC 4. The treatment of the BC 5. The treatment of VVA	1. In use 2. In use 3. Discountinued 4. Discountinued 5. Phase 3
Abbr.: BC- breast cancer; TAM - tamoxifene; VVA - vulvar vaginal atrophy		

Table 4. Effects of an ideal SERM, MHT and currently available SERMs in different tissues (adapted from *(Wend et al. 2012)*).

Compound	Breast	Bone	EM	GUT	CVS	HF
Ideal SERM *(Wend et al. 2012)*	Antagonist	Agonist ↓BMD	Antagonist	Agonist prevents VVA	Antagonist ↓ CVS diseases, stroke and VTE	Agonist prevents HF
MHT *(Santen et al. 2010 ; Wend et al. 2012)*	Agonist ↑BC risk	Agonist			Agonist ↑stroke, VTE	Agonist prevents HF
Tamoxifen *(Morello et al. 2002; Wend et al. 2012)*	Antagonist	Agonist	Agonist ↑EC			Antagonist
Raloxifene *(Collins et al. 2009 ; Wend et al. 2012)*	Antagonist	Agonist	Antagonist		Neutral	Antagonist
Bazedoxifene *(Wend et al. 2012)*	Antagonist	Agonist	Antagonist		Agonist	Antagonist
Lasofoxifene *(Cummings et al. 2010)*	Antagonist	Agonist	Antagonist		Agonist ↑VTE	Antagonist
Toremifene *(Vogel et al. 1993 ; Pyrhonen et al. 1999 ; International Breast Cancer Study et al. 2004 ; Tiitinen et al. 2004 ; Harvey et al. 2006)*	Antagonist	Antagonist				
Ospemifene *(DeGregorio et al. 2000; Voipio et al. 2002 ; Komi et al. 2004 ; Komi et al. 2005 ; Namba et al. 2005; Wurz et al. 2005 ; Komi et al. 2006; Burich et al. 2012)*	Antagonist *	Agonist	Neutral	Agonist		Neutral

* - data available only from rodent studies;
Colors indicate the effects : Antagonist/Agonist - *beneficial effects* and Antagonist/Agonist - *negative effects*
Abbr.: BMD - bone mineral density; BC - breast cancer ; CVC- cardiovascular system; EC_endometrial cancer; EM – endometrium; GUT – genitourinary tract; HF - hot flashes; VTE - venous tromembolic events; VVA – vulvovaginal athrophy

Discontinued SERMs – arzoxifene, droloxifene, idoxifene and levormeloxifene due to deleterious effects on the GUT, ↑HF and VTE

2.3 Experimental models for studies of the effects of MHT on breast tissue

Many methods and models exist to study the effects of hormonal treatment on the morphology and function of the micro-architecture and cell turnover in the human breast. Each "has its limitations in terms of validity in offering samples of normal tissue as well as being repeatable or not during the course of treatment" *(Conner 2007)*.

Clinical methods can be invasive, *e.g.* core needle biopsy and fine-needle biopsy (FNA), or noninvasive, *e.g.* mammography. In different studies, the participants in with changes are recorded and may vary from healthy volunteers to surgical patients operated on for mammoplasty reduction, MHT or oral contraceptive use, benign lesions or BC. The "ideal" method for elucidation of the effects of MHT on normal HBTs is a large-scale clinical study in a normal human population.

Preclinical studies consist of *in vivo* using experimental animals and *in vitro* studies using cell culture or tissue explants. *In vivo* studies, although helpful, have their limitations *(Burdall et al. 2003)* due to the biological differences, for instance, between human and rodent mammary glands *(Zhuang et al. 2003)*. Studies on non-human primates, with a similar reproductive physiology, mammary gland anatomy and peripheral steroid hormone metabolism *(Cline et al. 1998, Isaksson et al. 2003, Stute et al. 2004)* can also be used, however, these studies are very expensive.

Models used to grow and study human breast epithelium include, epithelial cell culture, mammary epithelial and stromal cell co-culture and organ culture *(Parmar et al. 2004)*. Cell culture systems use two main categories of cells – immortalized BC cells and primary cells directly obtained from human tissues. The vast majority of breast cell experiments are done using various types of BC cells. For many years, experimental studies with normal and BC cells contribute to the information concerning hormonal effects. These demonstrate the well-known proliferative effects, induced by estrogens that can be counteracted by the addition of progestogens *(Gompel et al. 1986)*. This has been presumed to be valid also in the clinical setting. The cell culture milieu, however, lacks stromal components, such as adipose and connective tissue, which play an important role in paracrine signaling.

Organ culture is the maintenance or growth of tissue pieces or the whole organ *in vitro* to study differentiation, preservation of the architecture, function and responses to external stimuli. The culture of any normal tissue "depends on a continuous series of cellular interactions in a microenvironment composed of various growth factors, hormones, and adhesion molecules as well as a complex extracellular molecular matrix" *(Kim et al. 2004)*. To successfully investigate the physiology of normal human breast and pathobiology of human BC, it is necessary to maintain or recreate the characteristic three-dimensional architecture of the breast tissue *in vitro (Kim et al. 2004)*. Thus, organ culture has an advantage compared to cell lines that the normal

architecture of epithelium is retained and epithelial-stromal interaction is maintained. Thus, the tissue-specific hormonal effects could be maintained more properly in tissue explant cultures compared to cultured cell lines.

3 AIMS OF THE STUDY

The purpose of the thesis was to investigate the proliferative, differentiating, and marker expressional effects on human breast tissue (HBTs) *in vitro* that 17β-estradiol (E_2), medroxyprogesterone acetate (MPA), testosterone (T), dihydrotestosterone (DHT) and SERMs (OSP, RAL and TAM) exert.

The specific aims of the study were:

1. To establish the conditions of an *in vitro* model, in which HBTs could be cultured for a long period, while still possessing most of the biological properties and responding to exogenous steroid hormone stimulation.

2. To characterize the proliferative and differentiating effects of E_2, MPA, androgens (DHT and T) and the SERMs: OSP, RAL and TAM on normal human breast epithelium.

3. To investigate the expression and regulation of specific markers as a response to hormonal stimulation.

4 MATERIALS AND METHODS

4.1 Patient groups and collection of breast tissue samples

HBTs were collected from women (*n*=72) aged 33–74 years undergoing either reduction mammoplasties or BC surgery at the Turku University Hospital, Finland (Table 5). None of the women had used MHT within 6 months before surgery. Normal tissues from BC patients were taken from the border area between normal tissue and excised tumor tissue. Histologically, these tissue pieces contained normal epithelium.

The tissues were transported to the laboratory in cold, phenol red-free DMEM/F12 medium (GIBCO, England) supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml). The cultures were started within 1–2 h of surgery. The tissues were cut in DMEM/F12 medium. First, breast tissue was carefully dissected under a stereo-microscope to exclude as much of the adipose tissue as possible, saving the collagenous connective tissue, since the ducts and lobules are found mostly in the dense collagenous stroma. The collagenous connective tissues were cut with fine scissors into pieces of approximately $2 \times 2 \times 2$ mm^3.

Table 5. Patient groups and breast tissue samples collected.

Groups	***n***	**Age** mean and range	**Status**	**Type of breast surgery**	**Type of tissue used**
Group I	3	36.6 (33–40)	premenopausal	reduction mammoplasties	normal
Group II	57	57.3 (55–64)	postmenopausal	reduction mammoplasties	normal
Group III	12	64.4 (60–74)	postmenopausal	breast tumor surgeries	peritumoral

4.2 Ethics

The study protocol was approved by the Joint Ethics Committee of Turku University and Turku University Central Hospital. Informed written consent was obtained from all patients before surgery.

4.3 Organ culture of human breast explants (I-IV)

The cell culture method of Trowell was used, with some modifications as described previously *(Trowell 1959, Martikainen et al. 1983, Nevalainen et al. 1997)*. Four to seven pieces of breast explant were transferred onto lens papers lying on stainless steel grids in Petri dishes and cultured for different periods. The explants were kept in a humidified atmosphere in mixture of 5% CO_2 and 95% air at 37ºC in phenol–red-free DMEM/F12 medium (GIBCO, Paisley, UK) supplemented (10%) with dextran-charcoal-stripped fetal calf serum (10%), penicillin (100 IU/ml), streptomycin (100 µg/ml), 0.5 ml ITS supplement (Sigma, Steinheim, Germany), 100 nM hydrocortisone (Sigma) and EGF (Sigma) at 10 ng/ml. This medium is referred to as basal medium. For the different experiments, the variety of SSH and SERMs were added to the basal medium: estradiol (E_2) (Sigma) at 10 nmol/L, 100 nM MPA (Sigma), testosterone (T) (17β-Hydroxy-3-oxo-4-androstene, *Sigma*) and dihydrotestosterone (DHT) (5α-androstan-17β-ol-3-one, *Fluka Chemie Ag*) at 1 nM, all SERMs (OSP, RAL and TAM) (they were kindly provided by Hormos Medical, Turku, Finland). Some compounds (*e.g.* MPA) had poor solubility in ethanol so all compounds were dissolved in dimethylsulfoxide (DMSO; Sigma). Control tissues were cultured in basal medium supplemented with DMSO. The medium was changed every second day and fresh steroid solutions were added. Two to three parallel dishes with samples obtained from each patient were cultured for every treatment group and every time point. The final concentration of DMSO in the culture medium was 0.03%. Explants were collected at 7, 14 and 21 days after the beginning of the culture.

4.4 Analyses of the morphology of breast tissue explants

4.4.1 Histology of human breast explants

Non-cultured (day 0) and cultured HBTs were fixed in 4% paraformaldehyde in phosphate buffer (PBS) overnight (o/n) at +4 °C, then dehydrated and embedded in paraffin according to routine procedures. Paraffin sections of 5 µm were cut from each piece and stained with hematoxylin and eosin (H&E) for histological examination and from same paraffin blocks, sections were cut for further analyses.

4.4.2 Morphometric analysis

All morphometric measurements were done on H&E-stained slides, using OsteoMeasure™ system (OsteoMetrics, Inc., Atlanta, USA). Morphometric measurements of ducts and acini were performed with the help of digitizing interactive video overlay drawing system run by this morphometry program. The acinar and ductal

profiles were measured by outlining the images on the monitor screen. In each sample, eight microscopic fields (four for ducts and four for acini) were randomly selected and examined at ×200 magnifications. The breast structures were quantified by digital tracing, and the wall thickness (µm) of ducts and acini were measured with exclusion of the lumens.

4.4.3 Whole Mounts

HBT was spread onto a glass slide, fixed in a 1:3 mixture of glacial acetic acid/100% ethanol, hydrated, stained overnight in 0.2% carmine and 0.5% $AlK(SO_4)_2$, dehydrated in graded solutions of ethanol, and cleared in xylene.

4.5 Immunohistochemical stainings

Immunohistochemistry was carried out using 4% paraformaldehyde-fixed, sections were cut from paraffin-embedded tissue samples sectioned at 5 µm and mounted on SuperFrost slides (Menzel-Glaser, Braunschweig, Germany). The tissue sections were first deparaffinized in xylene and rehydrated through graded ethanol. Antigen retrieval was achieved by heating the samples in 0.01 M citrate buffer (pH=6.0) with 0.5% Tween-20 (pH=6.0) for 15 min using a microwave oven or by using Digest-All ™ (Zymed) working solution for 20 min at 37 °C. Endogenous peroxidase activity was quenched by immersing the sections in 3% hydrogen peroxide (H_2O_2) in methanol for 20 min. The sections were then blocked with normal 2% horse or goat serum (depending on the primary antibody source) at RT for 30 min following incubation with Avidin/Biotin Blocking kit reagents (Zymed Laboratories Inc., CA) in accordance with the manufacturer's instructions (Table 3). The tissue sections were incubated with either mouse or rabbit primary antibodies at various dilutions overnight at +4 °C. For all subsequent washes and for dilution of the antibodies, 0.01 M PBS (pH=7.4) was used. A slide detected positive for antigen was used as a positive control, and a slide incubated without primary antibody was used as a negative control for each staining batch. The next day, after a further wash, biotinylated horse anti-mouse or goat anti-rabbit secondary antibodies (1:200, Vector, USA) were applied for 2 hours at RT. A Vectastain Elite Avidin-Biotin Complex kit (Vector Laboratories, Burlingame, CA) was used as the detection system and color development was carried out by using 3,3-diaminobenzidine (DAB kit, Vector Laboratories, CA). Counterstaining was undertaken with Mayer's hematoxylin, with a thorough rinse in distilled water, and then the tissue slides were briefly immersed in a bath of 0.037 M ammonia as bluing agent. Finally, all sections were dehydrated through graded ethanol, then incubated in xylene and mounted with Mountex (HistoLab; Gothenburg, Sweden).

All immunostainings were quantified in specific breast tissue structures by counting the proportion of immunostained cells in glandular compartments (inter- and intralobular ducts or terminal ductules and lobules). Only normal areas of breast tissues were evaluated. Areas with pathological changes (fibrocystic changes, hyperplasia, microcalcifications, and metaplasia) were excluded from the analysis. The expression of all markers was evaluated only in glandular epithelium – luminal epithelial and myoepithelial cells.

The pattern of immunostaining was nuclear or cytoplasmic for markers used in this study. For quantification of immunostaining an eyepiece grid of 10×10 squares at ×200 magnification was used to select positive cells for counting. Cells were considered positive if any DAB precipitate was observed in the nucleus. The immunostainings were evaluated according to the percentage of positive-staining cells and staining intensity in the nucleus. For assessment of the percentage of positive cells a Leica microscope connected to a computer, and Color vision software (Leica imaging System Ltd., Cambridge, UK), were used. Eight to ten fields of glandular tissue (epithelial cells – acinar and ductal cells) were assessed in each tissue section, excluding stromal cells. For each sample, as described previously, approximately 1,000 epithelial cells were scored across randomly selected areas of the tissue section to reach more reliable values *(Sadi et al. 1991, Hofseth et al. 1999)*. The scores of immunohistochemical markers were calculated as percentages of positive cells (100 × positive cells/total number of epithelial cells), presented as pooled data (acinar + ductal epithelial cells).

4.6 Western Blot Analyses

Cultured frozen HBT was homogenized in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH=8.0). The protein concentration was determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, CA). Next, 50 µg of protein was heated to 108°C for 5 min in Laemmli sample buffer with 2β-mercaptoethanol and loaded onto a 12% SDS-PAGE gel. After gel electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad). The primary antibodies used for the western blots are presented in Table 5. The secondary goat anti-mouse IgG-horseradish peroxidase (Bio-Rad) was used at a dilution of 1:10000. Detection was performed using ECL-Plus (Amersham, Piscataway, NJ). Band intensities were quantified using MCID Version 5.0 software and results are expressed as relative density of target/tubulin (loading control) ratios.

4.6 qRT-PCR

Total RNA from the frozen HBT was isolated using the TRIsure reagent (Invitrogen, USA) according to the manufacturer's instructions. 1 µg of total RNA was reverse transcribed with AMW Reverse Transcriptase (Finnzymes). The cDNA was diluted 1:10

in nuclease-free water, and 8 µl was analyzed in duplicate by RT-PCR in an CFX384™ RT-PCR Detection System (Bio-Rad, USA) using the DyNAmo™ SYBR® Green qPCR Kit (Finnzymes) with 0.4 µmol/l of each primer (Oligomer, Finland) (Tab.6) in a reaction mixture with a total volume of 20 µl. The negative controls contained water in place of first strand cDNA. The relative gene expression levels were normalized to a calibrator that was chosen to be the control group. The final results were expressed as *n*-fold differences in gene expression relative to keratin-18 and the control. The results were calculated using the $\Delta\Delta C_t$ method as follows: $n\text{-fold} = 2^{-(\Delta Ct\text{sample}-\Delta Ct\text{calibrator})}$, where the ΔCt values of the sample and controls were determined by subtracting the average Ct value of the housekeeping gene from the average Ct value of the different genes analyzed.

Table 5. Primers used in this study

Human Gene	Primers	Annealing temperature
AR	5'-CGTGGGGCGCCCCAGGCACCA-3' 5'-TTGGCCTTGGGGTTCAGGGGG-3'	60° C
β-actin	5'-CGTGGGGCGCCCCAGGCACCA-3' 5'-TTGGCCTTGGGGTTCAGGGGG-3'	60°C
Cyclin-D1	5'-TGCCAGAGGCGGAGGAGAACAA-3' 5'-GGAGGCAGTCCGGGTCACACT-3'	60°C
ER alpha	5'-TGGAGATCTTCGACATGCTG-3' 5'-GCCATCAGGTGGATCAAAGT-3'	62°C
Keratin-18	5'-ACAGTCTGCTGAGGTTGGAGCT-3' 5'-TCCAAGCTGGCCTTCAGATTTC-3'	60°C
TFF1	5'- GGTGTCCATGCTGGCCCTCG-3' 5'-ACACTCCTCTTCTGGAGGGACGTC-3'	60°C

4.7 Statistical analyses

Statistical analysis was performed using general linear mixed models and ANOVA. The effect of the treatment and time were also tested. First, various biomarker values (*e.g.* Ki-67, PCNA, ERα and etc.,) obtained from non-cultured samples (day 0) were compared between treatment groups. Next, the correlation of variables (various biomarkers) with time (certain days *in vitro*), treatment groups (control, E_2, *etc.*) and interaction between day of culture and treatment were used to estimate the significance. The variables obtained from cultured explants were pooled and used for calculation of means and standard deviations. In the analysis of biomarker values – proliferation markers, apoptosis markers, steroid hormone receptors, *etc.* groups and timepoints were fixed factors and the

subjects were random factors. This analysis was carried out separately for each time point depending on the experiment. The covariance structure for random effect was considered to be compound symmetry. Assumptions were checked using the analysis of residuals. All the results are expressed as means±SD and p-values less than 0.05 were considered to be significant. The Tukey–Kramer method was used to adjust the P-values of the pairwise comparisons, and the residuals were checked for justification of the analysis. The data are expressed as mean±SD. P-values less than 0.05 were considered to be statistically significant. Statistical analyses were carried out using the SAS/STAT (r) software, Version 9.1.3 SP4 of the SAS System, IBM SPSS Statistics version 19 and 20 for Windows.

Table 6. Primary antibodies used in this study.

Primary antibodies	Origin and clone	Manufacturer	Application	Dilution	Staining Pattern
Amphiregulin (AREG)	Mouse MoAb, Z432	SantaCruz, USA	WB	1:5000	Cytoplasmic
Apolipoprotein D (ApoD)	Mouse MoAb, 36C6	Novocastra, UK	WB IHC (HIER²)	1:5000 1:100	cytoplasmic
Androgen receptor (AR)	Rabbit PolAb, N-20	SantaCruz, USA	IHC (HIER²)	1:100	nuclear
anti-Cytokeratin 8&18	Rabbit PolAb,	Zymed, USA	EER	1:250	cytoplasmic
anti-PCNA	Mouse MoAb, PC10	Zymed, USA	IHC (HIER²)	1:200	nuclear
anti – pS2 peptide (TFF1)	Mouse MoAb, BC04	Zymed, USA	IHC (HIER²)	1:50	cytoplasmic
Anti-Estrogen Inducible Protein pS2	Rabbit MoAb, EPR3972	Abcam, USA	IHC (HIER²)	1:50	cytoplasmic
Caspase-3 (CPP32)	Mouse MoAb, 3CSP031	NeoMarkers, USA	IHC (HIER¹)	1:100	cytoplasmic
Cyclin D1	Rabbit PolAb, SP4	NeoMarkers, USA	IHC (HIER²)	1:50	nuclear
Cytochrome 450 aromatase (CYP19A1)	Mouse MoAb, H4	AbD Serotec	WB, IHC (HIER²)	1 :50 1:250	cytoplasmic
Estrogen Receptor-alpha (ERα)	Mouse MoAb, 1D5	Dako , Denmark	IHC (HIER²)	1:400	nuclear
Estrogen Receptor-beta (ERβ)	Mouse MoAb, EMR02	Novocastra, UK	IHC (HIER²)	1:800	nuclear
Keratin-14 (K-14)	Mouse MoAb, LL002	NeoMarkers, USA	IHC (HIER²)	1:400	cytoplasmic
Ki-67	Mouse MoAb, MM1	Novocastra, UK	IHC (HIER¹)	1:100	nuclear
Phospho-Histone H3 (PHH3)	Rabbit PolAb, Ser10	Cell Signaling,USA	IHC (HIER²)	1:100	nuclear
p21 $^{WAF1/Cip1}$	Mouse MoAb, DSC-60.2	NeoMarkers, USA	IHC (HIER²)	1:50	nuclear
p27 ^{Kip1}	Rabbit PolAb,	NeoMarkers, USA	IHC (HIER²)	1:50	nuclear
Progesterone receptor (A/B)	Mouse MoAb 16 & SAN27	Novocastra, UK	IHC (HIER²)	1:100	nuclear
Prostate specific antigen (PSA)	Rabbit PolAb, A0562	Dako, Denmark	IHC (HIER²)	1:800	cytoplasmic
Smooth muscle actin (SMA)	Mouse MoAb, 1A4	NeoMarkers, USA	IHC (HIER²)	1:200	cytoplasmic

MoAb – monoclonal antibodies; PolAb – polyclonal antibodies; WB – western blot; IHC – immunoshistochemistry; HIER¹-heat-induced epitope retrieval using a microwave own in 10mM Citric buffer (pH=6.0); HIER²-heat-induced epitope retrieval using a microwave own in 10mM Citrate buffer (pH=6.0); EER-enzymatic epitope retrieval

5 RESULTS AND DISCUSSION

5.1 Organ culture model for HBTs (I-IV)

I*n vitro* techniques, which evolved over the decades, are used to study mammary gland biology. These involve mechanical or enzymatic disaggregation of the breast tissue to form a cell suspension *(Hood et al. 1998)*. However, it is often difficult to localize molecular events to specific cellular components, such as epithelial and stromal cells *(Hood et al. 1998)*. The proliferative conditions and morbidities (hyperplasia, cancer, *etc.*) of the female endometrium and mammary gland correlate to hyperestrogenic states and/or use of exogenous estrogens or their combinations, thus methods, allowing evaluation of SSH effects on epithelial proliferation *in vitro,* are of particular clinical relevance *(Blauer et al. 2005)*.

The culture model, which is meant to mimic normal HBT responses, should include both the epithelial and stromal part of the tissue. Tissue cultures represent an *in vitro* model in which tissue integrity is well preserved. These models readily lend themselves to biochemical and histochemical examination and could be used to study effects of various chemical components *(Blauer et al. 2005)*. Due to the paucity of *in vitro* models for the HBTs, that will able to recapitulate steroid hormonal responses, defined culture conditions were used for this purpose. In this work, these conditions were established and the *in vitro* effects of SSH and SERMs studied on histology, differentiation, steroid hormone responsiveness markers, expression of steroid hormone receptors and cell cycle proteins, proliferation and apoptosis ratios in cultured HBTs. The study of the biology of normal HBT in intact mammary tissue was difficult because of the lack of a proper model. Normal human mammary gland tissue can be implanted into athymic nude mice *(Clarke et al. 1997c)*. By using this method, the proliferation, differentiation, and expression of ER and PR in human mammary gland were observed. Compared with the nude mice model, the long-term culture system reported here is relatively simple and cheaply carried out. This method also excludes any effects of a host animal on explants of human mammary tissues.

The culture periods of 7 and 14 days were mainly used, since previous experiment proved those time points to be long enough to study the effects of hormone addition to be clearly expressed and reliably evaluated. The 10 nM concentration of E_2 was chosen based on previous studies, which determined effective concentrations of these steroid hormones *in vitro (Zhuang et al. 2003)*. Pilot testing was done to determine the optimal concentration for studying the biological effects of androgens (DHT and T) and SERMs, in particular OSP, RAL and TAM on HBT. In further experiments, only effective doses were used.

5.2 Hormone regulation of HBTs

5.2.1 Histology of HBT explants treated with different compounds (I-IV)

Histological examination revealed that epithelial compartment of HBTs and stroma had retained their morphological structures throughout the culture period. HBT histology differed in the appearance of the epithelium between the different treatment groups. The acini of HBTs before and after the culturing in basal medium were small, compact and similar in appearance and the epithelial cells were arranged in two layers. By contrast, E_2 induced cell proliferation on explants as the epithelium often appeared hyperplastic, stratified and there was a loss of distinction between epithelial and myoepithelial cells. The size of the cells and the number of cell layers was increased. There was also a tendency of epithelium to overgrow and obliterate the basement membrane. In the explants cultured with MPA, the acini were large and the lumina were filled with secretion. In the explants cultured with E_2/MPA, the number of epithelial cell layers, in the ducts, was increased.

The HBTs cultured in the presence of 1 nM DHT and T maintained the glandular structure and integrity as well as showed morphological responses to androgens. In addition, HBTs expressed androgen stimulated proteins, such as Apo-D and PSA. After the combined treatment (E_2/DHT or E_2/T), the regressive changes observed in the presence of DHT or T, were prevented. The epithelium remained cuboidal, regular and organized, and the epithelial cells retained their polarity and height. Interestingly, HBTs treated with E_2/T had large luminae filled with secreted fluid.

OSP, RAL and TAM caused dose-dependent morphological changes of HBT structure. Explants treated with 1 nM of OSP, RAL and TAM had similar morphology as the controls retaining an epithelium that remained cuboidal, regular and organized, and the epithelial cells retained their polarity and height. HBTs treated with 10 nM of OSP, RAL and TAM had smaller acini, the cells were arranged in two layers only in OSP group, but the acini of HBTs treated with RAL and TAM were composed from one layer. A high dose (100 nM) of all SERMs, especially TAM, caused prominent involutive effects on the lobular structure of explants cultured for 14 days – acini were small and the lumens were not seen.

5.2.2 Histomorphometric analysis of HBT explants

Two morphometrical parameters - acinar and ductal wall thicknesses – were measured to evaluate whether hormonal treatment induced epithelial changes. Histomorphometrical analysis showed that the areas of breast epithelium (acini and

ducts) differed among treatment groups. According to previous studies *(Cline et al. 1998, Hofseth et al. 1999)*, similar histomorphometrical analysis was done on human and macaques mammary glands treated *in vivo* with estrogen and MPA. These studies demonstrate that glandular tissue areas were greatest in CE/MPA group *(Cline et al. 1998, Hofseth et al. 1999)*. These results are contrary to the ones obtained in this study, because it was found that the highest epithelial thickness was in the explants treated with E_2 only. Additionally, we evaluated the effects of androgens (DHT and T) on the thickness of acinar and ductal walls. Both androgens decreased the acinar and ductal wall thickness, significantly.

5.2.2 Effects of E_2 and MPA on proliferation in cultured HBTs (I)

The Ki-67 and PCNA are proliferation markers and their expression in cultured HBT was evaluated. In general, the results with PCNA were comparable to the Ki-67 results. The percentage of cells expressing PCNA and Ki-67 in HBTs treated with E_2 was significantly greater when compared to control group at days 7 and 14, as well with other treatment groups, such as MPA and E_2/MPA, showed also significant differences compared to control groups. Although the percentage of proliferating cells for the E_2 group were the highest after 7 and 14 days of culture, there were no statistical differences among the treatment groups E_2 *vs.* MPA, E_2 *vs.* E_2/MPA or MPA *vs.* E_2/MPA. Experimental studies report conflicting results regarding proliferation ratios in breast epithelial cells based on Ki-67 and PCNA immunostainings. Several reports show that E_2 increased breast epithelial cell proliferation in a more pronounced way than MPA or E_2/MPA. One clinical study, where postmenopausal women received topical treatments with gels containing placebo, E_2, progesterone (P) or E_2/P applied directly to their breasts for two weeks, showed that E_2-alone increased PCNA-positive cells in breast epithelium 100-fold, P-alone-15-fold and E_2/P combination-13-fold *(Foidart et al. 1998)*. *In vitro* results from this study are in accordance with these observations.

In this study, the proliferative response to SSH treatment, especially to E_2, was similar to that shown by other groups *(Laidlaw et al. 1995, Clarke et al. 1997a)*. In their *in vivo* study, pieces of normal HBTs were transplanted into immune-compromised mice, which were treated with E_2 and progesterone with doses to achieve the concentrations observed in the follicular or luteal phase of woman's menstrual cycle. The conclusion was that the luteal phase concentration of E_2 stimulates epithelial cell proliferation in HBTs xenografts, but treatment with P alone had no effect *(Clarke et al. 1997a)*.

A variety of methods and markers are used to identify proteins that are involved in regulation of cell proliferation. Ki-67 and PCNA recognizing antibodies can be used to visualise proliferating cells and both of these are widely used to measure relative levels of proliferative activity in rodents and human tissues. Ki-67 and PCNA were

used to obtain two measures of proliferation that could be compared to each other and to existing results *(Hofseth et al. 1999)*. Both of these markers are expressed in cycling cells, however, there are important differences in expression between them. However, there are differences in their expression pattern. Differences between the raw values obtained with PCNA and Ki-67 antibodies could be attributable to the following reasons *(Hofseth et al. 1999)*. Ki-67 is present in the mid- G_1, during S and G_2, with highest expression seen in M phase and rapid degradation at the end of M phase *(Nguyen et al. 2000)*. PCNA is detectable during the M phase to G_0 and/or G_1, and it has long half-life (Nguyen et al. 2000). This may explain why PCNA is more frequently expressed than Ki-67*(Nguyen et al. 2000)*.

According to some reports, oral E_2-alone associates with lesser proliferative effects than E_2/MPA in combination *(Cline et al. 1998, Hofseth et al. 1999)*. The discrepant results of present study and previous *in vitro* and *in vivo* findings may be in the differences in routes of administration and dosages of SSH used. After oral administration, hormones are metabolised in liver and in the intestinal tract to less active metabolites *(Kuhl 2005)*. After topical administration of gels, creams or patches containing sex hormones, peripheral aromatisation in fat and breast tissues may occur. On the other hand, when hormone containing gels were applied directly on breast skin, a high tissue concentration of E_2 was cumulated and the highest proliferation ratios were obtained *(Foidart et al. 1998)*. Finally, the concentrations in blood serum and target tissues may be different.

5.3.1 Cell cycle protein responses to the different hormonal treatment (II, III)

5.3.1.1 Cyclin-D_1 expression in cultured HBTs (II, III)

The highest numbers of cyclin-D_1–positive cells were in non-cultured peritumoral HBTs (peritum-HBTs), obtained from postmenopausal BC patients. In postmenopausal HBTs (postm-HBTs) stimulation with E_2, MPA and E_2/MPA for 14 days *in vitro* increased proportions of cyclin-D_1 positive cells. The expression cyclin-D_1 associates with age a positive correlation exists between the expression of cyclin-D_1 and increased age *(Loden et al. 2002, Walker et al. 2007a)*. Postm-HBTs are more sensitive to exogenous stimuli, such as hormone treatment, than premenopausal HBTs (prem-HBTs) in regards of the expression cyclin-D_1 that is one of key players in regulation of proliferation, at least *in vitro*.

The cyclin-D_1 gene is one of the most commonly overexpressed oncogenes in BC and its expression is elevated in 50% of cases of primary breast tumors *(Sutherland et al. 2004)*. Moreover, increased expression is detectable in premalignant proliferative lesions in which the proportion of cyclin-D_1 positive cells correlates with the degree of atypia *(Anderson et al. 2004)*. However, in normal human mammary epithelium taken

from adult nonpregnant, nonlactating women, only 0.3% of cells stain positively for cyclin D_1 *(Anderson et al. 2004)* and in lactating breast tissue - only 0.03% *(Shoker et al. 2001)*.

Cyclin-D_1 plays a role in mammary gland development. In experiments with rodents,D_1-null mice are viable and fertile and develop relatively normally, although they are smaller in general than wild-type littermates *(Fantl et al. 1995, Sicinski et al. 1995, Musgrove et al. 2004)*. However, they display specific defects in the development of their retinas and mammary glands *(Fantl et al. 1995, Sicinski et al. 1995, Musgrove et al. 2004, Caldon et al. 2010)*. In the absence of cyclin D_1, mammary ductal development proceeds normally, but lobuloalveolar development is impaired and the mice fail to lactate after their first pregnancy *(Fantl et al. 1995, Sicinski et al. 1995, Musgrove et al. 2004)*. This phenomenon is an epithelial-specific defect, since it can be recapitulated in epithelium transplanted onto wild-type mammary fat pads *(Fantl et al. 1999, Musgrove et al. 2004)*.

Cyclin-D_1 is overexpressed predominantly in ER-positive tumors, while cyclin E overexpression is confined almost exclusively to ER-negative tumors *(Sutherland et al. 2004)*. The D-type cyclins (cyclins D_1–D_3), which bind to and activate CDK 4 and 6 are essential for entry into G_1 from G_0 *(Anderson et al. 2004)*. Expression of these cyclins is induced by mitogenic factors, and the elevated levels remain only as long as the mitogens are present *(Anderson et al. 2004)*.

The mechanisms of androgen induced inhibition on HBT proliferation were further evaluated by studying the expression of cyclin-D_1, which has a crucial role in triggering cell cycle progression from the G_1 to the S phase. In this study, DHT decreased cyclin-D_1 mRNA and protein levels. This result is an agreement with earlier observations with BC cells *(Lanzino et al. 2010)*. Ligand-bound AR interacts with specific androgen response element sequences on the cyclin-D_1 promoter, which leads to the inhibition of cyclin-D_1 transcription *(Lanzino et al. 2010)*. In contrast, T increased cyclin-D_1 mRNA. This effect may partly result from androgen conversion to E_2 *(Somboonporn et al. 2004b)*. Unexpectedly, Bclt alone and or combined with T or DHT decreased cyclin-D_1 mRNA, which suggests promoter-specific Bclt effects and generally reflects the complex actions of ligand-bound AR on the cyclin-D_1 promoter *(Lanzino et al. 2010)*. The overall effects of T were anti-proliferative, opposed by Bclt which suggests that the regulation of cyclin-D_1 is not a critical mechanism of T-induced inhibition of proliferation. In combination cultures with E_2, both T and DHT clearly opposed the strong induction of cyclin-D_1 expression by E_2.

5.3.1.2 p21$^{WAF1/Cip1}$ response to hormonal treatment in HBTs (II)

The treatment with E_2 and MPA not only increased proliferation in HBTs, but induced the numbers of p21$^{WAF1/Cip1}$-positive cells. Besides the well known inhibitory action of p21$^{WAF1/Cip1}$on cell cycle, some reports suggest that the changes in the conformation of p21$^{WAF1/Cip1}$ may affect its functional activity and that p21$^{WAF1/Cip1}$ could also function as an assembling factor for cyclin D-CDK4,6 and become a positive regulator of the cell cycle *(Gartel 2006)*. Results suggest that p21$^{WAF1/Cip1}$ promotes rather than inhibits cell cycle progression in HBTs at least *in vitro*.

The protein p21$^{WAF1/Cip1}$ is a key mediator of p53-dependent cell cycle inhibition after DNA damage *(Gartel 2006, Caldon et al. 2010)*. p53 has many anticancer mechanisms, and it plays a role in apoptosis, genetic stability, and inhibition of angiogenesis. It becomes activated in response to cellular stress (UV, ionizing radiation, chemical agents, osmotic shock, *etc.*). Once activated, p53 increases the expression of p21$^{WAF1/Cip1}$, which binds to the cyclin D-CDK and cyclin E-CDK complexes, inhibiting their activity. The inhibitor p21$^{WAF1/Cip1}$ was initially considered to be a tumor suppressor because it inhibits proliferation *(Gartel et al. 2002, Gartel 2006)*. p21$^{WAF1/Cip1}$ may also act as an oncogene since it often displays cancer promoting and antiapoptotic activities *(Gartel et al. 2002, Shah et al. 2005, De la Cueva et al. 2006)*. This is supported by data from mouse models and from human samples *(Gartel 2006)*. However, the oncogenic activity of p21$^{WAF1/Cip1}$ is displayed only in certain tissues. Thus, in some human cancers, p21$^{WAF1/Cip1}$ expression is a predictor of favorable outcome, whereas in others, low levels of p21$^{WAF1/Cip1}$, especially after chemotherapy, predict a positive outcome *(Shah et al. 2005)*.

Generally, p21$^{WAF1/Cip1}$ acts as an inhibitor of apoptosis, but surprisingly, in some cases it could behave as anti-apoptotic agent *(Blagosklonny 2002, Gartel et al. 2002)*, and this may explain its role in tumorigenesis *(Gartel 2006)*. Thus, p21$^{WAF1/Cip1}$ has a dual role - it can act either as an oncogene or as a tumor suppressor gene depending on the tissues *(Shah et al. 2005)*.

5.3.1.3 p27^{Kip1} response to hormonal treatment in HBTs (II)

The results of this study indicate that postm-HBTs had a lower expression of p27^{Kip1}, another cell cycle inhibitor, in non-cultured and cultured samples in comparison to prem-HBTs. The stimulation with E_2 for two weeks decreased the percentage of p27^{Kip1}-positive cells in postm-HBTs, while in prem-HBTs, E_2 did not have a significant effect. This is in accordance with this study observation that post-HBT have higher proliferative activity and E_2 sensitivity than of prem-HBT.

The inhibitor p27^{Kip1} has significant homology to p21$^{WAF1/Cip1}$. Overexpression of p27^{Kip1} induces G_1 arrest in mammalian cells. It plays an important role in mammary

gland development and represses tumor development *(Chiarle et al. 2001, Alkarain et al. 2004, Musgrove et al. 2004)*. Nonexistent or low expression of p27 ^{Kip1} is a predictor of poor prognosis in human cancers *(Slingerland et al. 2000)*. Experiments with mice suggest that p27^{Kip} plays an important role in mammary development. In contrast to wild-type glands, p27^{Kip1}-null mammary glands did not proliferate or differentiate in organ culture, nor did lobuloalveolar development occur after steroid treatment *in vivo* *(Muraoka et al. 2001, Musgrove et al. 2004)*. Experiments in another laboratory with a different strain of p27^{Kip1}-null mice gave similar results, *i.e.*, lack of alveolar bud formation after steroid treatment *(Deans et al. 2004, Musgrove et al. 2004)*. Decreased proliferation in p27^{Kip1}-null mammary epithelium *in vivo* and purified p27^{Kip1}-null mammary epithelial cells in culture correlated with impaired mammary development *(Muraoka et al. 2001, Musgrove et al. 2004, Caldon et al. 2010)*.

5.4 Effects of androgens (DHT and T) on proliferation in HBTs (III)

The relative numbers of phospho-histone 3 (PHH3) positive cells were reported to be lower compared to those of Ki-67-positive cells, reflecting the expression pattern of the markers during the cell cycle *(Bossard 2006)*. The highest percentage of PHH3-positive cells was observed in the E_2 group when compared to the controls and other treatments, and similar results were obtained with Ki-67. The number of PHH3-cells was lower in androgen treatment groups (T and DHT) compared to the controls. The addition of antiandrogen bicalutamide (Bclt) to T or DHT opposed these androgen induced effects. The effects of E_2/T and E_2/DHT on proliferation were assessed only with Ki-67. The proliferation increased in E_2-treated HBT and decreased in a time-dependent manner in HBT treated with T and DHT for 7 and 14 days. The combination of T and DHT with E_2 opposed the E_2–alone effects as compared with the HBT cultured with E_2-alone at both 7 and 14 days.

Androgen effects on the proliferation ratios were determined by immunohistochemistry using Ki-67 and PHH3. Recently, PHH3 is an IHC prognostic marker for assessment of the proliferative activity for lymph node negative BC patients *(Baak et al. 2009)*. Recently, one of the most used markers, Ki-67 is a weaker predictor, because it stains not only cells in M and late G_2 phases, but also in G_1 and S phases *(Baak et al. 2009)*. Many Ki-67-positive cells may not survive the cell cycle and are driven into apoptosis, thereby diminishing the prognostic value of the Ki-67 *(Skaland et al. 2009)*. The optimal marker of proliferation should be expressed only in late G_2 and M phase because PHH3 is expressed during these phases *(Baak et al. 2007)*. E_2 stimulation of epithelial cell proliferation is considered to increase the risk for BC development *(Preston-Martin et al. 1990)*. However, conflicting studies show the effects of exogenous androgens on the proliferation of mammary epithelial cells and the associated risk of BC *(Hackenberg et al. 1988, Poulin et al. 1988, Marugo et al. 1992,*

Dimitrakakis et al. 2002, Dimitrakakis et al. 2003, Somboonporn et al. 2004a, Dimitrakakis et al. 2009). Results of this study demonstrate that androgens decreased proliferation in a time-dependent manner and effectively opposed the stimulatory effects of E_2.

Interestingly, Bclt increased the proportion of proliferating cells, which suggests that AR exerts an inhibitory effect on the basal levels of proliferation. Findings of this study agree with a previous study on HBTs *(Hofling et al. 2007)* which show that decreased Ki-67-cells in women treated with androgen-containing hormone therapy. Corresponding results obtained in non-human primate model studies in which androgen treatment decreased mammary proliferation *(Zhou et al. 2000)*.

5.5 Effects of SERMs (Osp, Ral and Tam) on HBTs in vitro (IV)

TAM used as a BC drug and RAL was originally developed for postmenopausal osteoporosis treatment and these have a strong inhibitory effect on human BC cells. However, there is much less information of the effects of different SERM molecules and even of TAM and RAL on normal human breast epithelium. The results demonstrate that OSP, RAL and TAM affect proliferation in a time and dose dependent manner and that they effectively oppose the stimulatory effects of E_2 at high concentration. The effects of TAM and RAL on normal HBT are not unexpected but they provide a good background and comparison for evaluating the effects of SERM molecules on breast tissue. OSP, for instance, functions in a tissue specific manner and may have either E_2 agonistic/antagonistic properties. These results are in agreement with previous studies which have evaluated the inhibitory effect of antiestrogens in primary human BC by a short treatment between diagnosis and surgery, an interval during which therapy is not conventionally given *(Clarke et al. 1993, DeFriend et al. 1994, Ellis et al. 1997, Makris et al. 1998)*. TAM reduces the levels of proliferating cells in breast epithelium (Ki-67 positive cells) after a median of 21 days in a placebo-controlled trial of 103 patients *(Clarke et al. 1993)*. A positive effect of TAM on ER-positive and negative BC was observed. The first clinical study to evaluate the effects of steroidal pure antiestrogen fulvestrant (ICI 182.780) (Fulv) was in a 7-day, no-treatment controlled, presurgical study in postmenopausal women with BC *(DeFriend et al. 1994)*. The antiproliferative effect of Fulv was confined to ER-positive tumors, where Fulv increased the levels of apoptosis *(Ellis et al. 1997)*. The TAM analogue idoxifene reduces Ki-67-positive cells by approximately 30% in post-menopausal women with ER-positive tumors, but in contrast to the study with Fulv, idoxifene did not have any effect on apoptosis *(Dowsett et al. 2000)*. Results from a short clinical study indicated that the numbers of Ki-67-positive cells decreased after 2 weeks of TAM treatment before surgery of the primary BC but not in patients who failed to respond to TAM *(Makris et al. 1998)*.

Few studies evaluated the effective doses of RAL and placebo for postmenopausal BC patients with primary tumors before BC surgery *(Dowsett et al. 2001, Lopes-Costa et al. 2010)*. Results indicated that the dose of 60 mg/day of RAL showed a significant antiproliferative effect in ER-positive BC, demonstrated by the decrease in levels of Ki-67 but there was no inhibitory effect of RAL on ER-negative BC *(Dowsett et al. 2001, Lopes-Costa et al. 2010)*. This provides evidence that RAL has a BC preventive effect in ER-positive postmenopausal women.

Previously, minimal data on OSP effects on human mammary gland is available. The results from this study show that OSP exerts inhibitory effects on normal HBT in a concentration dependent manner. Significant inhibition was not obtained at low OSP concentration (1 nM) as with RAL and TAM. OSP also inhibited E_2-induced stimulation on cell proliferation at the same level as RAL or TAM. The results are in accordance with previous studies. OSP prevents the growth of premalignant lesions and progression to invasive carcinoma in the adenoma/mammary intraepithelial neoplasia mouse model and slowed down tumor growth in MCF-7 xenograft models *(Qu et al. 2000, Namba et al. 2005, Burich et al. 2012, Wurz et al. 2013)*.

5.6 Steroid and SERM regulation of ERα, ERß and AR in HBTs (I-IV)

Immunohistochemical staining results showed that E_2 and androgen treatment markedly altered the percentage of ERα- and AR-positive cells in mammary epithelium. The androgen treatment increased the proportion of AR positive cells, whereas ERα-expressing cells were strongly decreased. The ERβ-expressing cells remained unaltered. Treatment with E_2 increased the number of ERα-positive cells, whereas ERβ- and AR-positive cells decreased. In the combination cultures, E_2 and the androgens opposed each other. The altered proportions of steroid receptor positive cells in the E_2 and androgen treatments could be explained by changes in receptor gene expression and/or the enrichment or reduction of receptor-positive cells, due to changes in proliferation and/or apoptosis of selected cell populations. DHT increases AR expression *(Lanzino et al. 2010)* and decreases ERα expression in primate mammary glands *in vivo (Zhou et al. 2000)* and in human breast cancer cells *(Macedo et al. 2006, Lanzino et al. 2010)*. The present experiments do not reveal the extent to which these mechanisms are involved in inducing changes in AR- and ER-positive cell percentages. The altered proportions of AR- and ER-expressing cells have an important role in E_2 stimulation response and androgen driven repression of cell proliferation in mammary epithelial cells and in mammary gland. Accordingly, one mechanism that could explain androgen mediated of proliferation is a strong decrease in ERα-positive cells. Whether AR-positive cells are able to divide in the mammary epithelium remains unknown, but recent reports demonstrate that androgens inhibit cyclin-D_1 expression in MCF-7 cells

in an AR-dependent manner, which may be an explanation for androgen-driven inhibition of proliferation in these cells *(Lanzino et al. 2010)*.

An increase in ERα-positive cells associated with stimulated proliferation and decreased apoptosis in E_2-treated explants. Conversely, decreased ERα positivity was linked to reduced proliferation and increased apoptosis in androgen-treated cultures. Previous studies *(Clarke et al. 1997c)* show that ERα-positive mammary epithelial cells do not proliferate and E_2-induced growth is mediated by paracrine stimulation of cell division in the adjacent ERα-negative cells *(Clarke et al. 1997c)*. In our HBT cultures, a critical mediator of ER action is AREG *(Ciarloni et al. 2007)*, which was induced by E_2. Because ERα-positive cells increased in the presence of E_2, it is likely that ER-negative cells rapidly differentiate into ERα-positive cells after cell division.

Interestingly, the response of ERβ-positive cells to the T, DHT and E_2 treatments resembled those observed in AR-positive cells, whereas the responses of ERα- and ERβ-expressing cells yielded opposite effects. The function of ERβ in breast tissue is largely unknown, but it is thought to have protective functions, similar to AR due to proliferation inhibition *(Gustafsson et al. 2000, Maehle et al. 2009)*. Correspondingly, the expression of AR and ERβ in tumor specimens associates favorable outcomes in BC patients *(Moe et al. 2007, Maehle et al. 2009, Park et al. 2010, Dimitrakakis 2011)*.

Results demonstrate that in addition to their direct inhibitory effects, T and DHT oppose the effects of E_2 on proliferation, apoptosis and target gene expression in HBT, as previously reported in various experimental *in vivo* and *in vitro* models *(Zhou et al. 2000, Dimitrakakis et al. 2003)*. The mechanisms by which androgens inhibit E_2 in breast tissue may include T- and DHT-induced decreases in ERα-positive cells and/or in ERα protein expression *(Zhou et al. 2000, Lanzino et al. 2010)* or increased apoptosis of ERα-positive cells. AR may strongly inhibit ERα activity by binding to estrogen response elements in a subset of ER target genes, thereby preventing ER activation of these genes *(Peters et al. 2009)*. Another mechanism that was recently suggested for maintaining the balance between ER/AR actions is the suppression of AR target gene expression by cyclin-D_1 *(Comstock et al. 2011)*. In addition to controlling the cell cycle, cyclin-D_1 has other functions including the modulation of nuclear receptor actions. Recent studies indicate that cyclin-D_1 can block the binding of AR in a subset of target genes, thereby inhibiting androgen action *(Comstock et al. 2011)*. If corresponding mechanisms work in the mammary epithelium, then E_2-induced cyclin-D_1 could effectively inhibit androgen regulation of selected target genes and their functions.

The effects of the SERMs: OSP, RAL and TAM on epithelial proliferation and gene expression of cultured HBTs also associate with changes in the proportions of ERα and AR expressing cells. While E_2 increased ERα positive cells, OSP, RAL and TAM all have a clearly antagonistic effect in decreasing ERα positive cells in a concentration dependent manner. The effect of TAM was the strongest. All SERMs used in this study also effectively opposed the effect of E_2. The number of ERα

positive cells in explants treated with Fulv alone or in any combination was very low which is obviously explained by the ability of Fulv to increase degradation of the ERα protein.

OSP, RAL and TAM had less effect on the relative numbers of AR positive than ERα positive HBT epithelial cells. At high concentrations, all SERMs maintained the AR positive cells at the level seen in the control but interestingly, at low concentration (1 nM) SERM decreased AR positive cells in a statistically significant manner although the effect was much smaller than that of E_2. At higher concentration (100 nM) OSP, RAL and TAM all opposed E_2-induced decrease of AR expressing cells in a statistically significant manner. Fulv also decreased AR positive cells and its effect was not opposed by the SERMs used. The effects of OSP, RAL and TAM on ERα and AR expressing cells were supported by their effects on the relative numbers of epithelial cells expressing TFF1 or Apo-D. The results suggest that the effects of OSP, RAL and TAM on epithelial cell proliferative correlates with AR level and in an inverse manner with ERα level. It is possible that regulation of the proportions of ERα and AR expressing cells forms a basis of SERM actions on breast epithelium.

5.7 Study limitations

The George E.P Box quote: "*all model are wrong, some models are useful*" might properly describe the limitations of either *in vivo* or *in vitro* experiments *(Hartung et al. 2009)*. Organ cultures of HBTs provide an *in vitro* model which enables studies on hormone regulation of normal and tumoral HBTs, but even at their best, they have certain limitations. Tissues are treated outside their normal "environment" under the artificial conditions (no blood supply, no normal supply of nutrients, *etc*.), which are not homeostatic (sudden exchanged of culture media, metabolisation can be simulated by addition of specific chemical agents, accumulation of waste products and metabolites, not sufficient oxygen supply, *etc*.) *(Hartung et al. 2009)*. The choice of culture conditions can affect the experimental outcomes, and cultured tissues become adapted to conditions *in vitro* and the changes which have allowed this ability may not occur *in vivo*. On the other hand, *in vivo* occurred responses to chemical active components cannot easily be mimicked *in vitro*.

Limitations of *in vitro* studies might be the high concentrations needed for an effective response to hormonally active compounds, such as SSH or SERMs *(Kramer et al. 2006)*, *e.g*. the clinically relevant blood concentrations achieved after E_2 and MPA as MHT admistration are 0.01–0.1 nmol/l for E_2 and around 4-10 nmol/L for MPA *(Mueck et al. 2003, Kramer et al. 2006)*. However, the assessment of blood level values may be limited, since tissue levels of estrogens are up to ten-fold higher than circulating concentrations. Moreover, it is well recognized that estrogens are synthesized in the breast tissue, at concentrations that are remarkably higher than blood levels achieved

under MHT [reviewed in *(Mueck et al. 2003)*]. Higher concentrations may be required *in vitro* in short-time tests in which the reaction threshold can only be achieved with supraphysiological dosages *(Kramer et al. 2005)*. Higher concentrations may also be reached *in vivo* in the vessel wall or organs compared to the concentrations usually measured in the blood *(Kramer et al. 2005)*.

A further limitation of this work and any culture model is the short incubation period of HBTs with the substrates under investigation, in comparison to the longer time period for which MHT is usually prescribed *(Kramer 2007)*. The longer duration of MHT may be an important factor for BC risk as indicated by the results the Women's Health Initiative study, where BC risk was significantly higher compared to placebo only in women who had been administrated combined MHT for 10 years or more, but not in those treated only for the duration of the study period, *i.e.* 5.2 years *(Kramer et al. 2006)*. Although *in vitro* experiments, even with human tissues, cannot replace clinical trials, they can be helpful research tools in predicting the effects of various treatments on HBTs.

6 SUMMARY AND CONCLUSIONS

The present study was performed to understand the effects of sex steroid hormones (E_2, MPA, DHT and T) and the SERMs: OSP, RAL and TAM on normal HBTs. The modified organ culture model was used to study the direct effects of these compounds on cell turnover (proliferation and apoptosis) and differentiation in HBTs without interfering systemic effects functions *in vivo*. The results of the present study led to the following main conclusions:

1.) Explant cultures of HBTs maintain the structural integrity of mammary tissue and tissue-specific, differentiated functions such as E_2 induced of expression of TFF1 and amphiregulin in an ER dependent manner, thus providing an appropriate model for studies on hormone regulation of HBTs.

2.) The E_2, MPA and their combination E_2/MPA exert stimulatory effects on HBTs. The results indicate that E_2, MPA and E_2/MPA enhance the proliferative activity of normal breast epithelial cells and decrease the number of apoptotic cells. The levels of cyclin D1 and p21 were increased and the proportion of p27-positive cells was increased. These changes occurred in both prem- and postm-HBTs but they were particularly prominent in the latter. The proliferative activity was higher in peritum-HBTs than in normal prem- or postm-HBTs. The proportion of ERα, ERß and PR positive cells was decreased in the presence of E_2, MPA and E_2/MPA in peritum-HBTs, but in normal postm-HBTs E_2 increased the relative number of ERα expressing cells while ERß positive cells decreased.

3.) The androgens T and DHT inhibited proliferation and increased apoptosis in the epithelium of HBTs from postmenopausal women and opposed E_2-stimulated proliferation and cell survival in an AR-dependent manner. Androgen treatment decreased the proportion of ERα positive cells whereas E_2 decreased that of AR positive cells.

The overall effects of the SERMs OSP, RAL and TAM on HBTs from postmenopausal women were antiproliferative but inhibition of proliferation was achieved at lower concentrations of RAL and TAM than with OSP. They all opposed E_2-stimulated proliferation. OSP, RAL and TAM also strongly decreased numbers of ERα expressing cells. Interestingly, the proportion of AR positive cells was not decreased in HBTs cultured with OSP, RAL and TAM.

Table 7. Summary of the effects of E_2, androgens and SERMs on normal postmenopausal HBTs.

	Estrogens	Androgens		SERMs					
	E_2	T	DHT	Ospemifene		Raloxifene		Tamoxifen	
	[10nM]	[1nM]	[1nM]	[1nM]	[100nM]	[1nM]	[100nM]	[1nM]	[100nM]
ERα	↑	↓	↓	↓	↓	↓	↓	↓	↓
ERß	↓	n	n	N/A		N/A		N/A	
AR	↓	↑	↑	↓	n	↓	n	↓	n
Ki67/PHH3	↑	↓	↓	n	↓	n	↓	n	↓
CPP32	↓	↑	↑	N/A		N/A		N/A	
H&E									

Abbreviations:
E_2 - 17ß-estradiol; T-testosterone; DHT-dihydrotestosterone; SERMs-selective estrogen receptor modulators;
↑- increased; ↓-decreased; n- no effects; N/A- data not available.
Proliferation was assessed by immunohistochemical stainings with Ki-67 and/or PHH3 (phospho-histone3) and apoptosis with Caspase-3 (CPP32) primary antibodies.

As the future prospective organ cultures of HBTs demonstrate the broad potential to study effects of novel hormonally active compounds (SERMs) or even response of tumoral HBTs to anti-cancer drugs. BC is a heterogeneous disease with very individual responses to specific treatment, and BC patients with same histological type of BC and grade are responding to therapy differently. Short-term cultures of the primary tumor tissue with certain anti-cancer drugs allow the determination of individual responses and test the susceptibility of individual tumors to various anti-cancer drugs. Thus, it would be possible to selective an appropriate treatment alternative.

In summary, the present findings support the notion that androgens in MHT protect HBT against the proliferative effects of E_2 and provide a more physiologically balanced hormonal environment for HBTs, *in vitro*. The results show that OSP, RAL and TAM also exert antiproliferative effects on HBTs and oppose E_2 action in HBTs. The study outcome evaluates the safety of currently available SERMs and suggests that a combination of an androgenic compound or an appropriate SERM with E_2 could be considered as components of MHT. Understanding hormonal and SERM effects in normal HBT may advance development of novel regimens of MHT with improved safety compared to currently available therapies.

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