1-1-2011

Estrogen sulfotransferase (sult1e1) expression and function in mcf10a-series breast epithelial cells: role as a modifier of breast carcinogenesis and regulation by proliferation state

Jiaqi Fu
Wayne State University,

ESTROGEN SULFOTRANSFERASE (SULT1E1) EXPRESSION AND FUNCTION IN MCF10A-SERIES BREAST EPITHELIAL CELLS: ROLE AS A MODIFIER OF BREAST CARCINOGENESIS AND REGULATION BY PROLIFERATION STATE

by

JIAQI FU

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2011

MAJOR: MOLECULAR AND CELLULAR TOXICOLOGY

Approved by:

_____

Advisor Date

_____

_____

_____

_____

DEDICATION

I dedicate this work to my parents for all the sacrifices they have made on my behalf.

To Luan, for his love and support during the preparation of this work.

ACKNOWLEDGEMENTS

I owe my deepest gratitude to Dr. Melissa Runge-Morris and Dr. Thomas A. Kocarek, for the encouragement and support throughout all these years of study. Their guidance helped me in all the time of research and writing of this thesis.

It is an honor for me to thank the members of my dissertation committee, Drs. Fred Miller, John J. Reiners Jr., Malathy Puthan Veedu Shekhar for all their time and insightful criticisms that made this thesis possible. I would like to show my gratitude to my fellow graduate students, the current and former lab members for all their help and encouragement over the years. In particular, thank you Mary Dorothy Gargano and Patricia A. Mathieu for all your support and willingness to help in any way possible.

Thanks to the support of National Institutes of Health National Institute of Environmental Health Sciences [Grant ES05823] which made this work possible. Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the projects.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

2-OH	2-hydroxylation
2-OHE	2-hydroxylation catechol estrogen
2-OHE2	2-hydroxyestradiol
4-OH	4-hydroxylation
4-OHE	4-hydroxylation catechol estrogen
4-OHE2	4-hydroxyestradiol
17β-HSD	17β-Hydroxysteroid Dehydrogenases
AhR	Aryl Hydrocarbon Receptor
ARNT	AhR Nuclear Translocator
BMI	Body mass index
BP	benzo[a]pyrene
BrU	bromouridine
cAMP	Cyclic AMP
CE	catecholestrogen
COMT	Catechol-O-methyltransferase
Ct	cycle threshold
CYP	Cytochromes P450
CYP19	Aromatase
CYP1A1	Cytochrome P450 1A1
CYP1B1	Cytochrome P450 1B1
DCIS	Ductal Carcinoma *in situ*
DHEA	dehydroepiandrosterone
DMEM/F12	Dulbecco's Modified Eagle Medium/Ham's F12
DMSO	Dimethyl Sulfoxide
DRE	dioxin response element or xenobiotic response element or AhR•ARNT heterodimer binding site
E1	estrone
E1S	Estrone Sulfate
E2	17β-Estradiol
E3	estriol
EREs	Estrogen response elements
EGF	Epidermal growth factor
ER	Estrogen receptor
ERDs	Estrogen receptor down-regulators
ERK	Extracellular signal-regulated kinases
GAPDH	Glyceraldehydes-3-phophate dehydrogenase
GPR30	G protein-coupled receptor 30
GPER	G protein-coupled estrogen receptor
HDACs	Histone deacetylases
HB-EGF	heparin-binding EGF
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HRT	hormone replacement therapy

ICI	ICI 182,780
IHC	Immunohistochemistry
MAPK	Mitogen-activated protein kinase
MEs	methoxyestrogen
MC	3-methylcholanthrene
MNF	3’-methoxy-4’-nitroflavone
OVX	Ovariectomized
PAHs	Polycyclic aromatic hydrocarbons
PAPS	3’-phopphoadenosine-5’-phosphosulfate
PI3k	Phosphoinositide 3-kinase
PBS	Phosphate-buffered saline
PR	progesterone receptor
RSK	Ribosomal S6 kinase
SERMs	Selective estrogen receptor modulators
STS	Steroid sulfatase
SULTs	cytosolic sulfotransferases
SULT1E1	Sulfotransferase family 1E; Estrogen sulfotransfrase
SULT1A1	Sulfotransferase family 1A
SULT2A1	Sulfotransferase family 2A
SULT2B1	Sulfotransferase family 2B
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDLU	Terminal duct lobular unit
TNBC	Triple negative breast cancer
TSA	Trichostatin A
TSC	Tobacco smoke condensate
UGTs	UDP-glucuronosyltransferases

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 1

INTRODUCTION

1. Breast cancer

Breast cancer is the leading type of cancer and the second most common cause of cancer death in American women [1]. The overall lifetime probability of developing breast cancer is 1 in 6, and about 1 in 8 women will develop invasive breast cancer in the United States [2]. According to 2010 estimates, approximately 207,090 new cases of invasive breast cancer would be diagnosed in women in the United States and about 39,840 women would die from the disease in 2010 [2].

Treatment options for women with breast cancer include surgery, radiation therapy, chemotherapy, hormone therapy, and targeted therapy. The treatment decision is made based on the size and location of the tumor in the breast, the results of lab tests done on the cancer cells, the stage or extent of the disease, and the age and general condition of the patient [3-7].

Surgery, consisting of lumpectomy (breast-conserving surgery) or mastectomy (removal of the whole breast), is usually the first line of treatment for breast cancer, especially early-stage primary breast cancer [7]. However, breast cancer can come back as a local recurrence (occur in the surgery area), regional recurrence (cancer has spread beyond the breast), or as a distant recurrence (also known as metastasis) somewhere else such as lymph nodes, the bones, liver, or lungs within the first two years if no further treatment is given after surgery [8;9]. Therefore, radiotherapy or chemotherapy has become an integral part of post-surgical breast cancer management [3;7]. It has been reported that between 5% and 33% of breast cancer patients develop

chronic lymphedema after surgery and radiotherapy or chemotherapy, accompanied by cognitive changes, early menopause, weight gain, hair loss and changes in skin tone and texture, which all result in significant psychosocial morbidity and decreased quality of life [10;11]. Thus, patients who prefer to have the best possible quality of life while they are being treated tend to choose less-aggressive treatments such as hormone therapy and targeted therapy.

Hormonal therapies, including selective estrogen receptor modulators (SERMs), aromatase inhibitors and estrogen receptor down-regulators (ERDs), are used to prevent early-stage, hormone receptor-positive breast cancer recurrence after surgery and to treat advanced-stage/metastatic hormone-receptor-positive breast cancer in both pre- and post-menopausal patients [12]. Targeted cancer therapies recognize and block specific characteristics of cancer cells and simultaneously activate defense responses. Therefore, targeted therapies are generally less likely than chemotherapy to harm normal, healthy cells. Moreover, novel conjugates of antibodies with cytotoxic agents that are activated after entering the cancer cell are under investigation [13].

Although these two kinds of less-aggressive treatment are widely used, hormone therapies primarily target estrogen receptor (ER)-positive breast cancer, and the majority of targeted therapies work specifically against HER2-positive breast cancer [12;13]. Neither of these approaches is effective against triple-negative breast cancers (TNBC) (ER-, progesterone receptor (PR)-, and HER2-negative). TNBC has become a focus of intense research because TNBC patients present at a younger age at onset and have a higher tumor grade, larger tumor size, and increased propensity to develop

metastases. Most importantly, they lack the three most significant therapeutic markers for clinical management resulting in the worst outcome when compared with other cancer subtypes, which motivates the investigation of novel approaches for the prevention and treatment of this aggressive form of breast cancer [14-18]. Another major clinical problem is that the majority of tumors will eventually manifest resistance during the course of hormone and targeted therapies [19-21]. Therefore, it is critical to identify new therapeutic targets both to improve the prognosis and survival rate of patients with all forms of breast cancer and to prevent breast cancer from developing by identifying women at increased risk and intervening to modify risk.

2. Estrogen and breast cancer

Based on the results of clinical and epidemiological studies, the well-established risk factors for breast cancer have been summarized as earlier age at menarche, nulliparity, later age at first full-term birth, later age at menopause, history of breast cancer in a first-degree relative, and socioeconomic status [22-26]. The majority of these risk factors indicate that prolonged cumulative exposure to endogenous estrogen may increase the risk of developing breast cancer [27;28]. The estrogen-sensitive nature of breast cancer has also been implicated by the fact that surgical removal of ovaries significantly reduces breast cancer risk in pre-menopausal women [29;30]. About 95% of breast cancer is initially hormone-dependent, which also indicates the crucial role of estrogen in breast cancer development and progression [31;32].

2.1 Critical estrogen exposure periods

Emerging evidence supports the hypothesis that there are critical periods during a woman's lifetime during which estrogen exposure increases breast cancer. The

intrauterine period, adolescence and full-term pregnancy are intervals in which mammary tissue undergoes a high rate of proliferation and extensive differentiation. Therefore, a high level and/or prolonged duration of estrogen exposure during these vulnerable periods may dramatically increase the risk of breast cancer.

Data from animal and human population studies provide leads to the critical role of estrogen exposure in uterus on one's risk for breast cancer development later in life. During pregnancy, the levels of intrauterine estrogen are at least 10 times higher than they are in women who are not pregnant, and the levels gradually increase throughout pregnancy [33-35]. The hypothesis that factors positively associated with intrauterine estrogen exposure such as gestational age, birth weight and birth length, are positively associated with breast cancer risk has been extensively investigated. Examples of findings from these studies are the following:

- Longer gestation is an indicator of prolonged exposure to pregnancy hormones and has been postulated to be associated with increased breast cancer risk. However, epidemiology studies have not provided consistent supportive results. Two matched case-control studies done in either women aged 14-37 years or who were twin births support a role for early estrogen exposure in the development of breast cancer [36;37]. However, three other cohort studies indicate an inverse relationship between gestational age and breast cancer risk [38-40].
- Increased birth weight and length have been demonstrated to be positively associated with prolonged in utero estrogen and other maternal hormone exposure [41-44]. Studies to date have generally suggested a positive correlation

between birth weight and length and breast cancer risk, especially in pre-menopausal breast cancer cases [36;38;45-48]. A nested case-control study within the cohorts of two Nurses' Health Studies indicates that pre-menopausal breast cancer risk is significantly associated with increased birth weight [46]. This finding is consistent with that reported in another British cohort study of 2167 women (including 59 breast cancer cases, 21 of which were diagnosed before menopause), in which women who had high birth weight were five times (relative risk=5.03; 95% confidence interval=1.13, 22.5) more likely to develop premenopausal breast cancer than were women who had low birth weight (P-value for linear trend=0.03) [48]. A record linkage study of 373 Norwegian breast cancer cases and 1150 age-matched control women provided strong evidence that breast cancer risk was positively associated with both birth weight and birth length (P for trend=0.02) [47]. The same author conducted another prospective population-based study in a cohort of 16,016 women in Norway (including 312 breast cancer cases) and reported a similar positive association between birth weight and length and breast cancer risk [45].

Animal studies also provide evidence that intrauterine factors influence future breast cancer risk [49]. Increased levels of estrogen due to high dietary fat intake in pregnant rats enhanced mammary tumor development in first-generation female offspring [49;50]. Similarly, a later study provided evidence that the plasma estrogen levels of pregnant rats were significantly elevated by feeding them a diet high in n-6 polyunsaturated fats. This finding indicated that the female offspring of high-fat-fed rats were more vulnerable to carcinogen treatment, as manifested by significantly higher

mammary tumor incidence and shorter latency, than were the offspring of pregnant rats fed a low-fat diet [50].

In addition to those studies demonstrating that increased exposure to estrogen in utero can result in increased breast cancer development during adulthood, two studies have demonstrated that offspring of women with preeclampsia, a pregnancy-induced condition characterized by decreased levels of pregnancy hormones [51;52], have a significantly lower breast cancer risk [40;53].

In summary, all of the findings described above suggest that prolonged exposure to estrogen and other hormonal factors particularly in utero may initiate the carcinogenic process in mammary gland tissue and affect breast cancer risk later in life.

The most dramatic development of breast tissue occurs during puberty when the ovaries begin to secrete estrogen and other hormones [54;55]. Therefore, breast tissue is most vulnerable to carcinogen exposure during this period. Breast cancer risk is closely related to the duration of estrogen exposure that is estimated by the ages of menarche and menopause. It has been estimated that breast cancer risk is reduced by 4 to 20% each year that menarche is delayed [56;57]. Similarly, epidemiology studies indicate a 1.5-fold increase in breast cancer risk for women who have an early age of menarche [58]. Another factor that can be translated into high estrogen exposure is body mass index (BMI). However, BMI is not always positively associated with breast cancer, and its association with breast cancer varies by menopause status. Numerous case-control studies and cohort studies suggest that BMI is nonlinearly inversely associated with breast cancer risk in pre-menopausal women, but nonlinearly positively associated with post-menopausal breast cancer risk [59-65]. Some of these studies

indicate that one's BMI at puberty is closely associated with breast cancer risk in adult life [61-65]. In a cohort study of 117,415 women (including 3340 cases of breast cancer), BMI at 14 years of age was inversely associated with increased breast cancer development during adulthood [65]. Four other studies also support this finding [61-64].

It has been suggested that pregnancy and lactation, especially the first full-term pregnancy, completes the full differentiation of breast tissue [66]. Therefore, the breast is highly susceptible to the influence of carcinogen during this period. Early age of first and second full-term pregnancy and the number of births have been associated with a long-term reduction in breast cancer risk, especially for postmenopausal women, according to a large scale study including 1.7 million Norwegian women born in the period 1925 to 1979 (including 22,890 breast cancer cases at ages 20–74 years) [67]. Two epidemiological studies showed that prolonged breastfeeding was associated with an additional protective effect against breast cancer development in premenopausal women [68;69]. In addition to the repeatedly demonstrated protective effect of early first pregnancy and breast-feeding in epidemiological studies, experiments in rodent breast cancer models have also confirmed the inverse association between age at first pregnancy and breast cancer risk [70-73]. The protective role of reproduction in the susceptibility to carcinogen-induced breast cancer is demonstrated by the finding that nulliparous rats are highly susceptible to the induction of mammary carcinomas by carcinogen 7,12-dimethyl benzanthracene [70-72]. Similarly, the study of Yang *et al* [73] also supports the protective role of parity and lactation against *N*-methyl-*N*-nitrosourea treatment induced mammary carcinogenesis.

Therefore, understanding the biology of estrogen in greater depth will facilitate the development of new interventions for breast cancer prevention and treatment.

2.2 Estrogen biosynthesis

The naturally occurring estrogens, 17β-estradiol (E2), estrone (E1), and estriol (E3), are C18 steroids derived from cholesterol, among which E2 is the predominant endogenous activator of ER-mediated cellular processes [19]. In premenopausal women, the primary source of estrogen is E2 produced by the ovaries with concentrations that fluctuate during menstrual cycles from 40 to 250 pg/ml [74]. However, in postmenopausal women, the serum E2 concentration is often lower than 20 pg/ml and E1 becomes the predominant form of estrogen [74]. Adipose tissue is another important source of estrogen, particularly in pre-pubertal girls and postmenopausal women [75].

Although circulating estrogen levels are decreased by 90% in postmenopausal women, the majority of breast carcinomas arise after menopause and the intra tumoral concentration of E2 is more than 10 times higher than in plasma [74;76-79]. Previous investigations also demonstrated that a large proportion (around 75% in premenopausal women and almost 100% in postmenopausal women) of the bio-available estrogen is derived from de novo biosynthesis in peripheral tissues. Overall, this finding highlights the importance of *in situ* metabolism of estrogen in cancer target tissues [80-83]. In the breast, three main pathways are involved in the *in situ* formation of estrogen [84-86]:

- **Aromatase (CYP19) pathway**: CYP19 catalyzes the rate-limiting step in the conversion of androstenedione taken up by the tumor from the blood into E1, which

is then converted into E2 by 17-β hydroxysteroid dehydrogenase type 1 (17-β HSD1) [87-89]. It has been demonstrated that *in situ* metabolism of estrogen through the CYP19 pathway is correlated with the risk of developing breast cancer [22;90]. CYP19 expression is significantly increased in breast carcinoma compared to nonmalignant breast tissue, and its expression also increases during breast cancer malignant progression, with the highest level in invasive breast carcinoma, which supports the therapeutic potential of targeting the CYP19 enzyme for breast cancer treatment [91].

- **17β-HSD pathway:** The enzymes of the 17β-HSD gene family are responsible for the interconversion of dehydroepiandrosterone (DHEA) and testosterone and of E1 and E2, which indicates that this enzymatic activity is required for the synthesis of all androgens and estrogens [18]. The distribution and activity of estrogenic 17β-HSD has been studied in 15 human tissues. Estrogenic 17β-HSD activity was detected in all tissues examined with the highest rates in placenta, liver, ovary, endometrium, testis and adipose tissue [92]. The mRNA levels of 17β-HSD1, which catalyzes the reduction of E1 to E2, was significantly higher in breast cancer lesions from postmenopausal patients than that in tumor from premenopausal patients, and the protein expression of this enzyme was detected in about 60% of breast carcinomas, which correlated with ER and PR levels [78;93]. 17β-HSD activity was significantly higher in tumoral tissues than in the normal surrounding mammary tissue [78;94;95].

- **Sulfatase pathway**: Steroid sulfatase (STS) converts estrone sulfate taken up by the tumor from the blood into E1, and then 17β-HSD1 converts E1 into E2. STS expression was detected in approximately 70% of breast carcinoma cases and was

higher in breast carcinoma lesions than in the surrounding normal tissue [96-99]. STS expression correlates with an increased risk of breast cancer recurrence, poor clinical outcome, and a significantly shorter relapse-free survival in breast cancer patients, which emphasizes the importance of sulfatase-mediated local production of estrogen in the malignant progression of breast carcinomas [96-99].

2.3 Estrogen carcinogenesis in breast cancer

Carcinogenesis is a multistage process that includes initiation and promotion [100-102]. The initiation of carcinogenesis is an irreversible, direct increase in chromosomal aberrations and mutation of oncogenes and tumor suppressor genes [100-102]. Promotion is an interruptible or reversible stage during which the initiated cells undergo clonal expansion [100-102]. It has been suggested that estrogens are unique carcinogens affecting both tumor initiation and promotion. There are mainly two mechanistic explanations for the involvement of estrogen in breast cancer processes.

The classical, well accepted mechanism for the role of estrogen in breast carcinogenesis is that estrogens stimulates cell growth, increases the rate of cell division and suppresses apoptosis, which can enhance opportunities for DNA mutation and render a spontaneous or chemically-induced mutation permanent [103-107]. These actions of estrogen can be achieved through pathways mediated by ER.

There are two ER subtypes, ERα and ERβ, which have different estrogen affinities, expression patterns and responses to ER modulators and anti-estrogens [108-110]. For instance, E1 and E2 have a higher affinity for ERα, while some phytoestrogens bind with higher affinity to ERβ [110]. Endothelial cells, bone and kidney express mainly ERβ, while breast cancer cells and ovarian stroma contain mostly ERα

[109]. Moreover, ERα and ERβ mediate different transcriptional effects due to the distinctive conformations of their major transactivation domains [111;112]. For example, E2 might activate transcription when it binds to ERα but display the opposite effect when it binds to ERβ [113]. Therefore, the effects of estrogens may vary in different tissues and cells, and may even be different in the same cell at different time points due to the structure of the hormone, the subtype of ER involved, the characteristics of the target gene promoter, and the availability of coactivators and corepressors.

ERs are mainly functional as ligand-dependent transcription factors [19]. Estrogens readily diffuse across the cell membrane and interact with ERs in the nucleus where the homodimeric complexes bind to estrogen response elements (EREs) and recruit coactivators or corepressors to regulate the transcription of estrogen-responsive genes involved in cell proliferation and cell survival [19]. However, recently it has been reported that one-third of estrogen regulated-genes do not contain conserved EREs in their promoter regions [114]. This finding led to the investigation of ERE-independent transcriptional activation by estrogen-ER complexes. In this circumstance, ER binds to alternative regulatory DNA sequences such as AP-1, SP-1, cyclic AMP (cAMP)-response element, and upstream stimulatory factor sites, or acts as a coactivator by interacting with other DNA-bound transcription factors, such as c-jun or c-fos proteins, to stabilize the DNA binding of the transcription factor complex or recruit other coactivators to the complex [115-117]. The expression of a variety of proteins involved in cell proliferation and metastasis, such as insulin-like growth factor receptor 1, cyclin D1, c-myc, collagenase, and the anti-apoptosis factor Bcl-2, are regulated in this manner [116;118-120]. Interruption of ERE-independent transcriptional activation,

especially at the AP-1 site, results in the *in vivo* and *in vitro* resistance of breast cancer cells to growth stimulation by estrogen or other growth factors, which indicates that these alternative ER signaling pathways may play an important role in estrogen-mediated breast cancer development and progression [121].

In addition to the above mentioned estrogen-dependent transcriptional activation, several kinases in the growth factor signaling networks, including mitogen-activated kinases (MAPK), Akt, p90 ribosomal S6 kinase (p90RSK), protein kinase A and c-Src, can also activate ER signaling through an estrogen-independent mechanism [122;123]. These kinases can directly phosphorylate several sites of ER such as S104/106, S118, S167, T331 and Y153 [124-130]. For example, both the PI3k-Akt and MAPK pathways can enhance transcriptional activation of ER by phosphorylating ER at serine 167 [124-126]. Additionally, the MAPK pathway can also activate cell proliferation and transform cells by phosphorylating ER at serine 118 [127-130]. Furthermore, these kinases can also indirectly stimulate ERα transcriptional activity by phosphorylating either coactivators for ERα, such as the p160 family coactivator AIB1, or other kinases, such as P90RSK, which can then phosphorylate ER [124;131;132].

These genomic signaling pathways take hours to days to alter responsive genes and produce effects on cells. However, there are some rapid responses to estrogen simulation that occur in minutes, which can not be accounted for by changes in gene expression mediated by nuclear ERs. These rapid responses include the generation of second messengers, such as cAMP, Ca^{2+}, and nitric oxide, as well as the activation of various receptor tyrosine kinase and protein lipid kinase pathways, such as the MAPK signaling pathway, which will affect cell adhesion, migration, survival and proliferation

[133-139]. These non-genomic effects of estrogen are mediated through a membrane-bound form of ERα, ERβ, or both, and its cross-talk with other signal transduction pathways, such as epidermal growth factor (EGF) receptor and insulin-like growth factor receptor-signaling pathways [133;134;140-144]. Alternatively, estrogens might act through non-ER-mediated pathways [145].

In addition to ERs, recent studies indicate that rapid effects of estrogen are also mediated by a novel transmembrane G protein-coupled receptor 30 (GPR30), also known as G protein-coupled ER, based on correlations of receptor expression with estrogen-mediated Erk-1/2 activation, association of receptor with local ER expression in breast cancer cell lines, and cellular estrogen binding [146-148]. GPR30 is also reported to mediate a c-fos–related, ERE-independent genomic signaling pathway of estrogen [149]. In support of the role of GPR30 in mediating estrogen signal transduction, several studies report that estrogen can bind to GPR30 with high affinity [148;150]. The activated GPR30 can then initiate intracellular second messenger signaling pathways and activate Src, which is involved in matrix metalloproteinase activation and heparin-binding EGF-like factor (HB-EGF) release [151;152]. Free HB-EGF can bind EGF receptor and activate multiple downstream events, such as (1) activation of phospholipase C that can produce inositol trisphosphate (IP3) to mobilize calcium; (2) activation of phosphoinositide 3-kinases and its downstream AKT pathway; and (3) activation of MAPK that can lead to the activation of numerous cytosolic pathways and nuclear proteins [151-154]. These findings indicate that GPR30 is involved in the regulation of cell proliferation, invasion, metastasis and target gene expression. Furthermore, decreased expression of GPR30 in human breast cancer and

the strong inverse association of GPR30 with cancer cell proliferation, migration, invasion, metastasis, differentiation and progression have been reported [149;155-166]. In addition, approximately half of classic ER negative breast tumors retain GPR30 expression, which indicates that these tumors (GPR30+/ER-) may remain responsive to estrogen-targeted treatment through the GPR30 signaling pathway [164]. Therefore, these important roles of GPR30 in estrogen-induced signal transduction and the strong association between GPR30 and breast cancer development and progression indicate that GPR30 may be a novel therapeutic target for breast cancer treatment, especially for ER negative breast cancer patients.

Another theory that can explain the involvement of estrogens in breast carcinogenesis is that reactive metabolites of estrogens, especially estrogen catechols and estrogen quinones, can directly induce mutations [167-175]. Enzymes that are involved in estrogen carcinogenic metabolism will be discussed in detail in the following sections.

2.4 Estrogen genotoxic metabolite metabolism

Once formed, estrogens are subjected to extensive local metabolism, such as oxidative and conjugation reactions, that can lead to either their deactivation and subsequent elimination or to the generation of genotoxic metabolites [176-178]. Therefore, further estrogen metabolism after biosynthesis is of great importance for both determining the estrogen levels in breast tissue and breast carcinogenesis. The main types of metabolic reactions include oxidation by cytochromes P450 (CYPs), *O*-methylation by catechol *O*-methyltransferase (COMT), glucuronidation by UDP-glucuronosyl transferases (UGTs), and sulfonation by sulfotransferases (SULTs).

Oxidation: Endogenous estrogens undergo oxidative metabolism catalyzed by various CYP enzymes in both liver and estrogen target organs (e.g., breast). (Fig. 1.1). However, since liver and estrogen target organs express different CYP enzymes, it is necessary to consider the specific CYPs that are expressed in human mammary tissue when considering the role of CYP-mediated metabolism in local estrogen regulation [176;179-182].

CYP1B1 is over-expressed in many kinds of tumors relative to normal tissues [183-186]. CYP1A1 is an inducible enzyme that has high catalytic activity for 2-hydroxylation (2-OH) of E2 and E1, while CYP1B1 has a distinct, selective activity for the 4-hydroxylation (4-OH) of E2 and E1 [187]. Because the expression level of CYP1B1 is higher than CYP1A1, 4-hydroxylation is the dominant pathway of E2 oxidation in human breast and uterus [188;189].

CYP enzymes also catalyze the further oxidation of the 2-OH and 4-OH catechol estrogens (2-OHE and 4-OHE) to reactive semiquinones and quinones that can directly form several types of DNA adduct [190-192]. Moreover, both catechol estrogens and their quinone/semiquinone metabolites can indirectly damage DNA and protein through redox cycling and generation of reactive oxygen species (ROS) [193-195]. Therefore, CYP-mediated estrogen metabolism can lead to the formation of both oxidative DNA damage and estrogen DNA adducts, which implies that these estrogen metabolites are potential initiators of tumor formation.

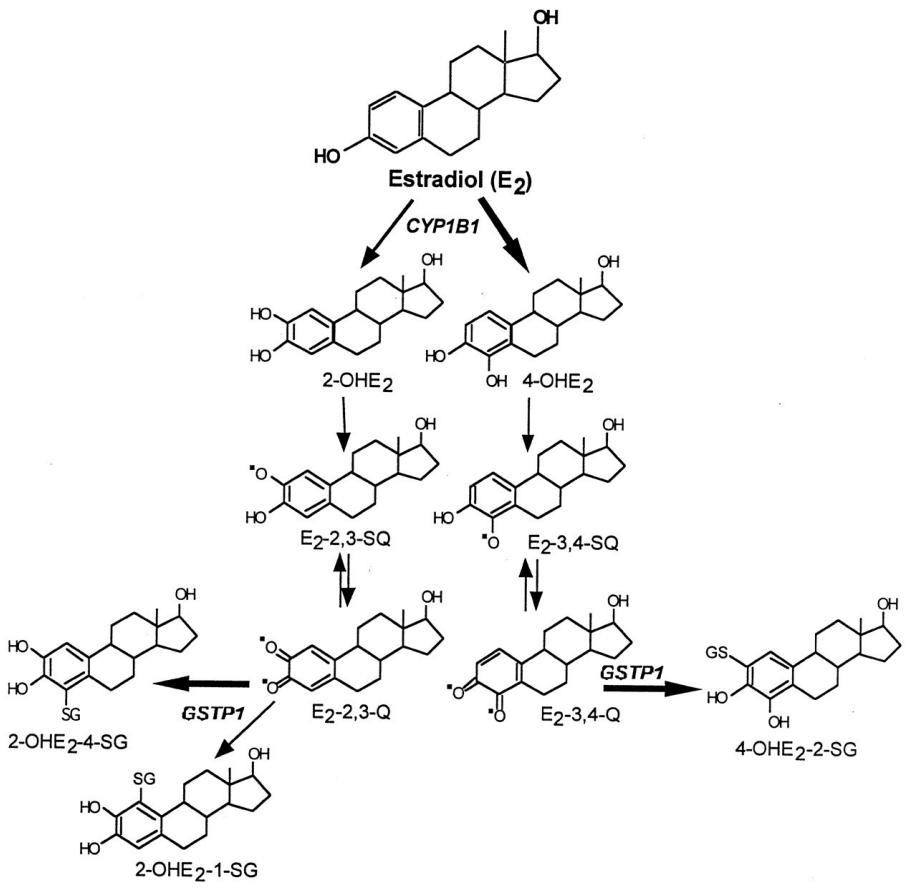


Fig. 1.1 Pathways of oxidative estrogen metabolism**.** CYP1B1 catalyzes the oxidation of E2 to catechol estrogen 2-OHE2 and 4-OHE2. The catechol estrogens are further oxidized to semiquinones (E2-2,3-SQ and E2-3,4-SQ) and quinones (E2-2,3-Q and E2-3,4-Q). GSTP1 catalyzes the conjugation of GSH to the estrogen quinones, leading to the formation of 2-OHE2-4-SG, 2-OHE2-1-SG, and 4-OHE2-2-SG. CYP1B1 preferentially forms 4-OHE2, and GSTP1 favors the formation of 4-OHE2-2-SG and 2-OHE2-4-SG as indicated by the larger arrows. (Figure from Hachey D L *et al*. Cancer Res 2003; 63:8492-8499)

Experiments on cell transformation, mutagenicity and carcinogenesis have suggested that 4-OHE2 is more carcinogenic than 2-OHE2 [193;196-200;200-202]. It has been found that 4-OHE2 is a stronger ER agonist than the parental E2, which makes this metabolite a potent promoter of tumor formation [203]. Elevated levels of 4-OH-estrogens have been measured in breast adenocarcinomas [204]. Treatment with

4-OHE2 enhanced adrenal tumor formation in the Syrian hamster [193;200], and neonatal exposure to E2, 2-OHE2, and 4-OHE2 induced endometrial carcinomas in 7, 12, and 66%, respectively, of treated CD-1 mice [202]. 4-OHE2 was reported to increase the production of free radicals that cause subsequent hydroxyl radical-mediated damage to DNA, whereas 2-OHE2 failed to induce oxidative DNA damage, possibly due to rapid methylation by COMT [200;201]. A higher 4-OHE2:2-OHE2 ratio has been detected in benign and malignant mammary tumors than in adjacent normal tissue [198;199]. The 2-hydroxylated estrogen metabolites, when compared with 4-OHE2, have a faster rate of metabolism by COMT, a more rapid clearance *in vivo*, and possess weaker hormonal potency in estrogen target tissues [205-210]. Moreover, 2-OHE2 and its methylated metabolite, 2-methoxyestradiol, have actually been shown to inhibit breast cancer growth and angiogenesis, both *in vivo* and *in vitro*, which may be an important reason for the lack of carcinogenicity of 2-OHE2. [196;196;197;197;211;212]. Therefore, all of these findings indicate that 4-OHE2 and CYP1B1, the main enzyme that catalyzes the formation of 4-OHE2, play an important role in breast carcinogenesis.

***O*-methylation**: The *O*-methylation of catechol estrogens is catalyzed by COMT, an S-adenosylmethionine-dependent methyltransferase enzyme [213]. The COMT gene is located on chromosome 22q11 and this enzyme exists in two forms, soluble cytosolic COMT, which is the main form of COMT, and membrane-bound COMT [213;214]. Recently, COMT has been closely associated with breast cancer. COMT inactivates catechol estrogens, the most carcinogenic and genotoxic estrogen metabolites, and therefore prevents both direct DNA damage and oxidative genomic

alterations [190-195]. The monomethylated estrogen metabolites have essentially no ER binding affinity, which suggests that COMT mediated *O*-methylation is a detoxification pathway for these catechol intermediates [215;216]. The methylated product of 2-OHE2, 2- methoxyestradiol, inhibits the proliferation of several cancer cell lines and is among the most potent endogenous inhibitors of angiogenesis, indicating that COMT may play a protective role against estrogen-induced breast cancer [196;197;217;218].

Glucuronidation: The superfamily of microsomal UGT enzymes catalyzes the conjugation of UDP-glucuronic acid to various endogenous and exogenous aglycones, including estrogen [219]. Steroid hormone glucuronidation has been identified in several human organs including the breast [220;221]. Glucuronide conjugates of estrogens and catechol estrogens are biologically inactive, more polar than the parent molecules, and readily excreted in urine and bile [220].

Sulfonation: Sulfate conjugation (sulfonation) is an important pathway in the biotransformation of many hormones, neurotransmitters, and xenobiotic compounds [222]. Sulfonation reactions are catalyzed by members of two distinct enzyme superfamilies: (1) the membrane-bound sulfotransferases, located in the Golgi apparatus, that catalyze the sulfonation of proteins, peptides and glycosaminoglycans and (2) the cytosolic sulfotransferases (SULTs) that metabolize xenobiotics and endogenous compounds such as estrogens [223-225]. The SULT superfamily is divided into families that are designated by Arabic numerals; SULT family members share at least 45% amino acid sequence identity [226]. Each family can then be further

subdivided into subfamilies that are designated by capital letters; SULTs in the same subfamily share at least 60% amino acid identity. Individual SULT enzymes within subfamilies are then identified using Arabic numerals [226-230]. SULTs catalyze the transfer of a $–SO_3$ group from the cosubstrate 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to the hydroxyl group of sulfonation targets [231].

Extensive biotransformation of endogenous and exogenous estrogens to sulfonated conjugates, especially the most abundant circulating estrogen sulfates, has long been recognized as a major route of estrogen metabolism in humans [232]. Estrogen sulfonation decreases estrogenic activity by facilitating estrogen excretion and blocking ER-mediated activity [233-235]. Estrogen sulfotransferase activity has been detected in various human tissues, including liver, small intestine, kidney, uterus, adrenal gland, and breast [236-238]. The *in situ* sulfonation of estrogen in estrogen target tissues contributes significantly to estrogen bioavailability in those tissues [239]. Several studies have associated SULT activities with breast cancer, although there are some contradictory findings [240-244].

There are mainly three SULTs, SULT1E1 (also known as estrogen sulfotransferase), SULT1A1 (as known as phenol-sulfotransferase) and SULT2A1 (also known as DHEA sulfotransferase), which can catalyze the sulfonation of E1 and E2 with different affinities [224;228;245]. Among the SULTs, SULT1E1 exhibits the highest affinity for estrogen, especially the potent E2*,* and is the only SULT that sulfonates estrogen at physiological nanomolar concentrations of the hormone [246-248]. SULT1E1 is highly expressed in normal human breast epithelial cells, but its expression is often attenuated in breast cancer and malignant breast cancer cell lines [249-251].

SULT1E1 immunoreactivity has been shown to correlate negatively with tumor size and to associate significantly with a reduced risk of recurrence or improved prognosis [97]. SULT1A1 and, to a lesser extent, SULT2A1 are the main forms of SULT expressed in breast tumors [249;252;253]. However, SULT1A1 sulfonates E2 at micromolar concentrations; the affinity of SULT1A1 for E2 is about 300-fold lower as compared to SULT1E1 [254]. Therefore, SULT1E1 is the primary SULT that regulates the availability of estrogens in the breast and consequently affects cell growth, and abnormal expression of SULT1E1 may contribute to the development and growth of breast carcinomas. This hypothesis has been supported by the finding that forced SULT1E1 expression in the human breast cancer cell line MCF7 significantly suppressed E2-stimulated cell proliferation and DNA synthesis, suggesting that breast tumors may maximize E2 levels *in situ* to promote tumor growth by suppressing the expression of SULT1E1 [255]. To date, little is known about the regulation of SULT1E1 expression.

3 MCF10A model

The MCF10A-derived lineage of human breast epithelial cell lines is a good model to study the molecular events that occur during breast cancer progression because these cell lines were all derived from a common genetic background and the model includes the full spectrum of neoplastic progression and incorporates aspects of both indolent preneoplastic diseases to aggressively neoplastic breast epithelial cell growth.

The pathology of breast cancer tumorigenesis is a multistep sequential process that begins with the development of hyperplasia, subsequent progression through ductal carcinoma *in situ* (DCIS), and finally malignant invasive carcinoma (Fig. 1.2). In

support of this progression model, several studies have demonstrated that the risk for later breast carcinoma and the frequency of molecular changes involving cell-cycle regulation and apoptosis that are found in malignant invasive cancer are both increased during progression in this continuum [256-262].

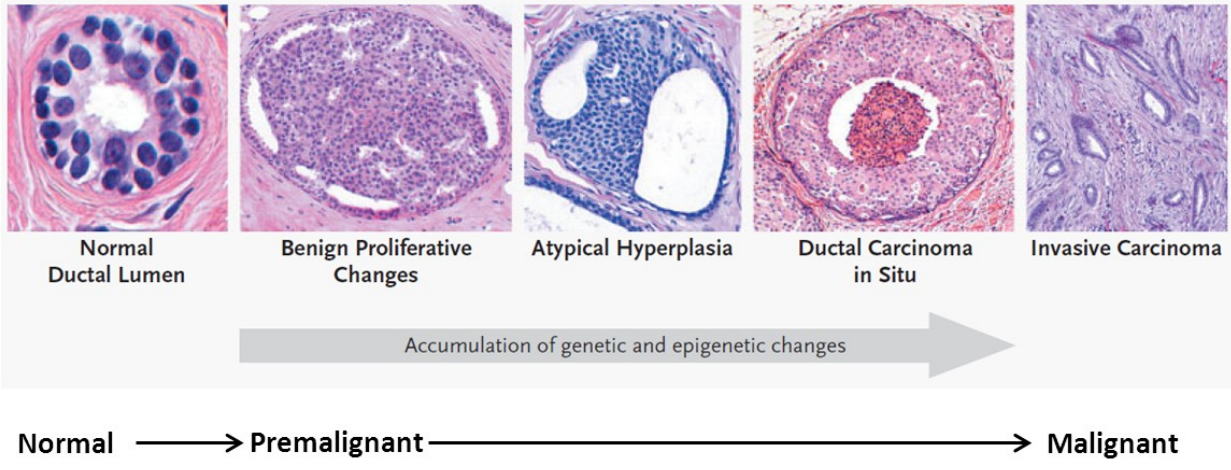


Fig. 1.2 The progression processes of human breast cancer. (Figure adapted from Burstein,H.J., *et al,* N.Engl.J.Med, 350, 1430-1441)

1) Hyperplasia: Within the mammary gland there is a vascularized fibro-fatty stroma supporting a complex network of branching ducts at the end of which is the functional unit of the breast, the terminal duct lobular unit (TDLU). It has been shown morphologically that most of the benign and malignant epithelial proliferations in the breast are derived from the TDLU [263]. The normal TDLU is lined by two cell layers consisting of an inner cuboidal epithelium, and an outer myoepithelial cell layer (also named the basal layer). These cells form a distinct luminal border around the empty space of the breast duct. Any proliferation that results in an increase of more than the normal 2 layer system is called hyperplasia [264].

Hyperplasia is usually the first step in the progression toward breast cancer and it can be further classified as 'typical' (also called usual ductal hyperplasia), including mild hyperplasia (2-4 layers of epithelial cells), moderate hyperplasia (4 or more layers of epithelial cells) and florid hyperplasia (Fig. 1.3) or 'atypical' [265]. About 60% of ‘typical’ hyperplasia has ER over-expression in most of the cells compared with normal epithelium [266-268]. However, only the atypical hyperplasia (AH) variety is of concern for possible breast cancer [269;270].

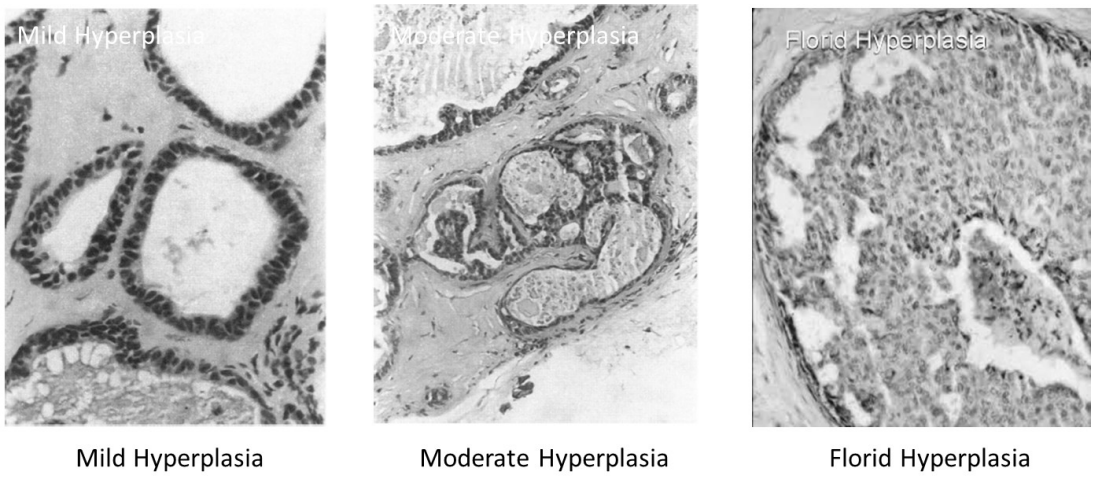
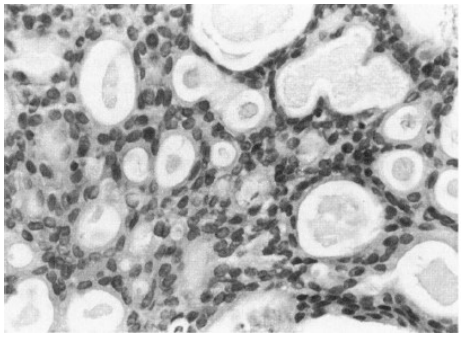


Fig. 1.3 Histological features of hyperplasia. Picture adapted from Dawson *et al* [319].

With florid hyperplasia, the lumen is distended and irregular, and the luminal spaces are filled or partially filled with proliferating epithelial cells [271;272]. The individual cells vary in size and shape from ovoid to spindle, elongated reniform, but with normal chromatin pattern and indistinct nucleoli [271;272]. Florid hyperplasia is associated with a 2-fold increased risk of breast cancer compared with the general population in the same age pool [256;271].

AH (Fig. 1.4) is an intermediate stage between hyperplasia of the usual type and DCIS. Therefore, AH contains cytological and architectural features of both of these other stages [273]. For example, AH has distended ducts, a population of relatively uniform small- or medium-sized round, cuboidal or polygonal cells, enlarged but evenly distributed hyper-chromatic nuclei, marked cellular proliferation and a regular micro-papillary configuration [273]. Mitoses, particularly abnormal forms, are infrequently seen [273].

AH is considered to be precancerous and can be detected more frequently in malignant breasts than in benign breast disease [274]. AH lesions have been reported to have an increased proliferation and growth advantage over normal epithelium, which has been attributed to the expression of a mutated, estrogen hypersensitive ER [275]. It is clear that AH is a rare condition only being seen in 4-5% of benign biopsies but it confers a 4-5 time increased chance of developing breast cancer when compared with an age-matched general population [276;277].



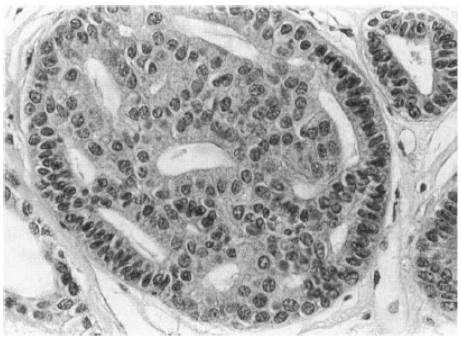
Atypical Hyperplasia

Fig. 1.4 Atypical Hyperplasia. Figure adapted from Dawson *et al* [319].

The specific criteria for distinguishing ‘typical’ hyperplasia from AH have been well established [256]. ‘Typical’ hyperplasia is characterized by cellular variability, nuclear overlap, and indistinct cell borders [256].

2) DCIS: ‘Typical’ and ‘atypical’ hyperplasia and DCIS have traditionally been categorized as intraductal proliferative lesions [276]. After the onset of mammography

screening, DCIS accounted for about 20% of all breast cancers detected in North America [278;279]. DCIS is a pre-invasive malignant proliferation of breast epithelial cells and has been demonstrated to be the evolutionary origin of invasive cancers because both of them present the same chromosomal alterations [280;281].



Ductal Carcinoma in situ

Fig. 1.5 Figure adapted from Dawson *et al* [319].

In this stage (Fig.1.5), ducts are grossly distended by tumor cells and the central area occasionally shows extensive comedo necrosis. DCIS shows a micro-papillary growth pattern and forms rigid intraluminal bridges with well-defined round spaces. The epithelial cells of DCIS tend to have distinct cell boundaries and become monomorphic. Chromosomal imbalance, prominent and multiple nucleoli, and enlarged nuclei are also seen in this stage. DCIS lacks evidence for invasion across the basement membrane, which can be confirmed by immunohistochemical (IHC) assessment of markers of the myoepithelial cell layer and the basement membrane surrounding the ductal lumen [282;282]. However, it has been reported that high-grade DCIS is remarkable for the break down

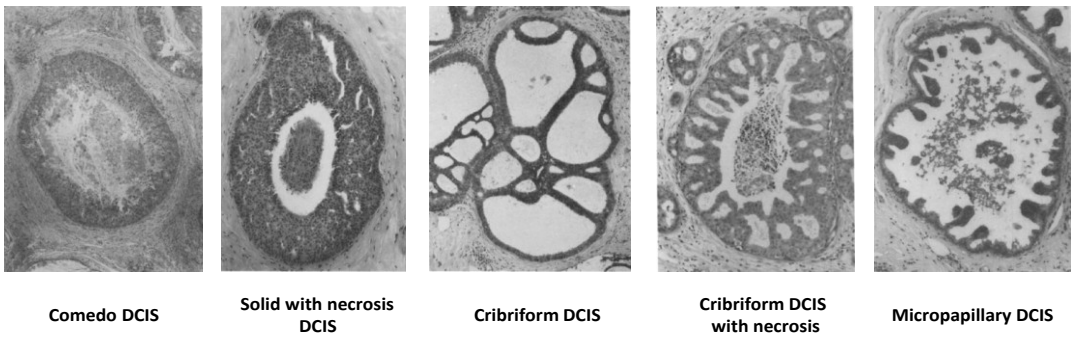


Fig.1.6 Comedo, solid, cribriform, and micropapillary ductal carcinoma *in situ*. Picture adapted from Bellamy *et al* [287].

of the myoepithelial cell layer and basement membrane surrounding the ductal lumen [283].

The expression profile of genes and proteins that are involved in cell proliferation, differentiation, signal transduction, interaction between cells and the surrounding extracellular matrix, and intracellular transport has been characterized and compared among different stages of breast cancer progression [284;285]. The data indicate that most of the critical changes during breast tumorigenesis happen in DCIS and prior stages [285]. A recent study showed that high grade DCIS had a greater tendency to be HER2 positive and basal-like than lower grade DCIS, which indicates that DCIS may be the precursor of basal-like invasive breast cancer [286].

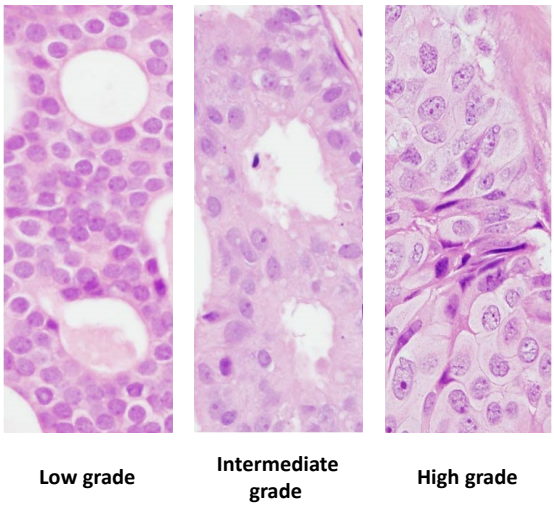


Fig. 1.7 Nuclear grading. Picture adapted from 'Breast Pathology on the Web'

It is well accepted that DCIS consists of a heterogeneous range of lesions with diverse histopathological features, genetic alterations, molecular biomarkers, and the chance of progression to invasive cancer. Various systems have been used to classify DCIS. The traditional classification is mainly based on architectural growth pattern and nuclear grading [287]. According to this system, DCIS can be further classified as comedo, solid, cribriform, micropapillary and papillary DCIS [287;288] (Fig. 1.6). However, this system provided poor reproducibility and inadequate ability to predict the potential for progression to invasive disease. Therefore, numerous updated

classification criteria have been proposed mainly based on lesion size, nuclear grading, necrosis, calcification, cytonuclear features and molecular bio-markers [289-293]. It is important to note that nuclear grading is the single criterion that has been shown to associate closely with recurrence after surgical excision of DCIS. As shown in Fig. 1.7, nuclei were graded into three levels:

Low grade: Nuclei are spherical, monotonous in appearance, small and centrally placed. Chromatin is finely distributed and mitoses are rare [294].

High grade: Nuclei are irregular in shape, pleomorphic, large and varied in size. Coarse chromatin, prominent multiple nucleoli and frequent mitoses are also commonly found in this level [294].

Intermediate grade: Nuclei show features in between those described for low and high grade [294].

In summary, based on the criteria described above, DCIS can be further classified into two grades. High grade DCIS is comprised of a group of atypical cells with high or intermediate grade nuclei and exhibits several growth and architectural patterns, including solid, micropapillary and cribriform. Comedo-type central necrosis with calcification is common in this grade. High grade DCIS tends to be ER-negative and HER2-positive and contains a series of other genetic changes [295-297]. Low grade DCIS contains a population of proliferative monomorphic cells with low grade nuclei, and the cells are generally arranged in micropapillary and cribriform patterns. This grade of DCIS is frequently ER-positive [298;299].

Low grade DCIS and AH are morphologically and histologically similar and hard to distinguish. However, low grade DCIS is associated with a significantly higher risk of

invasive breast cancer development compared to AH [270;271]. Therefore, it is clinically important to separate low grade DCIS from AH. The diagnostic distinction between AH and DCIS is fundamentally based on the extent of involvement (Fig. 1.8). If the proliferative cell amount is limited and does not congest the entire duct space, AH is identified [270;276]. DCIS is diagnosed if the cellular proliferation involves two or more adjacent duct spaces and is at least 2 mm in aggregate length [300].

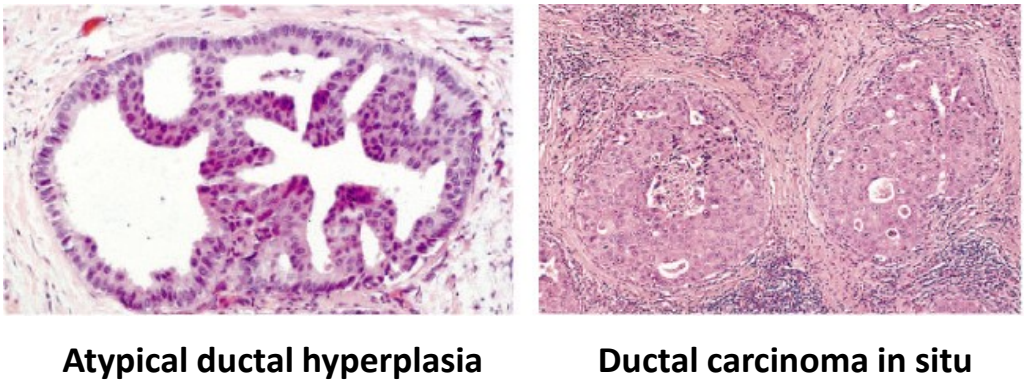


Fig. 1.8 The diagnostic distinction between atypical ductal hyperplasia and ductal carcinoma *in situ.* Picture adapted from Wiechmann,L. and Kuerer, H.M., 2008, Cancer, 112, 2130-2142.

3) **Invasive breast cancer**: Invasive breast cancer is an extremely heterogeneous disease in its rate of proliferation, nuclear morphology, stromal response, pattern of infiltration, degree of differentiation and clinical course. Different types of carcinoma have distinct prognostic outcomes and treatment options [301]. For example, tubular carcinoma and papillary carcinoma have a better prognosis compared with invasive ductal carcinoma, not otherwise specified [302].

Thanks to microarray technology, the molecular changes that enable normal epithelial cells to progress to invasive, metastatic disease are increasingly well understood [303;304] and the gene expression profiles are used to discriminate

different subtypes of breast cancer. According to ER-expression (normally expressed by luminal breast epithelial cells), breast cancer can be divided into two groups: ER-positive and ER-negative [284;303;305]. The risk factors, precursors, clinical behaviors and outcomes differ between ER-positive and ER-negative breast cancers [306-311]. The ER-negative group can be further subdivided into: basal-like, HER2-positive and normal-like tumors [284;303;305]. Basal-like breast cancers, a subtype originating from the basal epithelial layer, overlap with the so called triple-negative breast cancer (TNBC) (ER-, PR-, and HER2-negative). TNBC has become a focus of intense research since TNBC patients have a younger age at onset, higher tumor grade, larger tumor size, and increased propensity to develop metastases. Most importantly, they lack the three most significant therapeutic markers for clinical management resulting in the worst outcome when compared with other cancer subtypes [168;216;306;312;313].

The MCF10A-derived lineage of human breast epithelial cell lines is a good model to study TNBC because the resulting cancer cell lines of this lineage (MCF10CA) are triple negative [314;315]. This model mimics the development of human breast carcinoma from benign hyperplasia through AH to DCIS and eventually to malignant invasive tumors with the potential to metastasize. The parental **MCF10A cell line** was derived from spontaneously immortalized breast epithelial cells that were obtained from a donor with benign proliferative breast disease [315]. MCF10A cells are ER negative, near diploid and non-tumorigenic because MCF10A cells do not give rise to persistent lesions when xenografted into immunodeficient mice [315-317]. The MCF10A cell line has been used extensively as a model of normal breast epithelial cells because they maintain typical breast epithelial characteristics such as formation of acini in collagen,

lack of anchorage-independent growth, and requirement for hormones and growth factors to grow in culture [316].

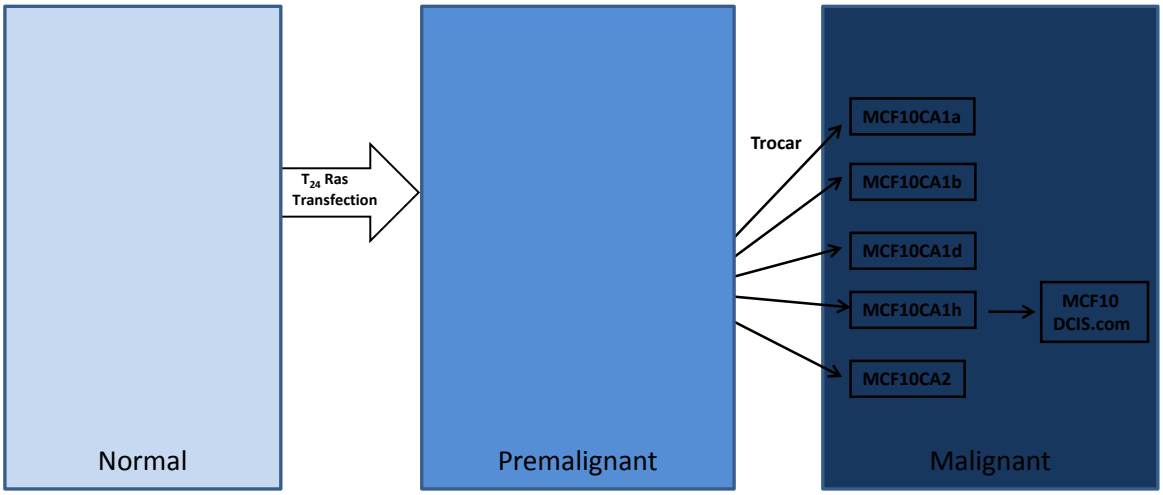


Fig. 1.9 The production of MCF10A lineage Human Breast Cancer Cell Lines. Figures adapted from [318,319]. The parental MCF10A cell line was derived from spontaneously immortalized breast epithelial cells that were obtained from a donor with benign proliferative breast disease. *In vitro* stable transformation of MCF10A cells with the mutated c-Ha-*ras* oncogene resulted in development of the premalignant MCF10AneoT cell line. Serial passage of xenograft lesions formed by MCF10AneoT cells led to the establishment of preneoplastic MCF10AT1 and MCF10AT1Kcl2 cell lines. A 292-day old MCF10AT1Kcl2 xenograft that progressed to adenocarcinoma was the source of the malignant MCF10CA variant series after serial passage of humor pieces. The MCF10DCIS.com cell line was developed by cloning of one of the malignant variants, MCF10CA1h

In vitro stable transformation of MCF10A cells with the mutated c-Ha-*ras* oncogene resulted in development of the premalignant **MCF10AneoT** variant cell line that slowly generates simple ductal lesions within 7-8 weeks when xenografted into nude mice (Fig. 1.9) [56]. Serial passage of xenograft lesions formed by MCF10AneoT cells led to the establishment of preneoplastic **MCF10AT1** and **MCF10AT1Kcl2** cell lines [318]. The MCF10AT1 cell line was established from a 100-day old MCF10AneoT squamous carcinoma explant lesion. When xenografted into nude mice, MCF10AT1

cells produced a spectrum of preneoplastic lesions, including AH and DCIS [317]. MCF10AT1 cells demonstrate a ~25% incidence of invasive cancers in xenograft lesions that develop over an extended period of time [319]. The MCF10AT1Kcl2 cell line was established from a 367-day old MCF10AneoT adenocarcinoma xenograft lesion. The MCF10AT1K.cl2 variant retained an MCF10AT1-type pattern of xenograft growth but also displayed chromosomal aberrations more characteristic of genetic instability and could form hyperplastic lesions more rapidly [318].

A 292-day old MCF10AT1Kcl2 xenograft that progressed to adenocarcinoma was the source of the malignant **MCF10CA** variant series after serial passage of tumor pieces. Rather than forming simple ducts, MCF10CA variants rapidly form highly proliferative invasive carcinomas at an incidence of 100% [318]. The **MCF10DCIS.com** cell line was developed by cloning of one of the malignant variants, MCF10CA1h [320]. MCF10CA1h was derived from a lesion formed by two successive trocar passages of a lesion formed by premalignant MCF10AT cells. Injection of MCF10DCIS.com cells into immunodeficient mice produces rapidly growing lesions that are consistent with the poor prognosis comedo-type DCIS and invariably progress to invasive cancer [320].

4. Statement of problem

Despite a growing understanding of SULT1E1's function in steroid hormone and drug metabolism, the role of SULT1E1 in the malignant progression of breast epithelial cells is still unknown. We previously reported that SULT1E1 mRNA was expressed in the "normal" breast epithelial MCF10A cells and in the preneoplastic MCF10AT1 and MCF10AT1Kcl2 cell lines [250]. However, the expression was "switched off" in the more

neoplastically progressed variant cell lines, beginning with the MCF10CA1a cell line [250]. Thus, MCF10AT1 cells that have preserved SULT1E1 expression and E2-ER regulated growth can be used to determine the impact of SULT1E1 expression on tumorigenicity. In the same study, we observed a proliferation state-dependent expression of SULT1E1 in MCF10A cells [250]. Therefore, the regulatory mechanism for this phenomenon was also investigated.

The results of this research will provide new information on the role of SULT1E1 in breast cancer progression. Since SULT1E1 has high affinity for physiological concentrations of estrogen, SULT1E1 may represent a good target for the prevention and treatment of breast cancer in humans.

Chapter 2 of the dissertation discusses the expression of estrogen-metabolizing genes including cytosolic sulfotransferases (SULT1E1, SULT1A1, SULT2A1, and SULT2B1), STS, CYP19, 17β-hydroxysteroid dehydrogenases (17βHSD1 and 2), CYP1B1, and catechol-*O*-methyltransferase (COMT) in the MCF10A-derived lineage cell culture model for basal-like human breast cancer progression and in ERα-positive luminal MCF7 breast cancer cells. Chapter 3 delineates the role of the aryl hydrocarbon receptor (AhR) in the transcriptional regulation of SULT1E1 in the non-tumorigenic MCF10A cell line. In chapter 4, the mechanism underlying tobacco smoke condensate (TSC)-mediated down-regulation of SULT1E1 expression in MCF10A cells is discussed. In chapter 5, the impact on tumorigenesis of SULT1E1 knock-down in the MCF10AT1 xenograft model is presented.

CHAPTER 2

Expression of estrogenicity genes in a lineage cell culture model of human breast cancer progression

INTRODUCTION

The estrogen sensitive nature of breast cancer was observed by Beatson over 100 years ago [321]. Prolonged cumulative exposure to estrogen during a woman's lifetime is a significant risk factor for the development of breast cancer [322]. Estrogen stimulates cell growth through ER-mediated events, which can enhance opportunities for DNA mutation. In addition, reactive estrogen metabolites generated by cytochrome P450-mediated catalysis, such as estrogen catechols and quinones, can directly induce mutations [167]. Particularly in post-menopausal women who lack ovarian sources of estrogen, the factors that govern intra-tissue metabolism of biologically active estrogen (i.e., estrogen "intracrinology") are important therapeutic targets and biomarkers for breast cancer progression [323]. Determinants affecting the amount and activity of estrogen in breast tissue include expression of the major forms of estrogen receptor (ERα and ERβ), cytosolic sulfotransferases (SULTs), STS, CYP19, 17β-hydroxysteroid dehydrogenase types 1 and 2 (17βHSD1 and 17βHSD2), CYP1B1, and COMT (Fig. 2.1 adapted from [246;324;325]).

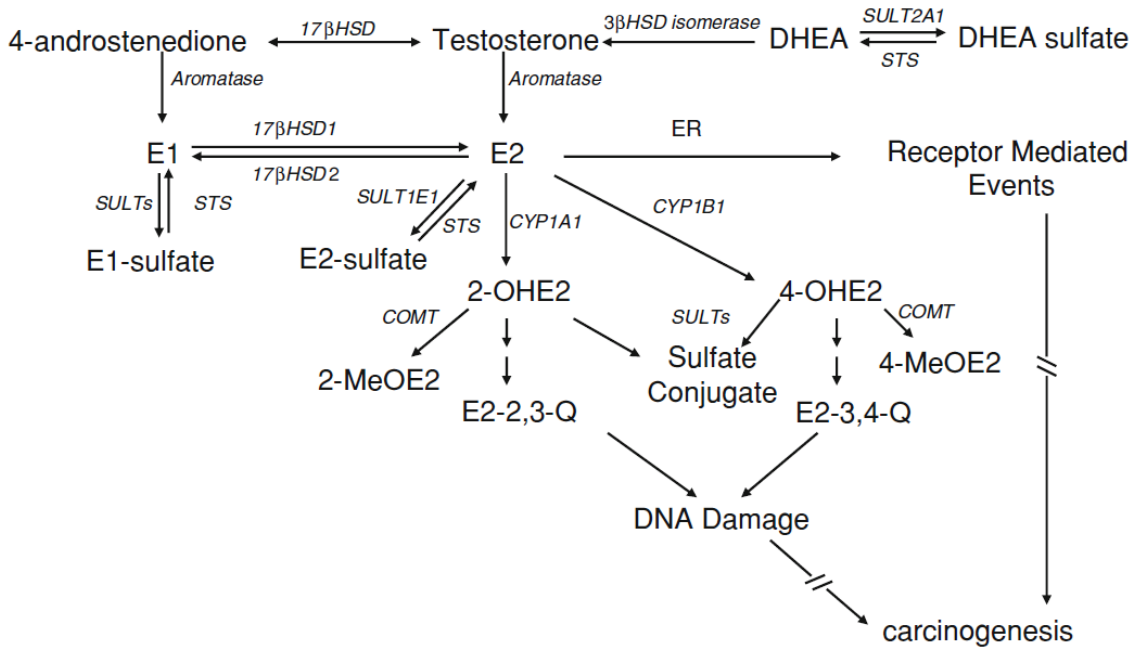


Fig. 2.1 Pathways of estrogen metabolism, bioactivation, and action. CYP19 (aromatase) catalyzes the aromatization of androstenedione and testosterone to form estrone (*E1*) and 17β-estradiol (*E2*), which can bind to and activate ER. Of the cytosolic SULTs, SULT1E1 preferentially catalyzes the sulfonation of E1 and E2 with high efficiency. Sulfonated estrogens are ER-inactive. Steroid sulfatase (*STS*) catalyzes the deconjugation of sulfonated steroids and favors the formation of biologically active estrogen. STS and 3-β-hydroxysteroid dehydrogenase (*3βHSD isomerase*) function in the formation of sex steroids from precursor hormones, such as dehydroepiandrosterone sulfate (DHEA sulfate) and dehydroepiandrosterone (*DHEA*). 17βHSD1 reduces E1 to the more potent E2, while 17βHSD2 oxidizes E2 to E1. The 4-hydroxylation of E2 is catalyzed by CYP1B1; E2 2-hydroxylation is catalyzed by CYP1A1. Catecholestrogens auto-oxidize to form mutagenic orthoquinone electrophiles that can be detoxified via *COMT*-mediated conjugation.

This study, published in Breast Cancer Research and Treatment [250], was designed to elucidate the expression of determinants of estrogen activity in MCF10A derived lineage cell culture model that captures the heterogeneity of breast cancer progression in humans.

The molecular phenotypic characteristics of the MCF10A lineage indicate that this is a model of basal breast cancer, in that the resulting MCF10CA1 cancer cell lines are triple negative (i.e., do not express ER, PR or HER2/neu) [314;315]. Nevertheless, xenograft studies have demonstrated that progression of preneoplastic MCF10AT1 cells is sensitive to estrogen manipulation [319;326-328]. Estrogen has been shown to alter signal transduction in triple negative MDA-MB-231 (do not express ERα; do express ERβ) and SKBR3 cells (express neither ERα nor ERβ), causing activation of the extracellular signal regulated kinases, ERK1 and ERK2 [147]. Both estrogen and EGF induced phosphorylation of c-raf and ERK1/2 while stimulating the proliferation of SKBR3 cells [329], and the two agents were more effective in combination. Importantly, growth of ERα-negative breast cells was induced by the same 17β-estradiol (E2) concentration (10^{-9} M) that stimulates ERα-positive MCF7 cells, whereas a higher concentration (10^{-6} M) inhibited the growth of MDA-MB-231, and SKBR3 cells, and this inhibition was additive with heregulin [330].

Although MCF7 requires supplementation with E2 to mimic pre-menopausal human serum levels (typically 400–1,500 pg/ml) to achieve xenograft growth in nude mice, MCF10AT1 forms lesions that progress to atypical hyperplasia, DCIS and invasive ductal carcinoma without E2 supplementation. Serum E2 levels in nude mice are typically less than 50 pg/ml [331], which mimics post-menopausal human serum levels [332]. However, MCF10AT1 cells form lesions consisting only of simple ducts in ovariectomized (OVX) nude mice [333], which have serum E2 levels of ~5 pg/ml [334]. Therefore, MCF10AT1 xenografts respond to very low doses of E2 in vivo, suggesting that the levels of estrogen-processing enzymes in breast cells may play a critical role in

determining growth response. Therefore, an analysis of the determinants of estrogenicity in MCF10A lineage cell lines was undertaken to provide insight into the dynamic changes in estrogen responsiveness that occur during basal breast cancer development.

Histone deacetylases (HDACs) are being increasingly recognized for their important roles as transcriptional modulators of pro-survival pathways during breast cancer progression [335]. HDAC inhibitors, such as Vorinostat, Valproic acid, and Panobinostat, are currently tested in many phase I and II clinical trials as single agents as wells as in combination schemes in the treatment for breast cancer. They have demonstrated to have promising antitumor activity, favorable clinical effects and, most importantly, encouraging activity in reversing hormone resistance [336-339]. However, their effects on the expression of estrogen metabolism machinery during breast cancer progression are unknown. Therefore, the effects of treatment with trichostatin A (TSA), a potent HDAC inhibitor, on ER and estrogen metabolism gene expression in the MCF10A lineage cell lines were also investigated.

Materials and methods

Materials. TaqMan Gene Expression reagents were purchased from Applied Biosystems (Foster City, CA). qPCR Human Reference Total RNA was purchased from Clontech Laboratories (Mountain View, CA). Culture media, sera, L-glutamine, sodium pyruvate, penicillin-streptomycin, anti-SULT1E1 antibody, recombinant human SULT1E1, Superscript II, and Lipofectamine 2000 were purchased from Invitrogen

Corporation (Carlsbad, CA). EGF was purchased from BD Biosciences (San Jose, CA). Recombinant human insulin (Novolin R) was purchased from Novo Nordisk Pharmaceuticals, Inc. (Princeton, NJ). Cholera toxin, hydrocortisone and TSA were purchased from Sigma–Aldrich (St. Louis, MO). Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ECL Plus Western Blotting Detection Reagents and Hybond-P membranes were purchased from GE Healthcare (Piscataway, NJ). Other materials were obtained from the sources indicated below.

Cell culture. MCF7 and MCF10A lineage (MCF10A, MCF10AT1, MCF10AT1K.cl2, MCF10CA1a, MCF10CA1d and MCF10DCIS.com) cell lines were obtained from the Cell Resources Facility of the Barbara Ann Karmanos Cancer Institute, Wayne State University. MCF10A lineage cell lines were cultured in phenol red-free Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM/F12) nutrient mixture (1:1) supplemented with 10 μg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, 5% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. MCF7 cells were cultured in phenol red-free Minimum Essential Medium supplemented with 10 μg/ml insulin, 1 mM sodium pyruvate, 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell lines were routinely maintained in T75 flasks in a 37°C humidified environment of 5% CO_2/95% air. For experiments, 250,000 cells were plated into 60 mm dishes. For pre-confluent cultures (~70% confluency), cells were harvested 2 days after plating. Confluency was reached approximately 5 days after plating. On day 7, confluent cultures were harvested for preparation of total RNA. Experiments were conducted at a cell density of 70% confluency unless otherwise indicated. For

TSA studies, 24 hr after plating, cells were treated with 0.1% dimethyl sulfoxide (DMSO control) or TSA.

TaqMan Gene Expression assays. Total RNA was prepared from individual dishes of cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA samples were reverse transcribed using Superscript II. Transcript levels were measured using the following TaqMan Gene Expression Assays: Hs01046818_m1 (ERα), Hs00230957_m1 (ERβ), Hs00193690_m1 (SULT1E1), Hs00419411_m1 (SULT1A1), Hs01105284_m1 (SULT2B1), Hs00234219_m1 (SULT2A1), Hs00165853_m1 (STS), Hs00240671_m1 (CYP19), Hs00166219_g1 (17βHSD1), Hs00157993_m1 (17βHSD2), Hs00164383_m1 (CYP1B1), and Hs00241349_m1 (COMT). Each PCR reaction included 2 μl of cDNA template, a primer/probe (5-carboxyfluorescein fluor, minor groove binder quencher) set, a primer-limited primer/probe (VIC-minor groove binder) set for 18S rRNA and Universal PCR master mix, and amplifications were performed using an ABI Prism 7500 Sequence Detection System. Thermocycling parameters were 94°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Cycle threshold (Ct) values were obtained using the SDS software package. For each sample**,** ΔCt was obtained by subtracting the Ct of 18S rRNA from the Ct of target mRNA. Then, ΔΔCt values were calculated by subtracting the ΔCt of the calibrator to which the other samples were compared from the ΔCt of each sample. Mean relative quantitative expression values were then calculated as $2^{-\Delta\Delta Ct}$.

Western blot and enzyme activity analyses. Cells in T75 flasks were washed with and scraped into ice-cold phosphate-buffered saline (PBS). Cells were pelleted and homogenized by sonication in buffer (200 μl per flask) consisting of 50 mM Tris–HCl,

25 mM sucrose, 1 mM EDTA and 1× Halt protease inhibitor (Thermo Fisher Scientific, Rockford, IL), pH 7.4. Homogenates were centrifuged at 20,000×*g* at 4°C for 20 min, and supernatants were used for western blot analysis. Protein concentrations were measured using the BCA Protein Assay (Thermo Fisher). Western blot analysis of SULT1E1 content was accomplished as described previously [340], using 30 µg of sample protein and polyclonal anti-SULT1E1 antibody. Uniform protein loading and transfer were verified using Ponceau S staining. SULT1E1 catalytic activity was measured in whole cell lysates prepared from MCF10CA1a cells as described [341].

Transient transfection analysis. The p2ERE-luc reporter was constructed by ligating a double-stranded oligonucleotide containing two vitellogenin ERE upstream of a minimal herpes simplex virus thymidine kinase promoter, which had been preligated into pGL3-Basic (Promega Corporation, Madison, WI). The sequences of the oligonucleotides are: 5′-CGCGTGTCCAAAGTCAGGTCACAGTGACCTGATTCAGGTC ACAGTGACCTGATCAAAGTTA-3′ and 5′-GATCTAACTTTGATCAGGTCACTGTGACC TGAATCAGGTCACTGTGACCTGACTTTGGACA-3′.

MCF7 cells (400,000) were seeded into 12-well plates and cultured in 2 ml phenol red-free Minimum Essential Medium supplemented with 10 µg/ml insulin, 1 mM sodium pyruvate and 10% charcoal-stripped fetal bovine serum. The following day, Opti-MEM containing a premixed complex of 4 µl of Lipofectamine 2000, 1.6 µg p2ERE-Luc and 1.25 ng pRL-SV40 (Promega) was added to each well. The following day, cultures (5 wells per group) were incubated with phenol red-free Minimum Essential Medium, supplemented as described above, containing 0.1% DMSO, 10 nM E2, 300 ng/ml TSA, or E2 and TSA in combination. After 48 hr, the cells were harvested

for measurement of firefly and *Renilla* luciferase activities using the Dual Luciferase Reporter Assay System (Promega) and a Dynex model MLX Luminometer.

Statistical analysis. Data were analyzed using the paired *t*-test or one-way analysis of variance followed by the Newman–Keuls test using Prism (GraphPad Software, San Diego, CA).

Results

The high catalytic efficiency of SULT1E1 toward E2 sulfonation supports a major

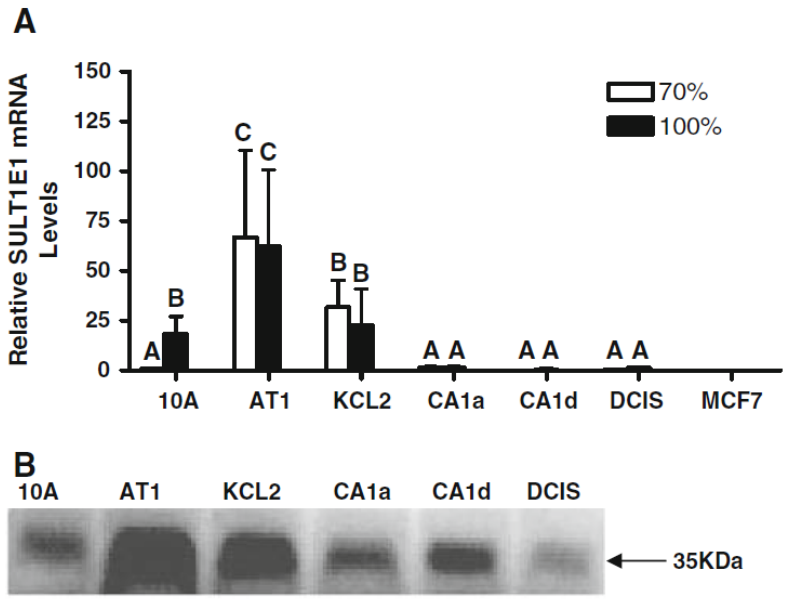


Fig. 2.2 Expression of SULT1E1 mRNA and protein in the MCF10A-derived lineage cell culture model for breast cancer progression and in MCF7 cells. A: A TaqMan Gene Expression assay was used to determine the relative levels of SULT1E1 mRNA in subconfluent (70%, *white bars*) and confluent (100%, *black bars*) MCF10A series cells (from *left to right*, MCF10A, MCF10AT1, MCF10AT1K.cl2, MCF10CA1a, MCF10CA1d and MCF10DCIS.com) and in MCF7 cells. SULT1E1 mRNA content is expressed relative to the level measured in subconfluent MCF10A cells, and all values represent the mean ± SEM of three independent cell culture experiments. Groups labeled with different letters are significantly different from each other ($P < 0.05$). B: Representative Western blots showing the relative amounts of SULT1E1 immunoreactive protein in subconfluent MCF10A-derived cell lines.

role for SULT1E1 in the *in situ* inactivation of E2 within the breast. SULT1E1 mRNA was detected in parental MCF10A cells, although the level of expression was highly dependent upon the confluency of the cultures. Relative to pre-confluent MCF10A cells, the amount of SULT1E1 mRNA in confluent cells was significantly increased by ~16-fold (Fig. 2.2A). SULT1E1 mRNA expression was robust in MCF10AT1 cells, and unlike MCF10A cells, was not significantly affected by confluency (Fig. 2.2A). SULT1E1 mRNA expression was also substantial in MCF10AT1K.cl2 cells, but was markedly diminished in MCF10CA1a, MCF10CA1d, MCF10DCIS.com, and MCF7 cells (Fig. 2.2A). SULT1E1 immunoreactive protein levels in the MCF10A lineage cell lines were in accord with the corresponding mRNA levels (Fig. 2.2B). Since STS and other cytosolic SULTs have been implicated in the modulation of breast intracrinology [79;97;254;341-343], the expression patterns of STS and SULTs 1A1, 2A1, and 2B1 were characterized. STS mRNA was detected across the MCF10A series cell lines, as well as in MCF7 cells (Fig. 2.3A). SULT1A1 and SULT2B1 transcripts were detected only in MCF7 cells, while the mRNA for SULT2A1, an enzyme that is highly expressed in human liver and adrenal gland [342], was not detected in any of the breast cell lines (Fig. 2.3B). 17βHSD1 catalyzes the reduction of estrone (E1) to the more potent E2, while 17βHSD2 catalyzes the oxidation of E2 to E1 [344]. 17βHSD1 mRNA was detected in all cell lines but was highest in the MCF10CA1a and MCF10DCIS.com cells (Fig. 2.3B). By contrast, 17βHSD2 mRNA was expressed most abundantly in the parental MCF10A cell line (Fig. 2.3B).

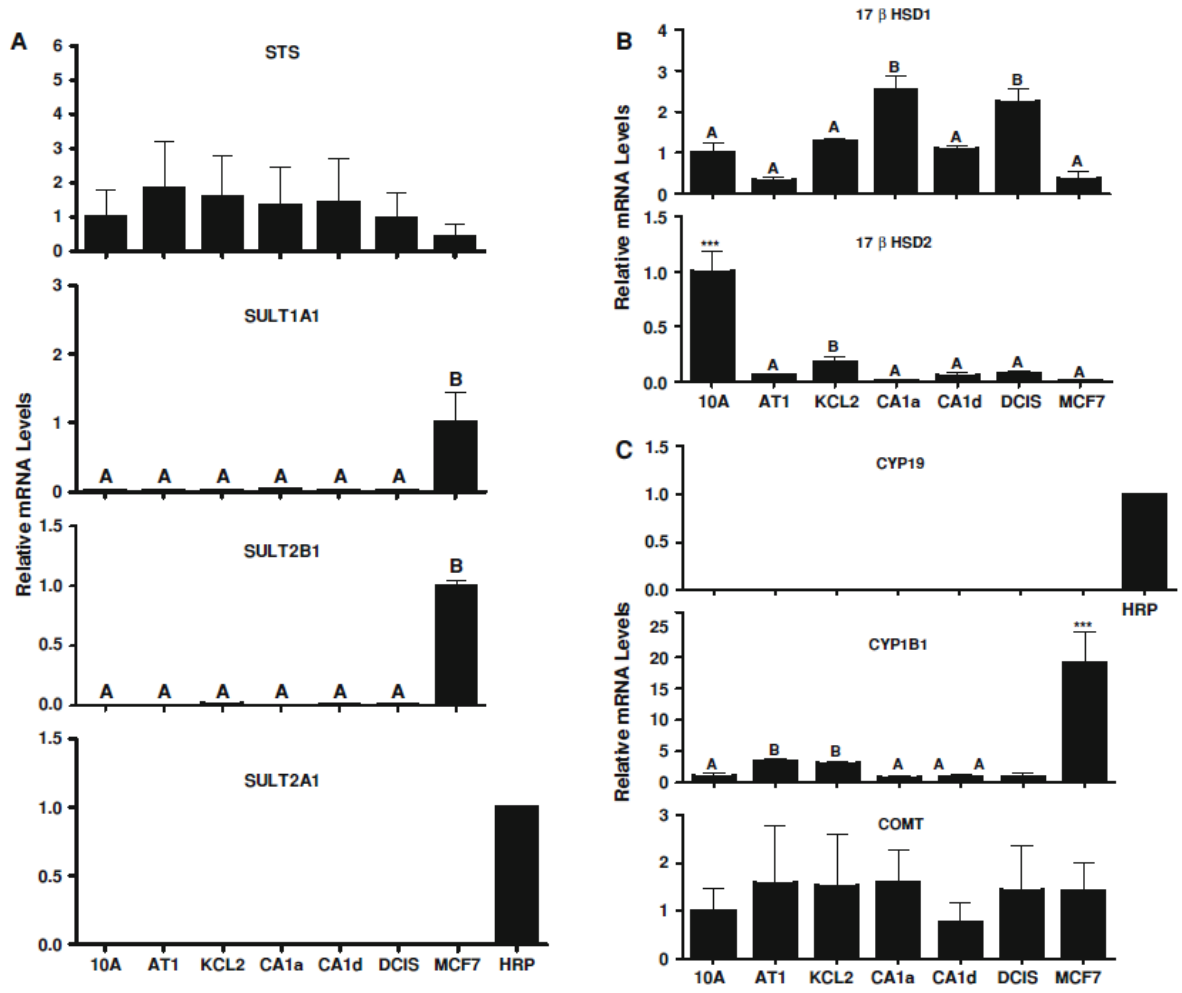


Fig. 2.3 Expression of estrogen metabolism enzymes in MCF10A series and MCF7 cell lines. TaqMan Gene Expression assays were used to determine the relative levels of STS, SULT1A1, SULT2B1, SULT2A1 (A), 17βHSD1, 17βHSD2 (B), CYP19, CYP1B1, and COMT (C) mRNA in pre-confluent MCF10A series cell lines and in MCF7 cells. The mRNA levels are expressed relative to their respective levels in MCF10A cells, MCF7 cells or a human RNA reference pool (*HRP*). All values represent the mean ± SEM of three independent cell culture experiments. For all transcripts except 17βHSD2 and CYP1B1: Groups labeled with different letters are significantly different from each other ($P < 0.05$). For 17βHSD2 and CYP1B1: *** Significantly different from all other groups, $P < 0.001$. When the group labeled with *** was omitted from the analyses, groups labeled with different letters are significantly different from each other ($P < 0.05$). For STS and COMT, no significant differences among groups were detected. For SULT2A1 and CYP19, mRNA levels were undetected in all cell lines.

The transcript for CYP19, which catalyzes the aromatization of androstenedione and testosterone to E1 and E2, respectively [345], was not detected in any cell line (Fig. 2.3C). CYP1B1 is the most active E2 hydroxylase [346], and high levels of E2 hydroxylation in estrogen-responsive tissues may play an important role in estrogen-related tumorigenesis [177]. By contrast, *O*-methylation catalyzed by COMT is an inactivation pathway for E2 catechols and quinones [177]. CYP1B1 mRNA levels were highest in MCF7 cells (Fig. 2.3C). Though CYP1B1 mRNA also appeared to be higher in MCF10AT1 and MCF10AT1K.cl2 cells than in the other MCF10A series cells (Fig. 2.3C), this difference was not maintained when the cultures were treated with DMSO (experiment shown in Fig. 2.6). COMT demonstrated relatively consistent mRNA expression across cell lines (Fig. 2.3C).

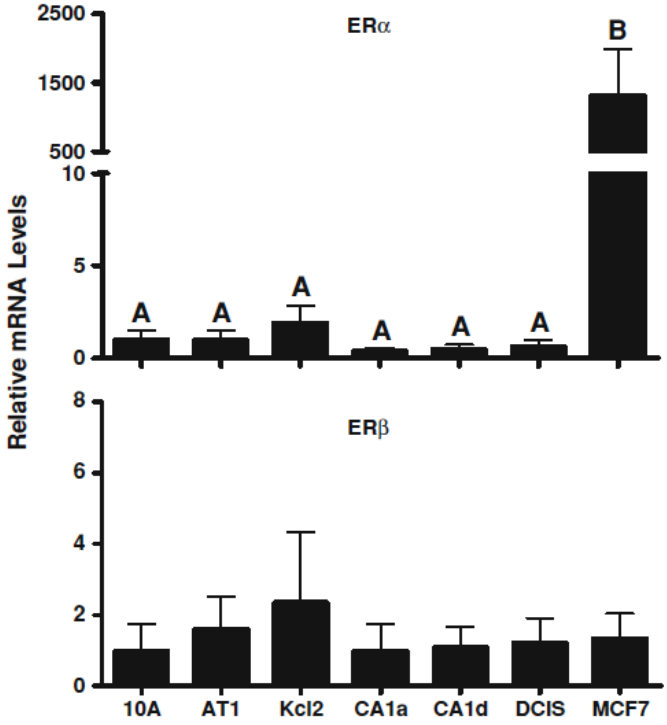


Fig. 2.4 ERα and ERβ mRNA expression in pre-confluent MCF10A series and MCF7 cell lines. TaqMan Gene Expression assays were used to determine the relative levels of ERα (*top*) and ERβ (*bottom*) mRNA. The mRNA contents of ERα and ERβ are expressed relative to their respective levels in MCF10A cells, and all values represent the mean ± SEM of three independent cell culture experiments. For ERα, groups labeled with different letters are significantly different from each other ($P < 0.05$). For ERβ, no significant differences among groups were detected.

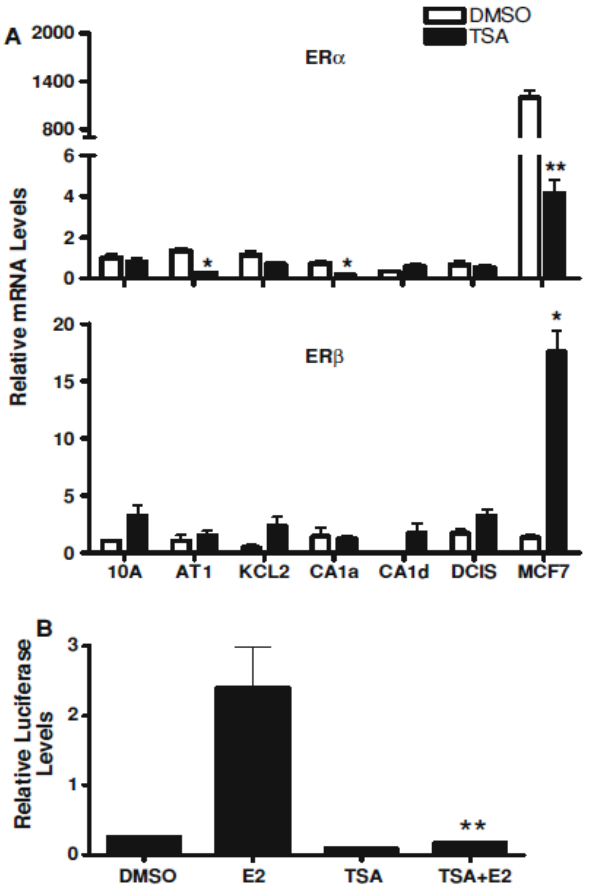


Fig. 2.5 Effects of TSA treatment on ERα and ERβ mRNA expression in MCF10A series and MCF7 cell lines and on E2-mediated activation of an estrogen-responsive reporter gene. a Cell lines were treated for 24 h with 0.1% DMSO (*white bars*) or 300 ng/ml TSA (*black bars*), and ERα and ERβ mRNA levels were measured with TaqMan Gene Expression assays. ERα and ERβ mRNA levels are expressed relative to the amounts measured in DMSO-treated MCF10A cells. All values represent the mean ± SEM of three independent cell culture experiments. *, ** Significantly different from the corresponding DMSO-treated group, $P < 0.05$ and $P < 0.01$, respectively. **b** MCF7 cells were transiently transfected with an estrogen-responsive luciferase reporter plasmid. After transfection, cells were treated with 0.1% DMSO, 10 nM E2, 300 ng/ml TSA, or E2 and TSA in combination for 48 h. After treatment, cells were harvested for the measurement of luciferase activities. Each *bar* represents the mean ± SEM of normalized (firefly/*Renilla*) luciferase measurements (5 wells per treatment group) relative to the activity measured in DMSO-treated cells. ** Significantly different from the E2-treated group $P < 0.01$

ER expression is a major determinant of estrogenic activity. As expected, the ER-positive MCF7 cell line expressed ERα mRNA at a level that was >100-fold greater than that detected in any of the MCF10A series cell lines (Fig. 2.4). ERα immunoreactive protein is reportedly undetectable in MCF10A cells [314], and in the present study ERα mRNA was detected at a low level in the MCF10A lineage cells (Fig. 2.4). By comparison, ERβ mRNA levels were not abundant either in MCF7 cells or in the MCF10A-derived series of cell lines (Fig. 2.4).

Since HDACs are important modulators of transcription and therapeutic targets in breast cancer [335;339], the effects of TSA treatment were characterized on ER and estrogen metabolism enzyme expression. As previously reported [347], TSA treatment of MCF7 cells produced a dramatic decrease (>99%) in ERα mRNA expression (Fig. 2.5A). The loss of E2 agonistic activity following TSA treatment was confirmed using an ER-responsive reporter (Fig. 2.5B). By contrast, TSA treatment increased ERβ mRNA levels in MCF7 cells by ~11-fold (Fig. 2.5A). TSA produced less pronounced effects on ERα and ERβ mRNA expression in the MCF10A lineage cell lines (Fig. 2.5A).

TSA also produced marked alterations in the mRNA expression of estrogen metabolism enzymes in the cell lines. In the neoplastic MCF10CA1a cell line, where SULT1E1 mRNA expression is substantially suppressed, TSA treatment produced a concentration-dependent increase in SULT1E1 mRNA expression that correlated with the induction of E2 sulfonation activity (Fig. 2.6A). TSA treatment increased SULT1E1 mRNA content by at least two-fold (2.3- to 26.1-fold) in all MCF10A series cell lines except MCF10AT1, although the increase was statistically significant only for

MCF10DCIS.com (Fig. 2.6B). TSA treatment also activated CYP19 expression in all of the cell lines (Fig. 2.6C). By contrast, TSA treatment uniformly suppressed STS and COMT expression (Fig. 2.6C). TSA treatment produced marked suppression of SULT1A1, SULT2B1 and CYP1B1 mRNA levels in MCF7 cells, while SULT2A1 expression was induced in TSA-treated MCF7 cells (Fig. 2.6C). TSA treatment also suppressed 17βHSD1 and 17βHSD2 expression in the MCF10A series cell lines that demonstrated constitutive expression (Fig. 2.6C).

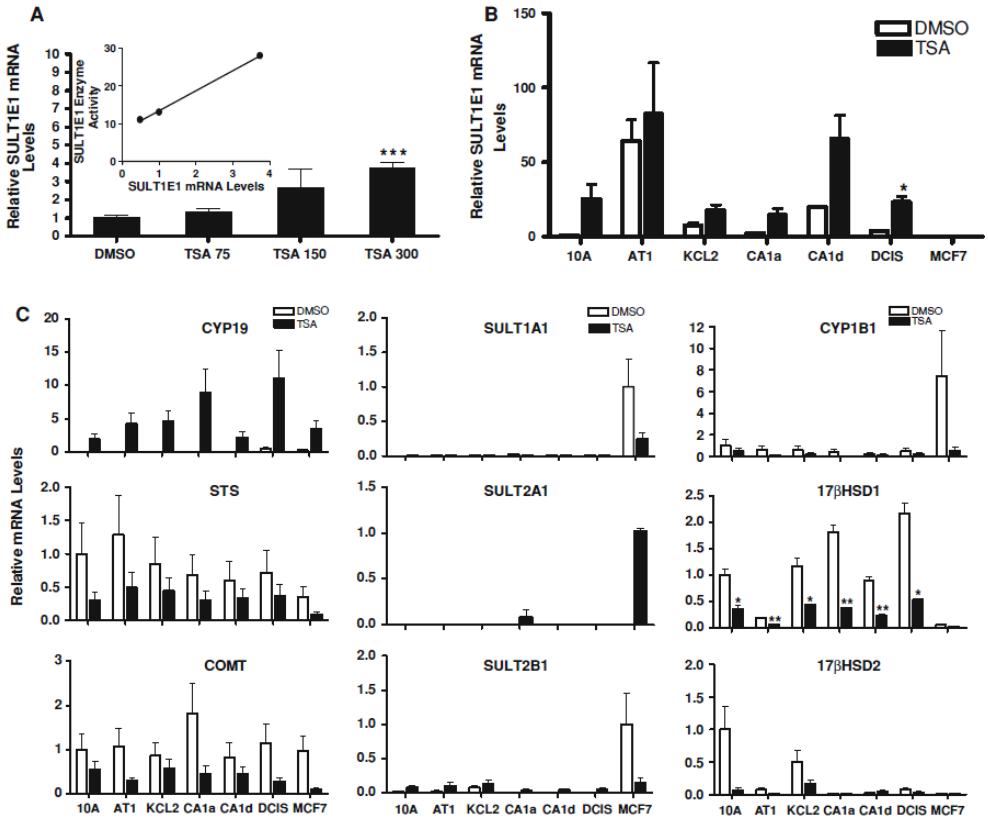


Fig. 2.6 TSA treatment effects on estrogen metabolism enzyme expression in MCF10A series and MCF7cell lines. A: Concentration-dependent effects of TSA treatment on SULT1E1 mRNA expression in MCF10CA1a cells. MCF10CA1a cells were treated for 24 h with 0.1% DMSO or with 75, 150 or 300 ng/ml TSA and harvested for measurement of SULT1E1 mRNA levels with a TaqMan Gene Expression assay. All values represent the mean ± SEM of three independent cell culture experiments relative to the amount measured in the DMSO-treated cells. *** Significantly different from the DMSO-treated group, $P < 0.001$. *Inset* Correlation of TSA-mediated changes in SULT1E1 enzymatic activity with changes in SULT1E1 mRNA levels. B: MCF10A series and MCF7 cell lines were treated for 24 h with 0.1% DMSO or 300 ng/ml TSA and SULT1E1 mRNA levels were measured. SULT1E1 mRNA contents are expressed relative to the level measured in DMSO-treated MCF10A cells. All values represent the mean ± SEM of three independent experiments. * Significantly different from the corresponding DMSO-treated group ($P < 0.05$). C: Cells were treated as indicated in B and estrogen metabolism enzyme mRNA levels were measured. STS, COMT, CYP1B1, 17βHSD1, and 17βHSD2 mRNA contents are expressed relative to the respective levels measured in DMSO-treated MCF10A cells. SULT1A1 and SULT2B1 mRNA levels are expressed relative to the levels measured in DMSO-treated MCF7 cells. SULT2A1 mRNA levels are expressed relative to the level measured in TSA-treated MCF7 cells. CYP19 mRNA levels are expressed relative to the level measured in TSA-treated MCF10A cells. All values represent the mean ± SEM of three independent experiments. *, ** Significantly different from the corresponding DMSO-treated group, $P < 0.05$ and $P < 0.01$, respectively

Discussion

Selective ER modulators, selective ER down-regulators and inhibitors of estrogen-producing enzymes, such as CYP19, STS, and 17βHSD1, represent important classes of drugs for achieving estrogen blockade in the treatment of hormone-responsive breast cancer [348-352]. In addition, HDAC inhibitors, which have been shown to down-regulate ERα mRNA expression in MCF7 cells [347], are being tested in phase II clinical trials that include patients with ER-positive metastatic breast cancer progressing on endocrine therapy [336]. The combination of HDAC inhibitor and tamoxifen were associated with low toxicity and reversed hormone resistance [336]. Despite an expanded range of targeted therapies, the critical alterations in breast intracrinology that predispose patients to breast cancer development remain to be elucidated.

In mammary tissue, stringent control of the powerful mitogen E2 is achieved through the tight balance of ERα and ERβ expression coupled with titration of E2 levels. Compared to ERβ, ERα is a high affinity receptor for estrogen [353]. Both ERα and ERβ bind to the same response elements but produce differential effects on target gene expression [354]. ERα is a recognized marker for E2-stimulated proliferation in breast cancer [355]. Although further studies are needed to better assess the role of different ERβ isoforms, emerging studies have associated ERβ with more aggressive breast cancer types and poor clinical outcome [356-358]. In the present study, ERα expression in the MCF10A lineage cell lines was low in comparison to its level in MCF7 cells, while ERβ mRNA expression was comparably low among the cell lines. As previously

reported [347], TSA suppressed ERα and induced ERβ expression in MCF7 cells. However, TSA produced only modest effects on ERα and ERβ expression in the MCF10A series of cell lines.

The pro-estrogenic machinery of the breast includes STS, CYP19, and 17βHSD1 [345;359]. Increased expression of STS occurs in 74% of breast cancer biopsies and its presence correlates with an adverse prognosis [97]. In the present analysis, STS mRNA levels were comparably detected across the MCF10A series of cell lines and in MCF7 cells, and TSA treatment consistently tended to repress STS expression.

CYP19 expression in breast tumor tissue facilitates a highly concentrated estrogen micro-environment. Like STS, elevated CYP19 expression in breast tumor tissue impairs prognosis [325]. Though CYP19 is more robustly expressed in breast stroma, the application of fine resolution techniques has revealed the presence of CYP19 in normal breast ductal epithelial cells and breast cancer cells, as well as in intra-tumor stromal cells and peri-tumoral adipose tissue [360]. In the absence of TSA treatment, CYP19 mRNA was not detected in MCF10A series cell lines or MCF7 cells. In contrast to a previous report demonstrating a repressive role for TSA on CYP19 expression in MCF7 cells [361], this investigation revealed a TSA-inducible effect on CYP19 expression in all of the cell lines examined.

There are at least fifteen 17βHSD enzymes that vary in catalytic range and efficiency [362]. 17βHSD1 and 17βHSD2 are involved in the interconversion of E2 with the less potent E1 [344]. Increased 17βHSD1 expression in breast cancer, either alone

or in combination with CYP19, enhances estrogen concentrations and negatively impacts prognosis [78;363], as does loss of 17βHSD2 expression [364]. In the present study, the mRNA expression of pro-estrogenic 17βHSD1 was detected in MCF10A cells but was more abundant in the neoplastic MCF10CA1a and MCF10DCIS.com variants. By contrast, 17βHSD2 mRNA was most prominent in parental MCF10A cells. TSA treatment produced only suppressive effects on 17βHSD1 and 17βHSD2 expression.

SULT1E1 is considered to be the predominant E2-inactivating enzyme in breast. A previous survey of cytosolic SULT expression concluded that human mammary epithelial cells expressed mainly SULT1E1, while breast cancer cell lines preferentially expressed SULT1A1 and only trace amounts of SULT1E1 and SULT2A1 [249]. In an independent study, SULT1E1 expression was reported in approximately 44% of human breast cancer biopsy specimens [97]. The present investigation revealed SULT1E1 mRNA expression in confluent MCF10A cells and in the preneoplastic MCF10AT1 and MCF10AT1K.cl2 cell lines. By contrast, SULT1E1 expression in the more neoplastically transformed MCF10A-derived cell lines and in MCF7 cells was markedly repressed. As previously described for CYP1A2 expression in MCF10A cells [365], SULT1E1 mRNA levels were more abundant in confluent than in proliferating MCF10A cultures. The constitutive expression of other steroid-metabolizing SULTs (i.e., SULT1A1, SULT2A1, and SULT2B1) was not a prominent feature across the MCF10A-derived cell lines.

4-Hydroxylated estrogen metabolites produced by CYP1B1 metabolism are particularly reactive and considered to be promutagenic [366]. CYP1B1 mRNA and protein expression has been reported in up to 73% of human breast cancer biopsies [367]. Increased CYP1B1 expression, coupled with the reduced expression of the

detoxicating enzyme COMT, is associated with amplified breast cancer risk [366]. In the current analysis, CYP1B1 mRNA was present at relatively low levels in the MCF10A-derived cell lines, but was more abundant in MCF7 cells, where its constitutive expression has been previously described [368]. In contrast to a previous report suggesting a stimulatory effect of TSA treatment on CYP1B1 expression in MCF7 cells [369], the present study revealed TSA-mediated suppression for both CYP1B1 and COMT in MCF7 cells. In contrast to the relatively restricted expression of CYP1B1, COMT mRNA was widely detected across cell lines.

In aggregate, it appears that normal or early preneoplastic breast epithelial cells act to minimize the mitogenic effects of E2 by retaining the expression of SULT1E1, a major E2- inactivating enzyme, and by preserving the expression of the anti-estrogenic dehydrogenase, 17βHSD2. Particularly in the MCF10A lineage model for breast cancer progression where ERα levels are held to a minimum, the expression of key E2-metabolizing enzymes is crucial for the establishment of the breast intracrine environment. Within the progression model, the expression of pro-estrogenic STS and 17βHSD1 is maintained, while the capacity for in situ estrogen production through aromatization is restrained. Based on the current study, several positive aspects of HDAC inhibitor treatment in humans might be anticipated. These include the up-regulation of SULT1E1 in neoplastic breast epithelial cells and also down-regulation of pro-estrogenic metabolic enzymes such as STS and 17βHSD1. With HDAC inhibition, the prominent expression of CYP1B1 that is observed in MCF7 cells becomes down-regulated. However, as a counter-weight, the expression of the protective enzyme COMT also becomes down-regulated.

The results of this study demonstrate that established breast cancer cell lines, such as MCF7, do not necessarily reflect the changes in estrogen metabolism that occur in breast epithelial cells as they progress from benign proliferative breast disease toward neoplasia. In order to harness the promise and potential power of combined or sequential metabolically-targeted therapies in breast cancer intervention, it will be become essential to understand and control the real-time dynamics that determine the delicate balance of intracrine metabolism within the breast.

CHAPTER 3

Regulation of SULT1E1 expression by confluency of MCF10A breast epithelial cells: Role of the AhR

INTRODUCTION

The SULTs are a family of conjugating enzymes that catalyze the transfer of a sulfuryl moiety from the activated physiological sulfate donor 3'-phosphoadenosine-5'-phosphosulfate to the hydroxyl groups of endogenous and xenobiotic substrates, including hormones, drugs, and procarcinogens [228;370]. One of the SULTs, SULT1E1, catalyzes the sulfonation of estrogen at physiological concentrations. SULT1E1 is an important determinant of a cell's response to estrogen because sulfonated estrogen cannot bind to ERs [371]. In this manner, SULT1E1 expression in breast epithelial cells likely limits the mitogenic effects of estrogen, thereby reducing the risk for breast cancer development [341]. SULT1E1 is expressed in human breast epithelial cells as well as in the MCF10A cell line, a model of normal human breast epithelial cells, but is down-regulated in many breast cancer cell lines, suggesting that this brake against estrogen mitogenicity is lost during neoplastic transformation [250;341].

During the characterization of estrogenicity gene expression in MCF10A lineage cell lines (Chapter 2), we observed that SULT1E1 mRNA content is markedly increased when replicating MCF10A cells become confluent (Fig. 2.2) [250], indicating that

SULT1E1 expression is regulated according to the confluency of these cells. By comparison, in an earlier study in which the expression of cytochrome P450 transcripts was profiled in MCF10A cells, two P450s, CYP1A1 and CYP1S1, were expressed in pre-confluent MCF10A cells but not in confluent MCF10A cells [365]. Since both of these P450s are transcriptional targets of the AhR [372], this finding suggests that AhR is active in pre-confluent MCF10A cells but inactive in confluent MCF10A cells.

AhR agonist treatments cause suppression of SULTs in hepatic systems. Treatment of female rats with 3-methylcholanthrene caused suppression of hepatic hydroxysteroid sulfotransferase expression in parallel with CYP1A1 induction [373], and treatment with β-naphthoflavone or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) caused suppression of hydroxysteroid sulfotransferase and aryl sulfotransferase expression in primary cultured rat hepatocytes [374]. In a microarray analysis of TCDD treatment effects on global gene expression in HepG2 human hepatoma cells, SULT1E1 mRNA content was decreased by 60% following treatment with 10 nM TCDD for 8 hr [375]. Approximately the same magnitude of suppression occurred when the cells were pre-treated with cycloheximide prior to TCDD treatment, suggesting that the reduction of SULT1E1 mRNA content was a direct effect of TCDD treatment on gene transcription and was not secondary to induction of a suppressive factor [375]. Most recently, TCDD treatment was reported to cause suppression of SULT1E1 expression in the livers of female C57BL/6 mice [376].

Taken together, these prior findings prompted us to hypothesize that AhR is the molecular switch that confers confluency-dependent expression of SULT1E1 in MCF10A cells. We propose that basally active AhR suppresses SULT1E1 transcription

in pre-confluent MCF10A cells, while in confluent cells AhR becomes inactive, thereby de-repressing SULT1E1 transcription.

Materials and Methods

Materials. TCDD was purchased from Midwest Research Institute (Kansas City, MO). 3'-methoxy-4'-nitroflavone (MNF) was purchased from ICC Chemical Corporation (New York, NY). Cell culture medium, L-glutamine, horse serum, penicillin-streptomycin solution, sodium pyruvate, Lipofectamine 2000, Superscript II, recombinant human SULT1E1, and anti-SULT1E1 antibody were purchased from Invitrogen Corporation (Carlsbad, CA). EGF was purchased from BD Biosciences (San Jose, CA). Recombinant human insulin (Novolin R) was purchased from Novo Nordisk Pharmaceuticals, Inc. (Princeton, NJ). Bromouridine (BrU), cholera toxin, doxycycline, hydrocortisone, and puromycin were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal AhR antibody (B-11), goat polyclonal ARNT1 antibody (C-19), rabbit polyclonal glyceraldehyde 3-phosphate dehydrogenase antibody (FL-335), and horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG, and donkey anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ECL Plus Western Blotting Detection Reagents and Hybond-P membranes were purchased from GE Healthcare (Piscataway, NJ). The microRNA mimic (c-301032-01-0005 for hsa-miR-100* and c-300578-05-0005 for has-miR-221) was purchased from Thermo Fisher Scientific. Other materials were obtained from the sources indicated below.

Cell culture. The MCF10A cell line was obtained from the Cell Resources Facility of the Barbara Ann Karmanos Cancer Institute, Wayne State University and cultured in phenol red-free DMEM/F12 Nutrient Mixture (1:1) supplemented with 10 μg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, 5% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell line was routinely maintained in T75 flasks in a 37°C humidified environment of 5% CO_2/95% air. For experiments, 125,000 cells or 1,000,000 cells were plated into 60 mm dishes. At these cell densities, approximately 3 days after plating, pre-confluency (~70% confluency) or confluency was reached, respectively, and the cells were harvested for preparation of total RNA. For TCDD or MNF treatment, 48 hr after plating, cells were treated with 0.1% DMSO (control), TCDD or MNF for 24h.

TaqMan Gene Expression assays. As described in Chapter 2.

Measuring SULT1E1 heterogeneous nuclear RNA (hnRNA). For measuring the amount of SULT1E1 heterogeneous nuclear RNA (hnRNA), total RNA was isolated using the RNeasy Mini Kit with on-column DNase I treatment, and samples of total RNA (1.5 μg) were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit. As negative controls, equivalent amounts of total RNA were “mock reversed transcribed” by performing the reactions in the absence of reverse transcriptase. PCR primers were designed using Oligo Primer Analysis Software, version 7.36 (Molecular Biology Insights, CA) and the human SULT1E1 structural gene sequence (NCBI Reference Sequence NC_000004, 70706930-70725870 complement). The sequence of the upper primer (5′-GCTGGTCATCCAAATCCTG-3′) was located within exon 5 and the sequence of the lower primer (5′-CAATTTGCCTTCTACATCTGGACA-3′) was

located within intron 5. Each PCR reaction contained 1 µl of reverse transcription reaction as template, 25 µl of 2x SYBR Green PCR Master Mix (Applied Biosystems), and 300 nM each of upper and lower primer in a volume of 50 µl. Samples were incubated at 95°C for 10 min, followed by 35 cycles of 95°C for 15 sec and 60°C for 1 min, followed by a melting curve of 95°C for 15 sec, 60°C for 1 min, ramp to 95°C with data collection every 0.3°C, and 95°C for 15 sec to ensure that a single product had been amplified. A commercial SYBR Green-based RT-PCR assay to detect TATA box binding protein was used for normalization (Qiagen, Valencia, CA). Following data acquisition, Ct values were determined and data were analyzed as described above. Control reactions containing aliquots of the mock reverse transcribed samples were performed to determine whether any fluorescent signal was derived from contaminating genomic DNA. To confirm amplification of the specific target fragment of expected size (203 nt), PCR products were run on a 2% agarose gel and visualized with ethidium bromide under ultraviolet illumination.

microRNA microarray analysis. Total RNA was prepared from different confluency MCF10A cells using Trizol reagent (Gibco). The quality of each sample used was checked using the RNA Nano Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The following microRNA microarray procedures were performed by the Microarray/Bioinformatics Core Facility. MicroRNA microarray analysis was performed using the microRNA Microarray System (Agilent Technologies, Palo Alto, CA). Each Human microRNA Microarray V2 (Cat. No. G4470B) slide contains 8 microRNA arrays. Each array consists of human and human viral microRNAs from the Sanger miRBASE 10.1. Agilent protocol “microRNA Microarray System” v. 1.5 was

followed during microRNA labeling and array hybridization. Samples of total RNA (100 ng) were treated with Calf Intestine Alkaline Phosphatase (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) at 37° C for 30 min. Ligation reactions were performed at 16° C for 2 hr. The labeled microRNA samples were then purified using Micro Bio-Spin 6 columns (Bio-Rad Laboratories, Hercules, CA), dried with a speed vac, and re-suspended in 18 µl of nuclease-free water. 4.5 µl of 10× GE Blocking Agent and 22.5 µl of Agilent 2× Hi-RPM Hybridization Buffer were added, and the samples were incubated at 100° C for 5 min and placed on ice for 5 min. Samples were immediately added to an array in an Agilent SureHyb hybridization chamber. The hybridization chambers were rotated at 20 rpm in a hybridization oven for 20 hr at 55° C. After hybridization, the slides were removed from the hybridization chamber and placed in a glass slide rack in a slide-staining dish for washing. The staining dish was placed on a magnetic stir plate, and stirred using setting 4. The slides were washed 5 min in GE Wash Buffer 1 and 5 min in pre-warmed 37° C GE Wash Buffer 2 (Agilent Technologies). Slides were slowly removed from wash 2 solution which allows for even drying across the slide. Slides were then scanned using the Agilent dual laser scanner. The photomultiplier tube settings were set at 100% and 5% for the Green channel. Tiff images were analyzed using Agilent's feature extraction software.

microRNA over-expression. MCF10A cells were seeded into 24-well plates (150,000 cells/well). The following day, the standard medium was replaced with Opti-MEM containing a premixed complex of 4 µl of the microRNA mimic (c-301032-01-0005 for hsa-miR-100* and c-300578-05-0005 for has-miR-221). The next day, cultures (3 wells per treatment group) were harvested for RNA. RNA samples (1.5 µg) were reverse

transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The levels of miroRNA were measured using the following TaqMan MiroRNA assays: has-miR-100* and has-miR-221.

BrU pulse-chase labeling. The BrU procedure was performed by collaborator Dr. Mats Ljungman at the University of Michigan. MCF10A cells grown to pre-confluency or confluency were incubated with 2 mM BrU in conditioned medium for 30 min to label nascent RNA. Cells were then washed 3 times in PBS and either collected directly (0 hr time point) or chased in conditioned medium containing 20 mM uridine for 2 or 6 hr at 37°C. Total RNA was isolated using TRIzol reagent and the BrU-containing RNA was isolated using magnetic beads (Dynabeads, Goat anti-mouse IgG, Invitrogen) conjugated to anti-BrdU monoclonal antibody (BD Biosciences). Conversion of the isolated BrU-containing mRNA into cDNA and real-time PCR analyses were performed by the Microarray Core of the University of Michigan Comprehensive Cancer Center according to protocols supplied by the manufacturer (SABiosciences, Frederick, MD). For the real-time PCR analyses, the Cancer Drug Resistance and Metabolism real-time RT PCR array (PAHS-004, SABiosciences) and the ABI 7900HT Sequence Detection System from Applied Biosystems were used. The data were analyzed using RT^2 Profiler PCR Array Data Analysis software (http://www.sabiosciences.com/pcr/arrayanalysis.php), and the data were normalized to the expression of 5 housekeeping genes present on the arrays. The housekeeping genes were: β2-microglobulin, hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L13A, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin.

Western blot analysis. For measurement of SULT1E1 protein content, MCF10A cells in T75 flasks were washed with PBS and scraped into ice-cold PBS. Cells were pelleted and homogenized by sonication in buffer (200 µl per flask) consisting of 50 mM Tris-HCl, 25 mM sucrose, 1 mM EDTA and 1x Halt protease inhibitor (Thermo Fisher Scientific, Rockford, IL), pH 7.4. Homogenates were centrifuged at 20,000xg, 4°C for 20 min, and supernatants were used for western blot analysis. Protein concentrations were measured using the BCA Protein Assay (Thermo Fisher Scientific). Western blot analysis was performed as described previously [250], using 60 µg of sample protein, SULT1E1 antibody at a dilution of 1:2000, secondary antibody at a dilution of 1:10,000, and enhanced chemiluminescence for immunoreactive protein detection. For western blot analysis of AhR and ARNT, MCF10A cells in 10 cm dishes were washed twice with PBS and then lysed using 700 µl of cold RIPA buffer (Thermo Fisher Scientific). For SDS-PAGE, 30 µg samples of the lysates were separated on 4-20% Precise Protein Gels (Thermo Fisher Scientific). Following transfer to polyvinylidene difluoride membranes, the blots were developed using AhR antibody (1:500) or ARNT antibody (1:400) and secondary antibodies at 1:10,000 dilutions. Western blots were normalized for variations in protein loading and transfer by reincubation with a GAPDH antibody (1:1000).

MCF10A cells stably expressing an AhR-responsive reporter. MCF10A cells were plated into 6-well plates (700,000 cells/well). The following day, 4 µg pGudLuc (provided by Dr. Michael Denison, University of California, Davis, CA), which contains 4 dioxin response element (DREs), and 0.65 µg pSV2neo (American Type Culture Collection, Manassas, VA) were cotransfected into MCF10A cells using Lipofectamine

2000. After 24 hr recovery in standard medium, transfected cells were re-plated into medium containing 550 µg/ml G418. After two rounds of limiting dilution cloning, individual cell clones were identified and expanded. For TCDD treatment, 50,000 or 500,000 cells were plated into 6-well plates and 48 hr after plating, cells were treated with 0.1% DMSO or TCDD for 24 hr. After treatment, growth medium was removed and cells were washed with PBS. Passive Lysis Buffer (Promega Corporation, Madison, WI) was added to each well (500 µl/well) and protein concentrations were measured in lysates using the BCA Protein Assay (Thermo Fisher). Firefly luciferase activities were measured in aliquots containing 10 µg protein using the Dual Luciferase Reporter Assay System (Promega Corporation, Madison, WI) and an LMAX II384 microplate reader (Molecular Devices Corporation, Sunnyvale, CA) equipped with SoftMaxPro software.

Conditional knockdown of AhR in MCF10A cells. A plasmid expressing a microRNA-adapted shRNA targeting human AhR in a doxycycline-inducible manner (oligo ID V2THS_132482, vector pTRIPZ) was purchased from Thermo Fisher Scientific, Open Biosystems Products (Huntsville, AL). For transfection, 700,000 MCF10A cells were plated into 6-well plates, and 4 µg/well plasmid was transfected into the cells using Lipofectamine 2000 24 hr after plating. Stably-transfected cells were obtained by incubation in culture medium containing 1 µg/ml puromycin followed by limiting dilution cloning. To achieve AhR knockdown, cells were treated with 1 µg/ml doxycycline for 96 hr.

Transfection of a SULT1E1 5'-flanking region-luciferase reporter plasmid. A fragment of the human SULT1E1 gene spanning from nt -7073 to +13 was amplified by

PCR using genomic DNA from the MCF10A cells line as template, forward primer: 5′-GGGGGTACCATTTGGCCTGCTATAACTGTATGCT-3′ (underscored sequence is a KpnI site), and reverse primer: 5’-GGGCTCGAGACTTCTGCATTTGGAATGTTTCTGG-3’ (underscored sequence is a XhoI site). The amplified fragment was ligated into the KpnI and XhoI sites of the pGL4.17[luc2/Neo] reporter plasmid (Promega Corporation, Madison, WI). The sequence of the SULT1E1 fragment was verified using the services of the Applied Genomics Technology Center, Wayne State University.

For stable transfection, 700,000 MCF10A cells were plated into 6 well plates. The following day, the cells were transfected with 4 µg of the SULT1E1 5’-flanking region-luciferase reporter plasmid using Lipofectamine 2000. After 24 hr recovery in standard medium, the transfected cells were re-plated into medium containing 550 µg/ml G418 and expanded.

For transient transfections, MCF10A cells or MCF10A cells engineered for conditional knockdown of AhR and treated with either 0.1% DMSO or 1 µg/ml doxycycline for 96 hr were seeded into 24-well plates (150,000 cells/well). The following day, the standard medium was replaced with Opti-MEM containing a premixed complex of 4 µl of Lipofectamine 2000, 0.8 µg SULT1E1-luciferase reporter, and 1.25 ng pRL-SV40 (Promega). The next day, cultures (3 wells per treatment group) were incubated with fresh medium containing 0.1% DMSO, 1-3 µM MNF, or 1 µg/ml doxycycline for 24 hr. The cells were then harvested for measurement of firefly and *Renilla* luciferase activities using the Dual Luciferase Reporter Assay System (Promega Corporation, Madison, WI) and an LMAX II384 microplate reader (Molecular Devices Corporation, Sunnyvale, CA) equipped with SoftMaxPro software.

Computational analysis of SULT1E1 5'-flanking region for DREs and site-directed mutagenesis. The region of the human SULT1E1 gene spanning from 10 kb upstream of the transcription start site (as indicated by the beginning of exon 1) through exon 1 was retrieved from NCBI (nt 70,725,767 through 70,735,870 of NC_000004) and was evaluated for the presence of DREs using MatInspector (Genomatix Software, Ann Arbor, MI) [377]. The V$AHRR (AHR-arnt heterodimers and AhR-related factors) matrix family was used for the search, and sites were considered to be matches if the calculated matrix similarity was greater than the optimized matrix threshold.

A single nt change (C to A) was introduced into the core region of a DRE predicted to be located at nt -3476 of the SULT1E1 gene within the context of the luciferase reporter plasmid containing 7073 of the SULT1E1 5'-flanking sequence. This nt change has been shown to abolish the ability of the AhR•ARNT heterodimer to bind to a DRE [378]. The nt change was introduced using the QuickChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA), forward primer: 5′-ACAGCAAAAACCTGGGAGTGCATGTGCACACAC-3′, and reverse primer: 5′-GTGTGTGCACATGCACTCCCAGGTTTTTGCTGT-3′. The presence of the mutation was confirmed by sequence analysis.

Statistical analysis. As described in Chapter 2

Results

As shown in Chapter 2, SULT1E1 mRNA is expressed at a higher level in confluent MCF10A cells than in pre-confluent cells [250]. To characterize the phenomenon more fully, we first confirmed the initial finding and then demonstrated that confluency-mediated up-regulation of SULT1E1 expression occurred at the protein level (Fig. 3.1A and 3.1B). We next addressed whether the confluency-mediated increase in SULT1E1 mRNA content was the result of increased

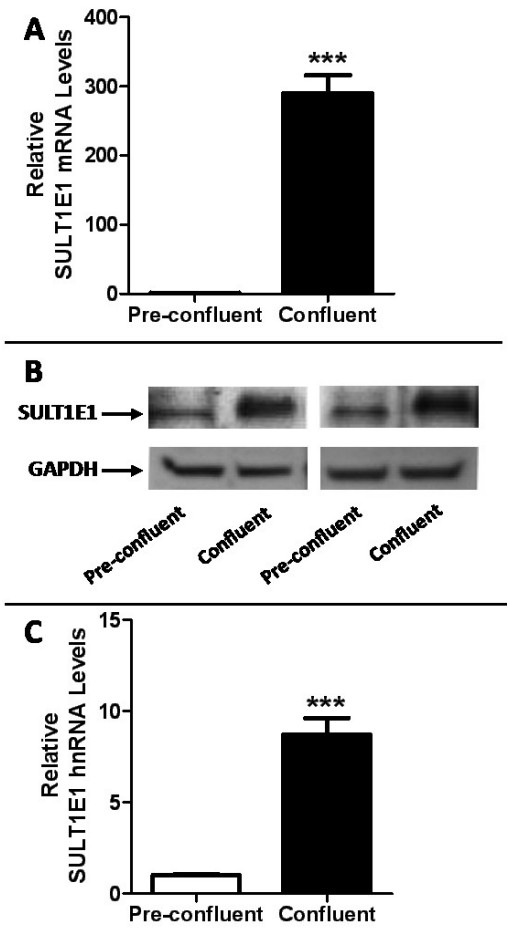


Fig. 3.1 SULT1E1 expression in pre-confluent and confluent MCF10A cells. MCF10A cells were harvested at approximately 70% and 100% confluency. A: SULT1E1 mRNA levels were measured in 6 independent experiments using a TaqMan Gene Expression Assay. B: SULT1E1 immunoreactive protein levels were measured in 2 independent experiments by western blot hybridization. C: SULT1E1 hnRNA levels were measured in 6 independent experiments using a SYBR green real-time RT-PCR assay. In panels A and C, data are expressed as mean ± SEM, and ***$p<0.001$ compared to pre-confluent cells.

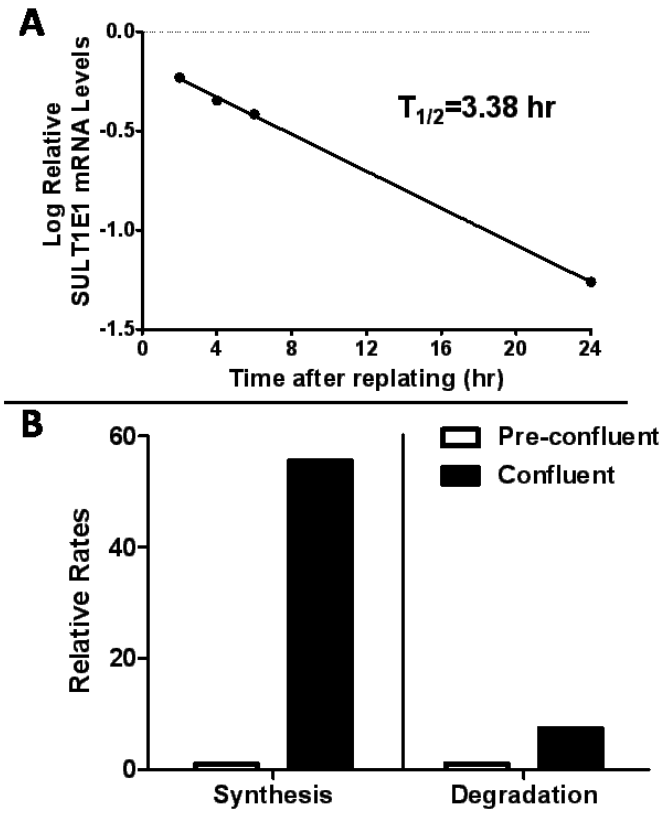


Fig. 3.2 Relative rates of SULT1E1 mRNA synthesis and degradation in pre-confluent and confluent MCF10A cells. A: Confluent MCF10A cells were subcultured and harvested at the indicated times post-plating for measurement of SULT1E1 mRNA levels. SULT1E1 mRNA content is expressed as the log of the fractional level measured in confluent MCF10A cells, and the first order half-life was calculated from the least squares line. B: Bromouridine labeling was used to measure relative rates of SULT1E1 mRNA synthesis and degradation in pre-confluent and confluent MCF10A cells. The data represent the averages from two independent experiments.

mRNA synthesis or decreased mRNA degradation. The relative levels of SULT1E1 hnRNA were measured in pre-confluent and confluent MCF10A cells as an approximation of transcription rate. SULT1E1 hnRNA levels were significantly higher (~8.7-fold) in confluent than in pre-confluent MCF10A cells (Fig. 3.1C). By measuring SULT1E1 mRNA content at different times after re-plating confluent MCF10A cells, the half-life of SULT1E1 mRNA in pre-confluent cells was estimated to be 3.4 hr (Fig. 3.2A).

Since microRNAs have emerged as important regulators of translation and mRNA decay, we analyzed the expression of microRNAs in MCF10A cells at different confluencies. Microarray analysis done by the Microarray/Bioinformatics core facility (Dr. Alan Dombkowski, Department of Pediatrics, Children's Hospital of Michigan) revealed that 85 microRNAs were significantly up-regulated and 24 microRNAs were down-regulated in confluent relative to pre-confluent MCF10A cells. Computational analysis of the SULT1E1 3'-untranslated region identified 4 candidate binding sites for confluency-regulated microRNAs. The confluency-related decrease in two of these microRNAs (miR-221 and miR-100*) corresponded with the confluency-induced increase in SULT1E1 mRNA observed in MCF10A cells (Fig. 3.3). However, overexpression of miR-100* or miR-221 did not suppress SULT1E1 expression in confluent MCF10A cells, suggesting that the down-regulation of these microRNAs is not responsible for confluency-induced SULT1E1 expression (Fig. 3.4).

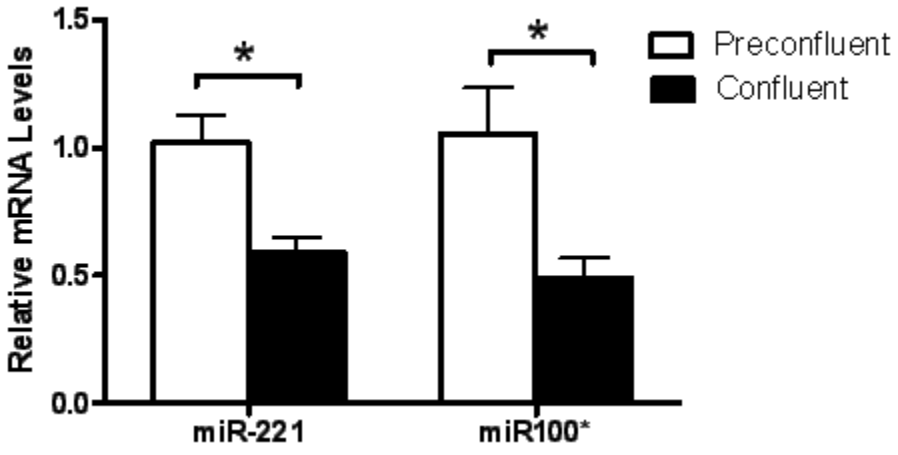


Fig. 3.3 Confluency-related decrease in miR-221 and miR-100*. MCF10A cells were harvested at approximately 70% and 100% confluency. The miR-221 and miR-100* mRNA levels were measured in 3 independent experiments using TaqMan MicroRNA Assays. Data are expressed as mean ± sd, and *p<0.05 compared to pre-confluent cells.

Our collaborator, Dr. Mats Ljungman of the Radiation Oncology Department, University of Michigan, recently developed an innovative approach to measure the relative rates of mRNA synthesis and degradation (Ljungman et al., submitted). We submitted material for this analysis and found that the rate of SULT1E1 mRNA synthesis was was 55.6-fold higher in confluent than in pre-confluent cells, while the rate of SULT1E1 mRNA degradation was 7.3-fold higher in confluent than in pre-confluent cells (Fig. 3.2B). Taken together, these results indicate that increased SULT1E1 mRNA stability cannot account for the increased SULT1E1 mRNA content that is seen upon cell confluence, and that confluence-mediated up-regulation is most likely due to increased transcription.

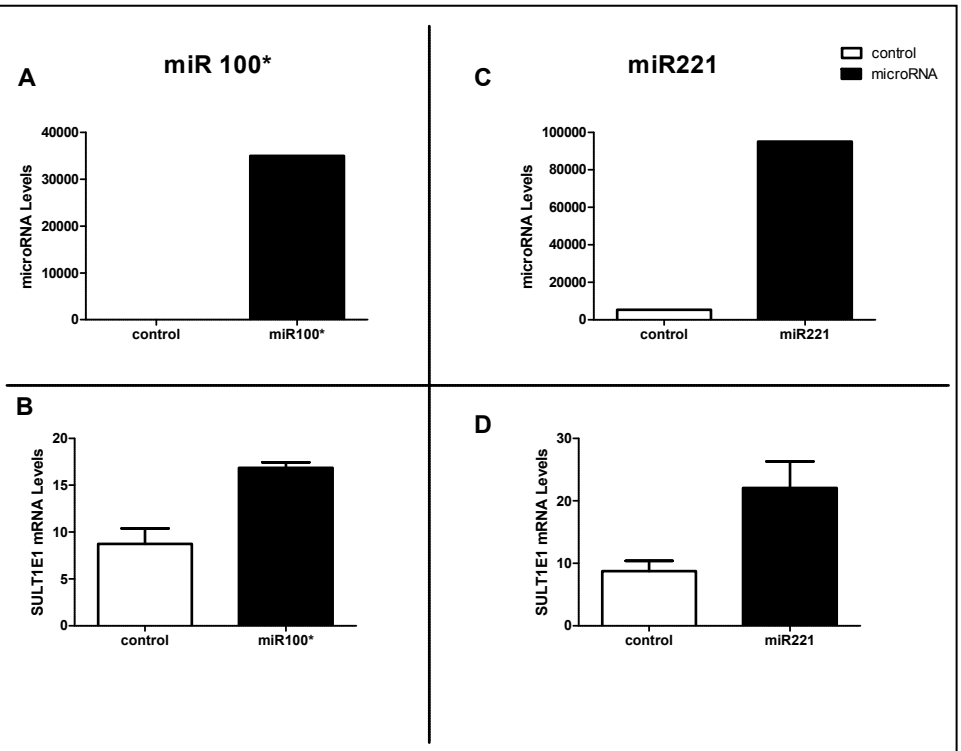


Fig. 3.4 Overexpression of miR-100* or miR-221 did not suppress SULT1E1 expression in confluent MCF10A cells. Confluent MCF10A cells were transiently transfected with miR-100* (**A, B**) or miR-221 (**C, D**). Forty-eight hours after transfection, the cells were harvested for the measurement of microRNA (**A, C**) and SULT1E1 mRNA (**B, D**) levels.

As described in the Introduction, confluence-mediated SULT1E1 up-regulation is mirrored by down-regulation of two CYPs, CYP1A1 and CYP1S1, that are known

transcriptional targets of AhR [365], suggesting that AhR might provide the mechanistic link between these phenomena. Using real-time RT-PCR, we confirmed that CYP1A1 mRNA content was significantly higher in pre-confluent MCF10A cells than in confluent cells (Fig. 3.5A). To evaluate further whether AhR activity varies as a function of MCF10A confluency, the cells were engineered to express firefly luciferase under the control of 4 DREs. TCDD treatment significantly increased luciferase reporter expression, demonstrating responsiveness of the engineered cells to AhR activation (Fig. 3.5B). Luciferase expression was significantly (~4.5-fold) higher in pre-confluent cells than in confluent cells, supporting the

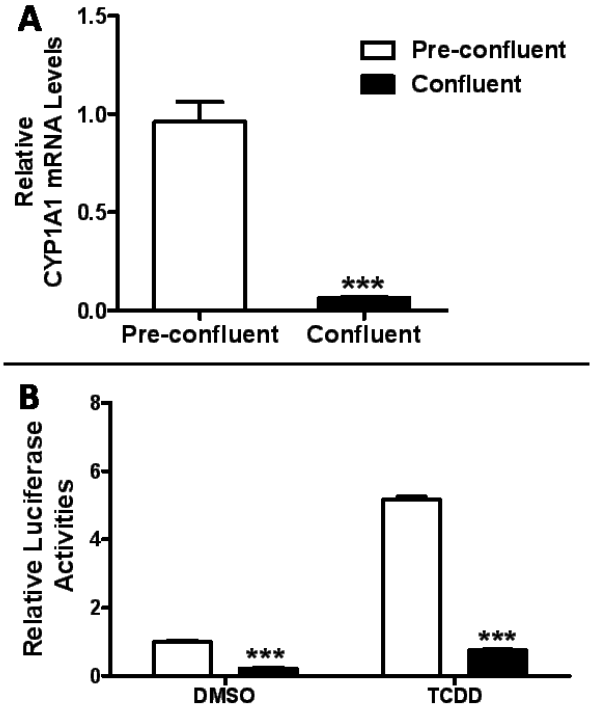


Fig. 3.5 Indices of AhR activity in pre-confluent and confluent MCF10A cells. A: MCF10A cells were harvested at approximately 70% and 100% confluency, and CYP1A1 mRNA levels were measured using a TaqMan Gene Expression Assay. Data are expressed as mean ± SEM of 3 independent experiments. B: MCF10A cells stably expressing an AhR-responsive luciferase reporter were treated for 24 hr with 0.1% DMSO or 30 nM TCDD prior to harvest at approximately 70% and 100% confluency for luciferase determinations. Data are expressed as mean ± sd, 3 wells per treatment group. *** $p<0.001$ compared to pre-confluent cells.

conclusion that AhR is basally active in pre-confluent MCF10A cells but less active in confluent cells (Fig. 3.5B).

The mRNA levels of AhR and its heterodimerization partner ARNT were ~3.0- and ~1.8-fold higher, respectively, in pre-confluent than in confluent cells, and the immunoreactive protein levels were correspondingly higher in pre-confluent cells (Fig.

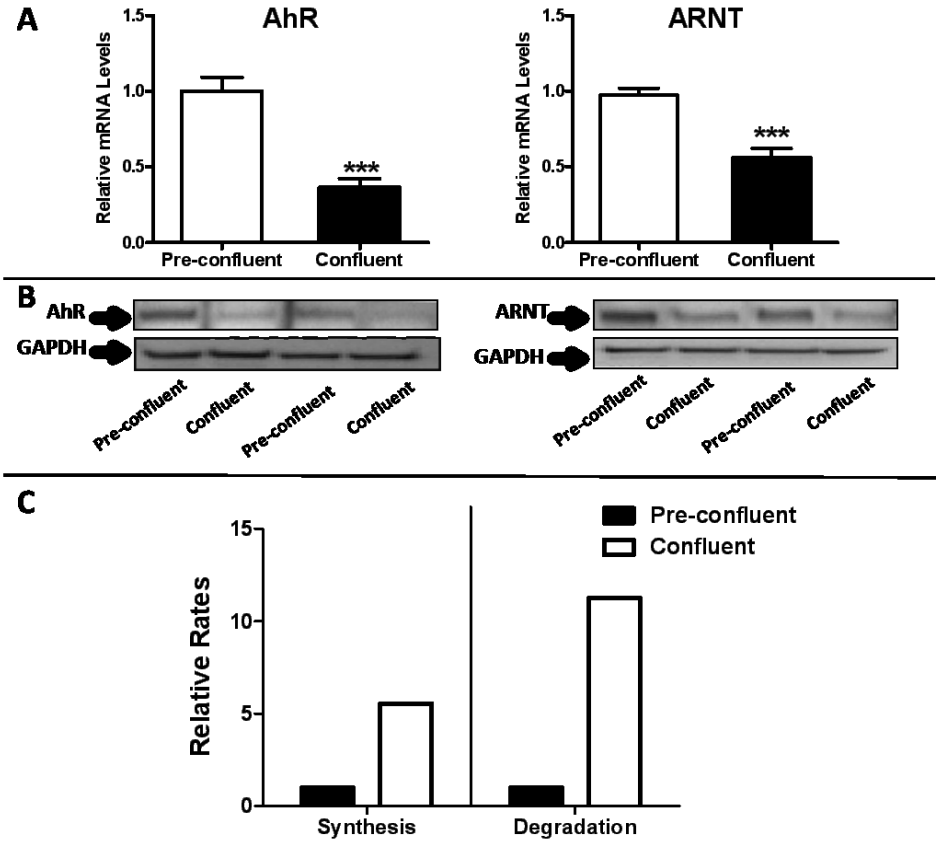


Fig. 3.6 Expression of AhR and ARNT in pre-confluent and confluent MCF10A cells. A: MCF10A cells were harvested at approximately 70% and 100% confluency. A: AhR and ARNT mRNA levels were measured in 6 independent experiments using TaqMan Gene Expression Assays. Data are expressed as mean ± SEM, and ***p<0.001 compared to pre-confluent cells. B: AhR and ARNT immunoreactive protein levels were measured in 2 independent experiments by western blot hybridization. C: BrU labeling was used to measure relative rates of AhR mRNA synthesis and degradation in pre-confluent and confluent MCF10A cells. The data represent the averages from two independent experiments.

3.6A and 3.6B). The higher levels of AhR mRNA and protein in pre-confluent than

confluent cells are likely attributable to differences in mRNA stability since, using the aforementioned bromouridine labeling technique, the rates of AhR mRNA synthesis and degradation were determined to be 5.5- and 11.3-fold higher, respectively, in confluent MCF10A cells than in pre-confluent cells (Fig. 3.6C).

To investigate the role of AhR in regulating SULT1E1 expression, we tested the effects of TCDD, a potent AhR agonist, and MNF, an AhR antagonist, on CYP1A1 and

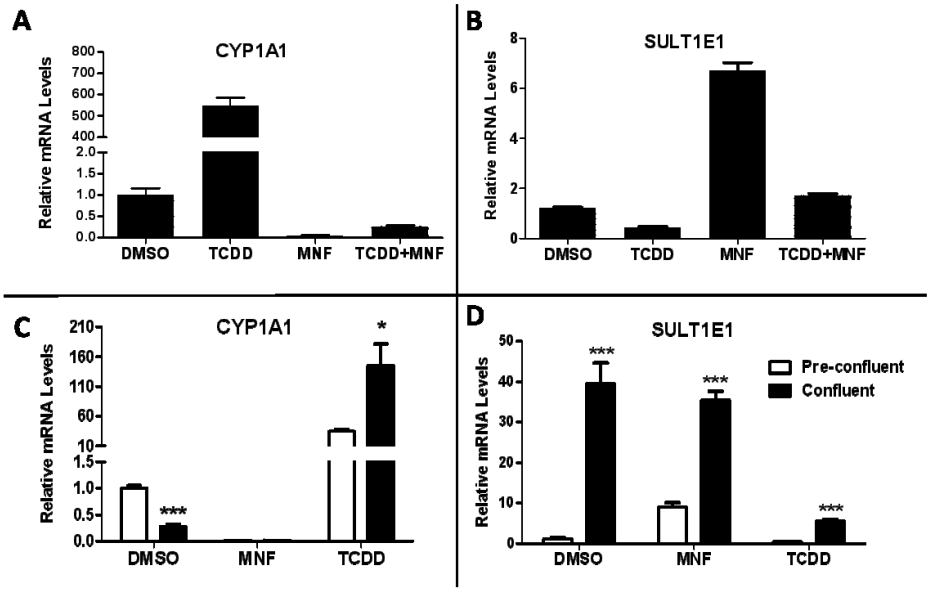


Fig. 3.7 Effects of AhR agonist and antagonist treatments on CYP1A1 and SULT1E1 expression in MCF10A cells. A and B: Pre-confluent cultures of MCF10A cells were treated for 24 hr with 0.1% DMSO, 30 nM TCDD, 1 μM MNF, or TCDD and MNF in combination and harvested for measurement of CYP1A1 (A) and SULT1E1 (B) mRNA levels. Data are expressed as mean ± SEM of triplicate assays. C and D: Pre-confluent and confluent cultures of MCF10A cells were treated for 24 hr with DMSO, MNF, or TCDD and harvested for measurement of CYP1A1 (C) and SULT1E1 (D) mRNA levels. Data are expressed as mean ± SEM of 3 independent cell culture experiments. *** P<0.001 compared to pre-confluent cells, * P<0.05

SULT1E1 expression in MCF10A cells. In pre-confluent cells, 30 nM TCDD treatment increased CYP1A1 mRNA content by 547-fold, while treatment with 1 μM MNF, a concentration sufficient to abolish TCDD-mediated induction, decreased basal CYP1A1 mRNA content by >99% (Fig. 3.7A). By comparison, TCDD treatment of pre-confluent

MCF10A cells decreased SULT1E1 mRNA content by ~57%, while MNF treatment increased the amount of SULT1E1 mRNA by ~6.7-fold (Fig. 3.7B).

Comparing the effects of TCDD and MNF treatments in pre-confluent MCF10A cells with those in confluent cells, TCDD-mediated CYP1A1 mRNA induction in confluent cells was not attenuated relative to the induction seen in pre-confluent cells,

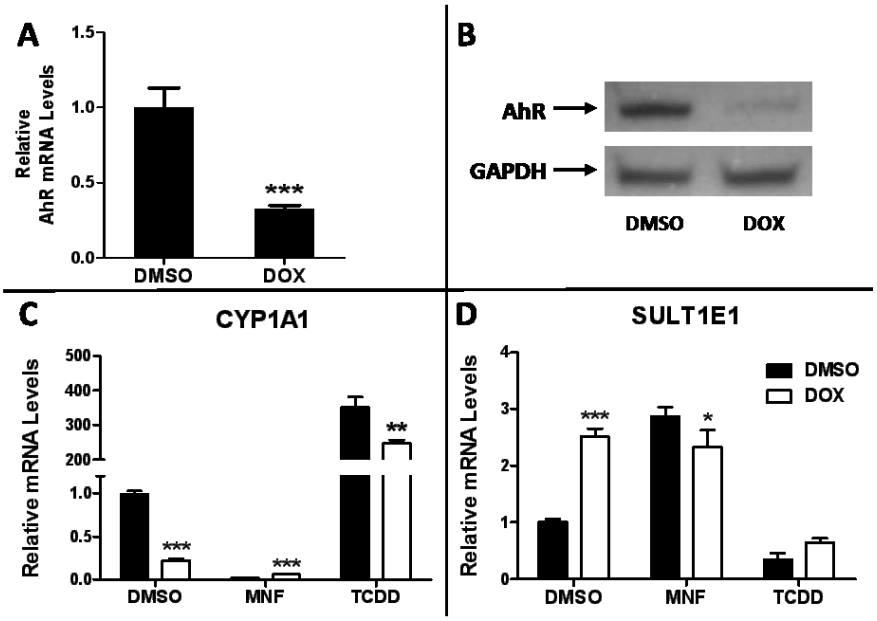


Fig. 3.8 Effect of AhR knockdown on CYP1A1 and SULT1E1 expression in pre-confluent MCF10A cells. A and B: Pre-confluent MCF10A cells engineered for conditional knockdown of AhR were treated for 96 hr with 0.1% DMSO or 1 μg/ml doxycycline and harvested for measurement of AhR mRNA (A) and immunoreactive protein (B) levels. In panel A, data are expressed relative to DMSO-treated cells as mean ± SEM of 3 independent cell culture experiments. ***p<0.001 compared to DMSO treated cells. C: Pre-confluent cells were treated for 96 hr with DMSO or doxycycline, either alone or in combination with 1 μM MNF or 30 nM TCDD, and harvested for measurement of CYP1A1 and SULT1E1 mRNA levels. Data are expressed as mean ± SEM of three independent cell culture experiments. ***P<0.001, ** P<0.01, * P<0.05 compared to DMSO-treated cells.

despite the lower levels of AhR and ARNT, demonstrating the high efficacy of this AhR agonist. As shown before, basal levels of CYP1A1 mRNA were significantly higher in pre-confluent cells than in confluent cells. MNF treatment abolished basal CYP1A1 expression in both pre-confluent and confluent cells (Fig. 3.7C).

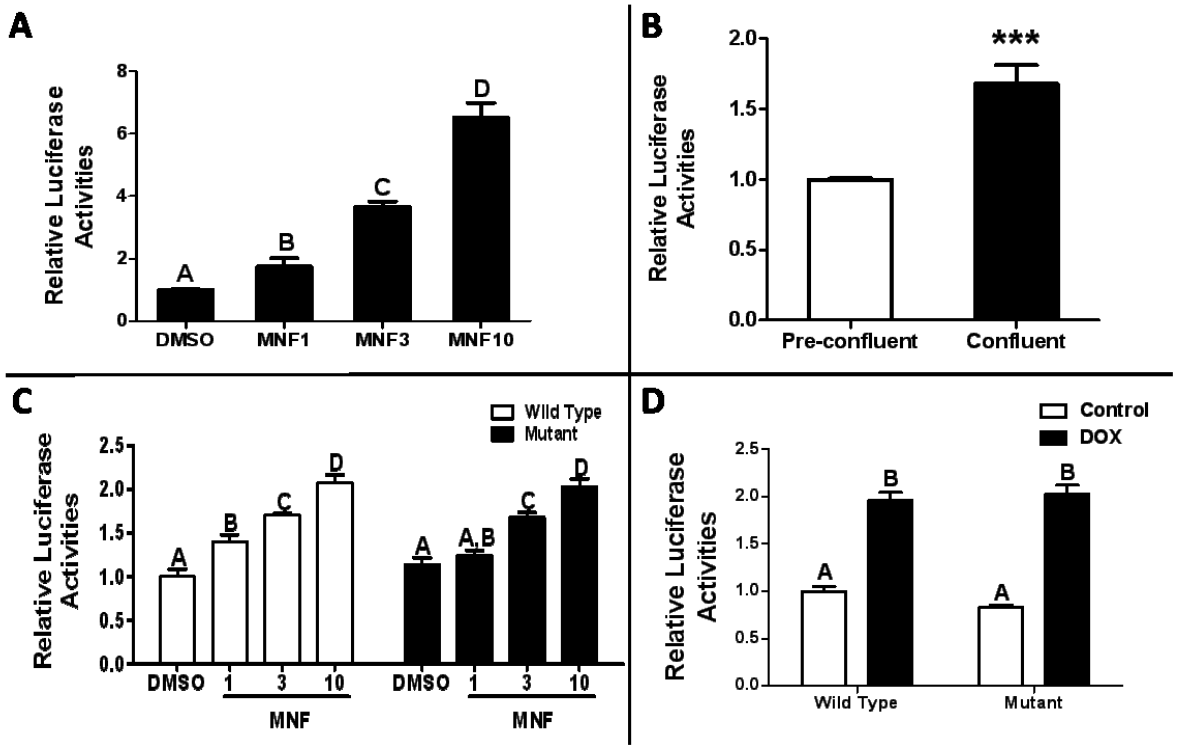


Fig. 3.9 Effects of MNF treatment, cell confluency, or AhR knockdown on luciferase expression from a reporter plasmid containing 7073 of the SULT1E1 5'-flanking sequence. A. MCF10A cells were stably transfected with the SULT1E1-luciferase reporter plasmid and pre-confluent cells were treated with 1 to 10 μM MNF for 24 hr, after which they were harvested for measurement of firefly luciferase activity and protein content. Each bar represents the mean ± sd of normalized luciferase activity (3 wells per treatment group). Groups that do not share a capital letter are significantly different from each other ($p<0.001$). B. Pre-confluent and confluent MCF10A cells stably transfected with the SULT1E1-luciferase reporter were harvested for measurement of luciferase activity as described above (A). ***$p<0.001$ compared to pre-confluent cells. C. Pre-confluent MCF10A cells were transiently transfected with the SULT1E1-luciferase reporter containing either an intact (white bars) or mutated (black bars) predicted DRE at -3476, treated for 24 hr with 1 to 10 μM MNF, and harvested for measurement of luciferase activities. Each bar represents the man ± sd of normalized (firefly/*Renilla*) luciferase activities (3 wells per treatment group). Groups that do not share a capital letter are significantly different from each other ($p<0.05$). D. Pre-confluent MCF10A cells engineered for conditional knockdown of AhR were treated with either 0.1% DMSO or 1 μg/ml doxycycline for 96 hr and transiently transfected with SULT1E1-luciferase reporter containing intact or mutant DRE. Twenty-four hr after transfection, cells were harvested for measurement of firefly and *Renilla* luciferase activities. Groups that do not share a capital letter are significantly different from each other ($p<0.001$).

For SULT1E1, mRNA levels were significantly higher in confluent cells, and TCDD treatment suppressed expression in both pre-confluent and confluent cells. MNF treatment increased SULT1E1 expression in pre-confluent cells but not in confluent cells (Fig. 3.7D), suggesting that confluency and MNF treatment increase SULT1E1 expression through a common mechanism.

As a complementary approach, MCF10A cells were engineered for conditional (doxycycline-mediated) knockdown of AhR expression. A significant reduction of AhR mRNA content (by ~68%), accompanied by a marked decrease in AhR immunoreactive protein level, was achieved when the engineered cells were treated with doxycycline for 96 hr (Fig. 3.8A and 3.8B). Doxycycline treatment also caused a significant reduction in TCDD-inducible CYP1A1 mRNA expression (Fig. 3.8C), although the reduction was only ~30%, again indicating that this potent and efficacious AhR agonist can produce a substantial signal even in the presence of reduced AhR levels.

When pre-confluent cells were treated for 96 hr with doxycycline, CYP1A1 mRNA levels were significantly reduced (by ~78%) while SULT1E1 mRNA levels were significantly increased (by ~2.5-fold) (Fig. 3.8C and 3.8D). The doxycycline-mediated increase in SULT1E1 mRNA content was comparable to the increase that was produced when cells with intact AhR expression (i.e., not treated with doxycycline) were treated with MNF. Also, co-treatment with doxycycline and MNF did not produce an additive effect, indicating that the effects of doxycycline and MNF on SULT1E1 expression were mediated through the common mechanism of AhR disruption.

Computational analysis of 10Kb of the SULT1E1 5’-flanking region identified two candidate DREs, one at 8138 and one at 3476 nt upstream of the transcription start site.

A fragment containing 7073 nt of the SULT1E1 5'-flanking region was ligated into a luciferase reporter plasmid, and this plasmid was used for stable or transient transfection of MCF10A cells. Treatment of stably transfected, pre-confluent MCF10A cells with MNF caused a concentration-dependent increase in reporter gene expression (Fig. 3.9A). Also, luciferase expression was significantly higher in confluent than in pre-confluent stably transfected MCF10A cells (Fig. 3.9B), indicating that the information responsible for both confluence- and MNF-inducible SULT1E1 transcription is contained within the 7 kb 5'-flanking region. MNF treatment also increased luciferase expression in pre-confluent MCF10A cells that had been transiently transfected with the 7 kb SULT1E1 reporter plasmid (Fig. 3.9C). Transfection of a SULT1E1 plasmid containing a site-directed mutation in a core nucleotide of the DRE at -3476 did not attenuate MNF-mediated reporter induction (Fig. 3.9C). Also, transient transfection of the 7 kb SULT1E1 reporter plasmid into the MCF10A cells that had been engineered for conditional AhR knockdown resulted in doxycycline-inducible reporter expression, and mutation of the DRE did not affect this up-regulation (Fig. 3.9D). These findings indicate that AhR inhibition/suppression-mediated SULT1E1 up-regulation is not mediated through the DRE at -3476.

Discussion

The impact of manipulations that alter cell-cell or cell-matrix contacts on AhR target gene expression was first investigated by Sadek and Allen-Hoffmann [379], who reported that the suspension of cultured human keratinocytes caused increased expression of CYP1A1 and other AhR target genes. Further studies using Hepa1c1c7 murine hepatoma cells and variants defective in AhR signaling confirmed that cell suspension caused activation of the AhR [380]. Subsequently, Monk et al. [381] reported that suspension of cultured rat keratinocytes caused transient AhR activation and CYP1A1 induction, and that co-treatment with the AhR antagonist, α-naphthoflavone, inhibited suspension-mediated CYP1A1 induction.

Cho et al. [382] then demonstrated that either suspension or monolayer culture at low confluency caused activation of the AhR in C3H10T1/2 fibroblast clonal sub-lines. An important conclusion from these studies was that disruption of cell-cell contact, rather than removal from the substratum, was responsible for AhR activation. Relative to confluent C3H10T1/2 cells, in ~70% confluent cells, there was ~4-fold activation of an AhR-responsive reporter, which is approximately the same magnitude of CYP1A1 mRNA and pGudLuc up-regulation that we observed in confluent versus pre-confluent MCF10A cells. In very low (5%) confluence C3H10T1/2 cells, AhR-responsive reporter expression was activated ~13-fold, which was approximately the same magnitude that occurred after TCDD treatment [382]. Treatments with several inhibitors of processes involved in AhR•ARNT complex formation inhibited AhR activation whether it was produced by TCDD treatment or loss of cell-cell contact, indicating that these stimuli

induced AhR•ARNT complex formation through the same mechanism. However, some treatments that interfere with the transcriptional activity of AhR•ARNT complexes produced stimulus-dependent effects on AhR activation, suggesting that the AhR•ARNT complexes are regulated differently after TCDD treatment and loss of cell-cell contact. A notable difference from the findings of Monk et al. [381] was that α-naphthoflavone treatment blocked TCDD-mediated AhR activation but not activation by loss of cell-cell contact. Consistent with the findings of Monk et al., we found that AhR inhibitor treatment (MNF) reduced the level of CYP1A1 expression in pre-confluent MCF10A cells.

Most recently, Ikuta et al. [383] reported that cell density influenced the subcellular distribution of AhR and AhR activity. AhR was predominantly nuclear in the HaCaT human keratinocyte cell line at low confluence, both nuclear and cytoplasmic at pre-confluence, and predominantly cytoplasmic at confluence. They also used a cell-scrape model of *in vitro* wound healing to demonstrate that AhR became activated in the loosely-associated cells at the border of the wound margin [383]. These investigators hypothesized that loss of cell-cell contact activates signaling events, possibly mediated through p38 MAPK, that increase the phosphorylation of AhR at its nuclear export signal, which causes AhR to accumulate in the nucleus [383].

AhR function has also been linked to the cell cycle. For example, Santini et al. [384] used centrifugal elutriation to isolate populations of TCDD-treated human monocytic U937 cells in different phases of the cell cycle, and reported that late G_1/early S phase cells had CYP1A1 mRNA contents that were ~1.4- and 3-fold higher than the contents of asynchronous/early G_1 and G_2/M cultures, respectively. These

studies suggest that the transcriptional activation of AhR target genes by TCDD is cell cycle-dependent and suppressed in G_2/M cells. However, Cho et al. reported that absence of cell-cell contact in low-density culture induced AhR activation was cell cycle independent because three G1/S phase inhibitors treatment did not affect this cell-confluency-mediated AhR up-regulation [382]. Similarly, Ikuta et al. confirmed this finding by showing that BrdUrd labeled S-phase cells had the same nuclear localization of AhR as cells in other cell cycle phases [383].

One possible mechanism for AhR-mediated transcriptional suppression is through the binding of the activated AhR•ARNT complex to an inhibitory DRE. Safe and co-workers have reported that certain genes (i.e., cathepsin D, c-fos, pS2, and Hsp27) contain pentanucleotide GCGTG sites that correspond to the core DRE motif and function as inhibitory DREs in that the binding of liganded AhR to these sites inhibits estrogen-mediated transcriptional activation by disrupting the binding of ER or other transcription factors to activating sites that are located in proximity to the DREs (for review, see [385]). Computational analysis of 10 kb of the SULT1E1 5'-flanking region identified two high-scoring AhR•ARNT binding sites: one at 8138 and one at 3476 nt upstream of the transcription start site. Of note, both of these DRE sites were identified as matches to the V$AHRARNT.03 matrix, which was compiled using, among other sequences, the inhibitory DRE sites contained in the cathepsin D and Hsp27 genes. Although a reporter construct containing ~7 kb of SULT1E1 5'-flanking sequence, and therefore the DRE at –3476, showed significant up-regulation in response to MNF treatment, AhR knockdown, or cell confluency, site-directed mutagenesis of the DRE

did not affect the up-regulation, suggesting that this DRE does not play a role in the negative regulation of SULT1E1 transcription.

It is therefore probable that AhR suppresses SULT1E1 transcription by modulating the activity of some other transcription factor. AhR has been shown to interact physically with a variety of transcription factors or transcription factor modulatory proteins, including nuclear receptors ERα, COUP-TFI, and ERRα1 [386]; NFκB subunits RelA and RelB [387;388]; the cell cycle regulatory protein Rb [389]; and the apoptosis regulatory transcription factor E2F1 [390], thereby modulating their activities either positively or negatively. AhR also engages in cross-talk interactions with MAP kinases [390], which might modify the activities of MAP kinase-regulated transcription factors in MCF10A cells.

Our results add to the growing number of mechanisms by which AhR modulates estrogenic activity. Anti-estrogenic effects of AhR ligands, in particular, have been extensively studied, and several mechanisms underlying such effects have been reported (for review, see [385]), including (1) AhR-mediated induction of one or more enzymes (e.g., CYP1B1) that metabolize estrogen and thereby reduce tissue active estrogen concentrations [391]; (2) AhR-mediated induction of a transcription inhibitory factor [392]; (3) an inhibitory action mediated by the non-productive binding of liganded AhR to an ER target gene, which prevents ER from binding [393]; (4) association of liganded AhR with ER, thereby disrupting a transcriptionally productive interaction between ER and Sp1 [394]; (5) AhR -mediated reduction of cellular ER levels, by either suppression of ER transcription [395] or acceleration of ER degradation through AhR -mediated recruitment of both ER and proteosome machinery [396]; and (6) AhR-

mediated transcriptional activation of its target genes, resulting in competition for recruitment of the limited pool of co-activators that are shared by the Ah receptor and ER. In this regard, ARNT is said to function as a co-activator for ER, but with selectivity for ERβ. Thus, ARNT recruitment to AhR-target genes can reduce the transcription of ER-target genes [397]. Concerning pro-estrogenic effects, Ohtake et al. [398] reported that AhR ligand treatment can induce ER-mediated transcription through the formation of an AhR•ARNT•ER complex. By this mechanism, the ligand-bound AhR•ARNT heterodimer “hijacks” ER to enable the transcription of ER target genes in the absence of an ER ligand [399]. In other studies, AhR ligands have been found to activate ER-mediated transcriptional activity without a requirement for the AhR [400;401]. Abdelrahim et al. [401] reported that AhR ligands 3MC and PCB126 were both capable of activating ER in MCF7 breast cancer cells, while Shipley and Waxman [400] found that 3MC, but not PCB126 or TCDD, functioned as an ER agonist in Ishikawa uterine cancer cells. In another study, Boverhof et al. [402] reported that TCDD treatment of OVX mice altered the expression of numerous uterine genes that were comparably regulated by 17α-ethynylestradiol. Co-treatment of mice with the pure anti-estrogen ICI 182,780 (ICI) inhibited both the 17α-ethynylestradiol- and the TCDD-mediated effects on these genes [402]. In another study in mice, AhR was shown to be required for the expression of CYP19 in the ovary, and treatment with the AhR ligand 9,10-dimethyl-1,2-benzanthracene increased ovarian expression of CYP19 [403]. By demonstrating that AhR activation suppresses expression of SULT1E1, a major estrogen-inactivating enzyme, our study provides another mechanism by which AhR can regulate estrogenicity.

We suggest that AhR-mediated regulation of SULT1E1 plays an important role in modulating estrogen mitogenicity in normal breast tissue. When the breast cells are in a non-proliferative state, it is essential that the growth stimulatory effects of estrogen be held to an absolute minimum. Therefore, cell-cell contact triggers molecular events that include inhibition of AhR activity and up-regulation of SULT1E1 activity. When the breast cells switch to a proliferative state, a lessening of cell-cell contact causes activation of AhR activity and suppression of SULT1E1 expression, resulting in increased active estrogen levels in the breast microenvironment.

CHAPTER 4

TSC down-regulates SULT1E1 expression in MCF10A human breast epithelial cells through an AhR-mediated mechanism

Introduction

Smoking, one of the leading environmental etiologic factors that is associated with human cancers in different organs, causes diseases not only in the directly exposed tissues of the respiratory tract but also in distant organs such as bladder and pancreas [222;404-407]. Heart disease, which is the primary cause of death in developed countries, and about 90% of lung cancer, which is a leading cause of cancer-related death resulting in 1.2 million deaths annually, have been closely associated with tobacco smoking [408-410]. In addition, some evidence supports an association between tobacco smoking and breast cancer. For example:

Tobacco smoke contains carcinogens, including polycyclic aromatic hydrocarbons (PAHs), aromatic amines, and *N* -nitrosamines, which have been shown to play important roles at different stages of breast cancer development [404;409;411-413]. These lipophilic carcinogens are able to pass through the alveolar membrane and be transported to the breast through the circulatory system [414;415]. Moreover, these fat soluble compounds can be stored in breast adipose tissue and undergo further metabolism *in situ* by breast epithelial cells, which has been supported by the finding that the breast fluid of non-lactating tobacco smokers contains mutagenic tobacco

compounds and their metabolites, such as nicotine and its major metabolite, cotinine [416;417]. These carcinogens and their electrophilic intermediates, such as benzo[a]pyrene (BP), crotonaldehyde (2-butenal), and acetaldehyde, covalently bind to nucleic acid and form carcinogen-DNA adducts, which may lead to mutation of oncogenes and tumor suppressor genes and malignant cell transformation [418-422]. Accumulated *in vitro* exposure to BP, a PAH found in tobacco smoke, transforms normal human breast epithelial cells to a precancerous stage [423].

Tobacco smoke may produce genotoxic effects in breast tissue because it increases the prevalence and spectrum of *p53* mutations and alters the expression of genes involved in DNA repair and carcinogen metabolism, including CYP family members, catechol-O-methyltransferase, glutathione-*S*-transferases and *N*-acetyltransferase [424-429].

Epidemiological studies indicate that tobacco smoking exerts anti-estrogenic effects that manifest clinically in an early natural menopause, a lowered risk of cancer of the endometrium, and an increased risk of some osteoporotic fractures in female smokers. Moreover, women who smoke may have a reduced risk of uterine fibroids, and endometriosis [430-433]. However, a recent study has indicated that a low dose of TSC can exert estrogen-like effects both *in vitro* and *in vivo* [434]. TSC has been demonstrated to bind and transcriptionally activate ER [435]. Cadmium, a toxic heavy metal that occurs in nature and in high levels in cigarette smoke, has been shown to increase the steady state levels of ER regulated genes such as pS2 and cathepsin D in human breast cancer cells [436]. These findings indicate an estrogenic effect of TSC.

Therefore, the role of tobacco smoke in the endocrine modulation associated with breast cancer needs further study.

SULT1E1 is an enzyme that can decrease the hormonal activity of estrogens by facilitating their excretion and blocking their ER-mediated activity [233-235]. Since estrogen plays a central role in the development and progression of breast cancer according to epidemiologic, clinical, as well as molecular and cell biology studies, SULT1E1 is well positioned to be a main estrogen modulator and therefore closely associated with breast cancer etiology [437;438]. Our data (see Chapter 3) suggest that SULT1E1 expression is suppressed by AhR activation. AhR mediated gene transcription has been shown to be activated by TSC, and tobacco smoke contains PAHs that are known activators of the AhR [435;439]. These findings suggest that TSC may suppress SULT1E1 expression through an AhR-mediated mechanism and thereby alter estrogen-dependent breast cancer development. In the current study, we investigated the effects of TSC treatment on CYP1A1, a well-characterized AhR target gene [439], and SULT1E1 expression in the “normal” nontumorigenic human breast epithelial cell line MCF10A.

Materials and Methods

Materials. TSC was prepared by Dr. Deepak Bhalla (College of Pharmacy and Health Sciences, Wayne State University). The rest of the materials are as described in Chapter 2.

Cell culture. As described in Chapter 2.

Gene expression analysis. As described in Chapter 2

Transient transfection analysis. As described in Chapter 2.

Statistical analysis. As described in Chapter 2.

Results

CYP1A1 has been shown to participate in metabolic activation of PAHs, a typical

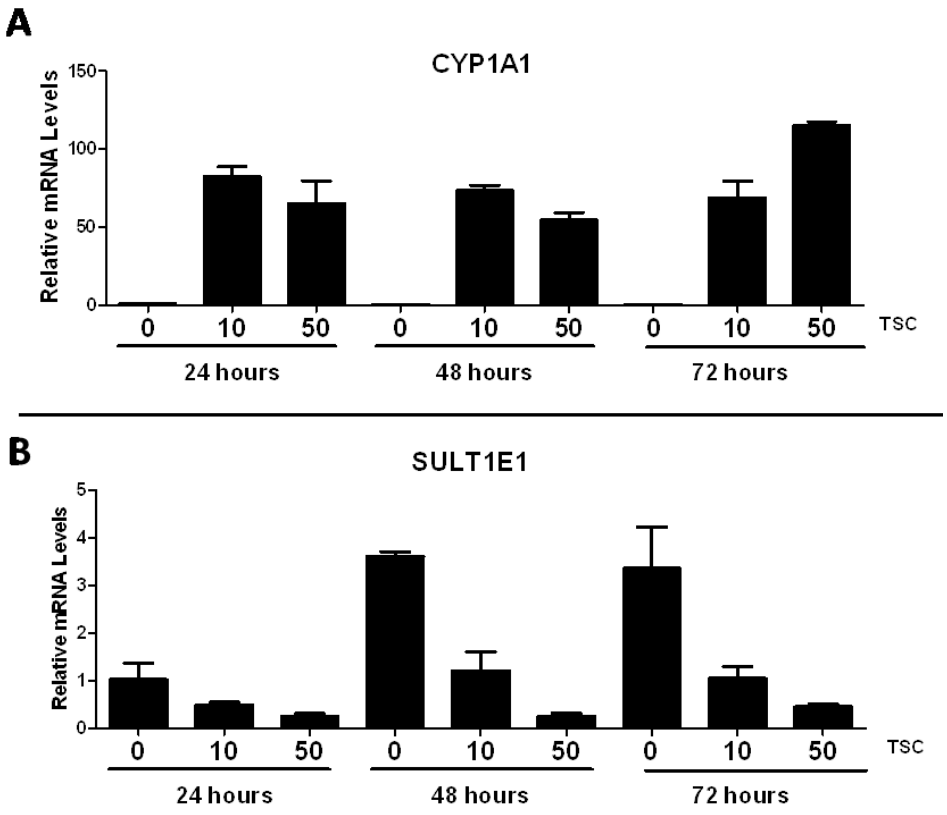


Fig. 4.1 Time-dependent effects of tobacco smoke condensate (TSC) treatment on CYP1A1 (A) and SULT1E1 (B) mRNA expression in MCF10A cells. Pre-confluent cultures of MCF10A cells were treated for 24, 48, or 72 hr with 0, 10 or 50 µg/ml TSC, and CYP1A1 (A) and SULT1E1 (B) mRNA levels were measured using TaqMan Gene Expression Assays. CYP1A1 and SULT1E1 mRNA levels are expressed relative to 24-hr vehicle treated controls, and all values represent the mean ± SD of technical replicates of the same sample.

class of carcinogenic compounds present in tobacco smoke [404;413;435;440]. CYP1A1 expression is inducible by both PAHs and TSC through activation of the AhR [435;439].

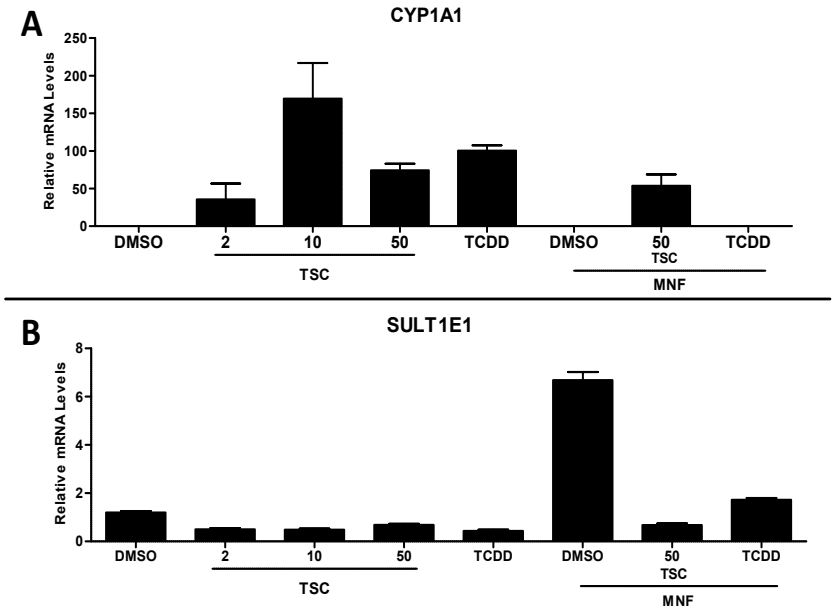


Fig. 4.2 Effect of AhR antagonist treatment on TSC-mediated regulation of CYP1A1 (A) and SULT1E1 (B). MCF10A cells were treated for 24 hr with 0.1% DMSO; 2, 10 or 50 µg/ml TSC; or 30 nM TCDD, alone or in the presence of 1 µM of the AhR antagonist 3'-methoxy-4'-nitroflavone (MNF). After treatment, the cells were harvested for measurement of CYP1A1 (A) and SULT1E1 (B) mRNA levels using TaqMan Gene Expression Assays. The mRNA contents were expressed relative to the levels measured in untreated MCF10A cells and all values represent the mean ± SD of technical replicates of the same sample.

Therefore, CYP1A1 expression was used as an indicator for AhR functional activity in the present study. Treatment of MCF10A cultures with 10 or 50 µg/ml TSC for 24 hr increased CYP1A1 mRNA content >50-fold, and this increase persisted for 72h (Fig. 4.1A). When the same samples were used to examine SULT1E1 mRNA content it was found that TSC treatment attenuated the expression of SULT1E1 in a concentration-dependent manner at all time points tested (Fig. 4.1B). Treatment with 10 µg/ml TSC resulted in 50%, 67% and 70% decreases in SULT1E1 mRNA expression after treatment for 24 hr, 48 hr, and 72 hr, respectively (Fig. 4.1B), while 50 µg/ml TSC treatment decreased

SULT1E1 expression by 75%, 94% and 85%, respectively, at those three treatment times (Fig. 4.1B).

To evaluate whether AhR activation is the mechanistic reason for TSC-mediated SULT1E1 suppression, the effects of TCDD, a potent AhR agonist, and MNF, an AhR antagonist with little or no agonist activity [441], on CYP1A1 and SULT1E1 mRNA levels were tested in MCF10A cells. We first verified that treatment of MCF10A cells with the prototypical AHR agonist, TCDD, resulted in an ~100-fold up-regulation of CYP1A1 (Fig. 4.2A), while treatment with the AhR antagonist, MNF (1μM), decreased the CYP1A1 mRNA level by about 99% relative to vehicle (DMSO) control (Fig. 4.2A). As expected, TCDD treatment suppressed SULT1E1 expression by 57% and MNF exposure induced a ~5-fold up-regulation of SULT1E1 expression (Fig. 4.2B). Treatment with 2, 10 and 50 μg/ml TSC resulted in an induction of CYP1A1 mRNA

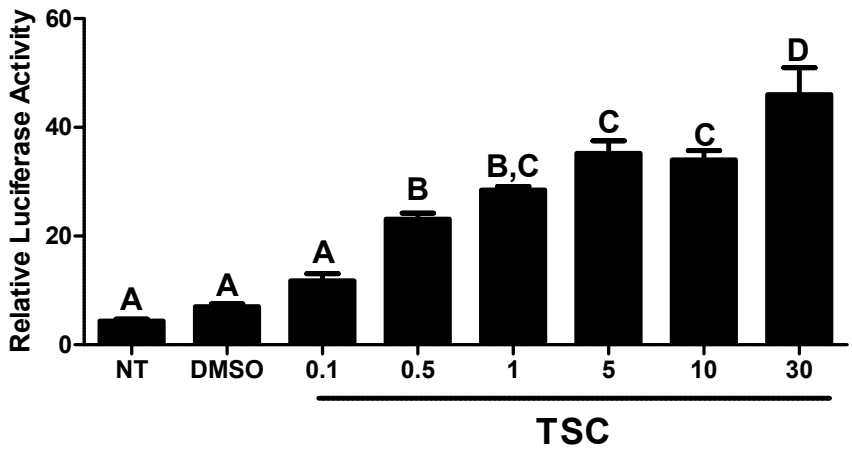


Fig. 4.3 Effect of TSC treatment on expression of an AhR-responsive reporter. MCF10A cells were transiently transfected with pGudLuc reporter containing a 484-bp fragment from the upstream region of the CYP1A1 gene that includes four dioxin responsive element (DRE) [9]. Twenty-four hours post transfection, cells were treated with nothing (NT), 0.1% DMSO, or 0.1–30 μg/ml TSC for 24 h and harvested for measurement of luciferase activities. Each bar represents the mean ± S.D. of normalized (firefly/Renilla) luciferase measurements (3 wells per treatment group). Groups that do not share a capital letter are significantly different from each other ($p \leq 0.05$).

levels of 35-, 169-, and 74-fold, respectively (Fig. 4.2A). By comparison, TSC treatment decreased SULT1E1 expression by at least 44% at all of the concentrations that were examined (Fig. 4.2B). Co-treatment with MNF abolished TCDD-mediated CYP1A1 induction and SULT1E1 suppression, but only attenuated ~30% of the 50 µg/ml TSC-mediated CYP1A1 inductions and had no effect on the suppression of SULT1E1 by TSC (Fig. 4.2).

To confirm the ability of TSC to activate the AhR in MCF10A cells, cells were transfected with a luciferase receptor plasmid containing four dioxin-response elements (i.e., AhR binding sites), and luciferase activity was measured after treatment with 0.1-30 µg/ml TSC for 24h. TSC concentrations of 0.5, 1, 5, 10, 30 µg/ml significantly enhanced luciferase reporter expression by 3.3-, 4-, 5-, 4.9-, and 6.6-fold, respectively (Fig. 4.3), indicating the activation of AhR. Effects of MNF treatments on the TSC concentration-

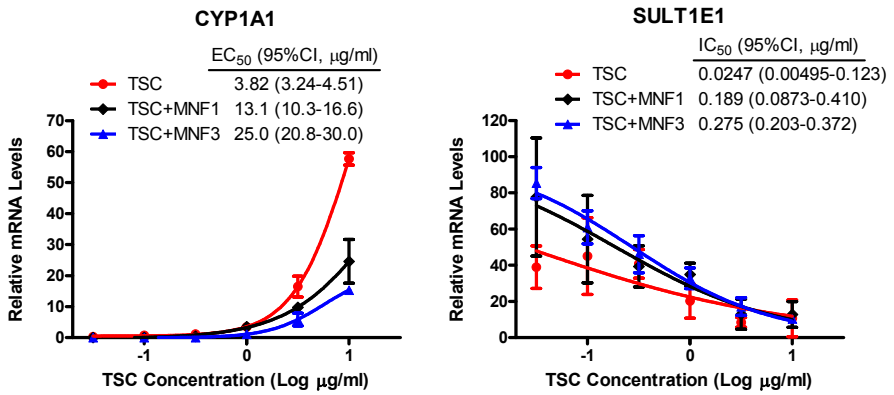


Fig. 4.4 Effect of MNF treatment on the TSC concentration-response curves for CYP1A1 induction and SULT1E1 suppression. MCF10A cells were treated for 24 h with 0.03–10 µg/ml TSC in the absence or presence of 1 or 3 µM MNF. After treatment, the cells were harvested for measurement of CYP1A1 and SULT1E1 mRNA levels using TaqMan Gene Expression Assays. The mRNA contents were expressed relative to the levels measured in untreated MCF10A cells and all values represent the mean ± SD of technical replicates of the pooled sample of 3 wells per treatment group. The fitted sigmoid curve for the data is shown, together with the calculated EC_{50} or IC_{50} value and 95% confidence interval (95% CI).

response curves for CYP1A1 induction and SULT1E1 suppression are shown in Fig. 4.4 MNF treatment inhibited TSC-mediated CYP1A1 induction and SULT1E1 suppression and shifted the TSC concentration-responsive curves rightwards (Fig. 4.4), suggesting a role for the AhR in TSC-mediated CYP1A1 and SULT1E1 regulation.

Discussion

It is well known that the etiology of human breast cancer is affected by complex inherited and environmental factors [442;443]. To date, genetic studies have identified and confirmed germ-line mutations in high-penetrance genes, such as BRCA1, BRCA2, PTEN, and TP53; moderate-penetrance genes, such as ATM, BRIP1, CHEK2, and PALB2; and around 20 common low-penetrance variants in 19 genes or loci that contribute to a woman's risk of breast cancer [444]. The development of microarray approaches enabled genome-wide analysis of breast cancer and identified numerous genetic and epigenetic alterations involving a small number of altered signaling pathways (PI3K, NK-κB, FGF, etc.) [445]. In addition to these genetic and well accepted hormone-related risk factors discussed in the previous chapters, a large number of environmental chemicals are suspected of playing a role in breast cancer [26;446;447]. For example, traffic emission, the major source of air pollution in urban areas, has been associated with increased breast cancer risk among women living close to industrial sites and heavy traffic in Long Island, New York [448]. Traffic emissions contain many potential carcinogens, such as PAHs, which are also major compounds found in tobacco smoke [449].

Tobacco smoke has been associated with increased risk of breast cancer [450]. Various factors such as duration and intensity of smoking [451] and tobacco smoking during first pregnancy [452] were found to influence the smoking induced risk of breast cancer. Moreover, a direct relationship was observed between tobacco smoking and metastasis in breast cancer [453]. However, the results of epidemiological studies to date have not provided conclusive information about the association between tobacco smoking and breast cancer, with some epidemiology studies reporting a positive association [300;451;452;454-456] and some reporting a negative association [457;458]. The inconsistencies among studies are likely due to the fact that the quantity and duration of smoking, age at initiation of smoking, exposure to second hand smoke, hormonal profile and gene polymorphisms of the study population are all extremely difficult to assess accurately. Investigation of the interaction between tobacco smoke and genes that are involved in bioactivation or detoxification processes, in endocrine modulation, in DNA repair, and in cell cycle and apoptosis control will provide more information about the role of environmental exposure to tobacco smoke in breast carcinogenesis. TSC was used as a model to study the effect of tobacco smoke exposure. Calculations by Holden et al. [459] suggest that the concentration of condensate in the epithelial lining fluid would be 0.01-1% when one cigarette was smoked.

AhR signaling is known to activate both phase I enzymes (most notably CYP1A1) and phase II enzymes (mainly glutathione S-transferase Ya) in response to tobacco smoke, which will either bioactivate procarcinogens in tobacco smoke or sequester and detoxify reactive electrophiles [460-463]. It is therefore plausible that

AhR-mediated processes have a measurable and biologically significant effect on the carcinogenicity of tobacco smoke. BP, an important component of tobacco smoke, induced tumors in wild-type mice, but not in AhR null animals [464]. The results of Chapter 3 indicated that the expression of SULT1E1 was subjected to AhR regulation. To investigate the inhibitory effect of TSC on SULT1E1 expression, we utilized a chemical agonist (TCDD) and antagonist (MNF) to alter AhR signaling in MCF10A cells. We found that TSC treatment increased the expression of CYP1A1, a well-characterized AhR target gene, but suppressed the expression of SULT1E1. Moreover, the potency by which TSC suppressed SULT1E1 suppression was greater than the potency of CYP1A1 induction. We then confirmed that TSC treatment caused AhR activation, as indicated by significant concentration-dependent activation of a transiently transfected AhR-responsive reporter plasmid. Co-treatment with the AhR antagonist MNF inhibited TSC-mediated CYP1A1 induction, as indicated by MNF-dependent rightward shifts of the TSC concentration-response curve. Treatment with MNF alone increased SULT1E1 expression and caused rightward shifts of the TSC concentration-curves, suggesting a role for the AhR in TSC-mediated SULT1E1 suppression. Further studies are needed to determine whether tobacco smoke exposure causes SULT1E1 suppression in the breast *in vivo*, and whether such an effect plays significant role in the development of breast cancer.

CHAPTER 5

Knockdown of estrogen-inactivating enzyme SULT1E1 by RNAi accelerates tumorigenesis in vivo

INTRODUCTION

Breast cancer is the leading type of cancer and the second most common cause of cancer death in American women [1]. Breast cancer incidence rates have decreased about 2 percent per year since the early 2000s, possibly due to the use of screening mammography and discontinuation of postmenopausal hormone replacement therapy (HRT) [465-470]. The latter change probably had a greater effect in that women who took HRT to relieve menopausal symptoms had an increased risk of developing breast cancer and there was a rapid decline in breast cancer incidence after discontinuation of HRT [465-467]. These findings indicate that exogenous estrogen exposure influences the development of breast cancer. Moreover, increased cumulative endogenous exposure to estrogen resulting from early onset of menarche, later onset of menopause, nulliparity, and late age of first pregnancy has also been associated with increased breast cancer risk according to the results of clinical and epidemiologic studies [22-26]. In addition to the above mentioned evidence linking breast cancer incidence to estrogen exposure, clinical and laboratory studies have shown that estrogen can directly stimulate the proliferation of human breast cancer cells *in vitro* and drive the growth of breast tumors *in vivo* [29;30;471-474]. This involvement of

estrogen in the development and progression of breast cancer provides the conceptual basis for the potential use of estrogen modulators as treatments for breast cancer [475;476].

Extensive biotransformation of endogenous and exogenous estrogens to estrogen conjugates, especially to abundant circulating estrogen sulfates by SULTs, has long been recognized as a major determinant of estrogen levels in humans [178;232]. It has been demonstrated that normal human breast epithelial cells possess endogenous SULT1E1 activity at physiologically significant levels [341;477]. In contrast, many breast cancer cell lines appear to have lost the expression of SULT1E1 and therefore lack the high affinity estrogen sulfonation pathway [249;250]. Moreover, SULT1E1 has been shown to regulate the *in situ* bioavailability of estrogen in human breast, especially in postmenopausal women [79]. In another study in which specimens of human breast carcinoma tissues were used to examine the expression of SULT1E1, it was found that SULT1E1 mRNA was expressed in both carcinoma and intratumoral stromal cells [97]. It was also demonstrated that SULT1E1 immunoreactivity was inversely correlated with tumor size or lymph node status [97]. Moreover, SULT1E1 immunoreactivity was significantly associated with a decreased risk of recurrence or improved prognosis [97]. These studies indicate that SULT1E1 regulates the availability of the estrogen in the breast and consequently affects cell growth. We propose that SULT1E1 silencing might be a critical event in the transition of normal breast epithelial cells to cancer cells by facilitating a breast microenvironment that favors increased estrogenic stimulation.

The MCF10A-derived lineage of human breast epithelial cell lines is a good model to study the molecular events that occur during breast cancer neoplastic progression. These MCF10 cell lines were all derived from a common genetic background and the model includes the full spectrum of neoplastic progression and incorporates aspects of both indolent preneoplastic diseases to aggressively neoplastic breast epithelial cell growth. In the current research, we use the premalignant MCF10AT1 cell line, which expresses SULT1E1 at a relatively high level (Chapter 2) [250], as a model to determine the impact of SULT1E1 silencing on xenograft progression. Understanding the function of SULT1E1, the major estrogen-inactivating enzyme, will provide new information on *in situ* estrogen metabolism during human breast cancer development. The results of this research will have implications for breast cancer prevention and treatment.

Methods and Materials

Materials. Cell culture medium, L-glutamine, horse serum, penicillin-streptomycin solution, sodium pyruvate, Lipofectamine 2000, Superscript II, recombinant human SULT1E1, and anti-SULT1E1 antibody were purchased from Invitrogen Corporation (Carlsbad, CA). EGF was purchased from BD Biosciences (San Jose, CA). Recombinant human insulin (Novolin R) was purchased from Novo Nordisk Pharmaceuticals, Inc. (Princeton, NJ). Doxycycline, E2, ICI 182780, cholera toxin and hydrocortisone were purchased from Sigma-Aldrich (St. Louis, MO). The pS2 antibody and orseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Santa

Cruz Biotechnology (Santa Cruz, CA). FuGENE 6 Transfection Reagent was purchased from Roche Molecular Biochemicals. ECL Plus Western Blotting Detection Reagents and Hybond-P membranes were purchased from GE Healthcare (Piscataway, NJ). All materials that were used to produce recombinant lentiviruses targeting human SULT1E1 gene were purchased from Addgene (Cambridge, MA), unless otherwise stated. Other materials were obtained from the sources indicated below.

Cell culture. The MCF10AT1 cell line was obtained from the Cell Resources Facility of the Barbara Ann Karmanos Cancer Institute, Wayne State University and cultured in medium as described in Chapter 2. The human HUVEC cell line was obtained from ATCC (Manassas, VA) and maintained in F-12K medium supplemented with 0.1 mg/ml heparin, 0.05 mg/ml endothelial cell growth supplement (ECGS) and 10% fetal bovine serum at 37 °C with 5% CO_2.

Transient transfection analysis. The p2ERE-luc reporter was constructed by ligating a double-stranded oligonucleotide containing two vitellogenin EREs upstream of a minimal herpes simplex virus thymidine kinase promoter, which had been preligated into pGL3-Basic (Promega Corporation, Madison, WI). The sequences of the oligonucleotides are: 5′-CGCGTGTCCAAAGTCAGGTCACAGTGACCTGATTCAGGTCACAGTGACCTGATCAAAGTTA-3′ and 5′-GATCTAACTTTGATCAGGTCACTGTGACCTGAATCAGGTCACTGTGACCTGACTTTGGACA-3′.

MCF10AT1 cells (200,000) were seeded into 24-well plates and cultured in 0.5 ml phenol red-free DMEM/F12 Nutrient Mixture (1:1) supplemented with 10 μg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, 10% charcoal-stripped fetal bovine serum. The following day, Opti-MEM containing a

premixed complex of 4 µl of Lipofectamine 2000, 0.8 µg p2ERE-Luc and 1.25 ng pRL-SV40 (Promega) was added to each well. The following day, cultures (3 wells per group) were incubated with phenol red-free DMEM/F12 Medium, supplemented as described above, containing 0.1% DMSO, 0.1, 1 or 10 nM E2. After 48 hr, the cells were harvested for measurement of firefly and *Renilla* luciferase activities using the Dual Luciferase Reporter Assay System (Promega) and a Dynex model MLX Luminometer.

Heterotypic culture of MCF10AT1 and HUVEC cells. Heterotypic three dimensional cultures were prepared based on the methods previously reported by Dr. Malathy P. V. Shekhar [478]. In brief, 55,000 parental MCF10AT1 cells or MCF10AT1 cells that were engineered for conditional knockdown of SULT1E1 expression were mixed with an equal number of HUVEC cells and seeded as a single-cell suspension into 96-well plates with a solidified layer of phenol red-free Matrigel (100 µl/well) (Collaborative Biomedical Products, Bedford, MA). MCF10AT1 cells and HUVEC cells were pre-labeled with fluorescent cationic membrane tracers, DiI and DiO (Molecular Probes, Inc., Eugene, Oregon), respectively, prior to coculturing. For E2 or ICI treatment, 24 hr post plating, the co-culture system was treated with E2 or ICI alone or in combination in charcoal stripped medium supplemented with EGF and basic fibroblast growth factor (bFGF). Cultures were maintained at 37°C for 7 days with daily media changes, and cell viability was measured with cellTiTer 96 cell proliferation assays according to the manufacturer's directions (Promega Corp., Madison, WI). For the trypan blue exclusion assay, 100,000 parental MCF10AT1 cells or SULT1E1 knockdown (siSULT1E1) MCF10AT1 cells were mixed with an equal number of HUVEC cells and seeded as a single-cell suspension into 24-well plates with solidified layer of phenol red-free Matrigel

(400 µl/well). After the same E2 or ICI182,780 treatment for 7 days, the medium was removed and wells were rinsed with PBS. Matrigel was digested and cells were recovered with Cell Recovery Solution (Collaborative Biomedical Products, Bedford, MA) according to manufacturer's instruction. Cells recovered were then treated with trypsin to get single cells. The number of viable cells was determined by trypan blue solution in a hemocytometer.

SULT1E1 conditional knockdown. To prepare a recombinant lentivirus expressing shRNA oligonucleotides targeting SULT1E1 in a conditional manner, oligonucleotides corresponding to the most effective shRNA that was tested,

5'-CGCGTCGCCAGAAATTGACGCCCTTCATCTCGAGATGAAGGGCGACAATTTCTGGCTTTTTTAT-3'

and 5'-CGATAAAAAAGCCAGAAATTGTCGCCCTTCATCTCGAGATGAAGGGCGTCAATTTCTGGC-3'

(Integrated DNA Technologies, INC), were ligated into the MluI and ClaI sites of pLVTHM lentiviral plasmid, and then the fragment containing the H1 promoter and the shRNA sequence was subcloned into the pLVCT-tTRKRAB Tet-on lentiviral plasmid opened with MscI and FspI enzymes. This plasmid was cotransfected into 293T cells together with psPAX2 (plasmid expressing lentiviral packaging elements) and psMD2.G (plasmid expressing vesicular stomatitis virus envelope protein) using FuGENE 6 Transfection Reagent. Two days following transfection, medium containing lentivirus was collected, filtered and stored at -80 °C. The viral titer was estimated by transducing MCF10AT1 cells with several dilutions of virus and visualizing green fluorescence, which was also expressed by the lentiviral vector. For infection, MCF10AT1 cells were plated into 24-well plates (200,000 cells/well). After 16 hr, medium containing recombinant lentivirus was added (the multiplicity of infection=1). Following 16h of incubation, the cells were washed and subcultured, and doxycycline (Dox, Sigma) was

added to half of the infected cells at a final concentration of 50 ng/ml. Five days later, the cells were harvested and sorted by fluorescence-activated cell sorting. Then individual clones were prepared by limiting dilution and evaluated for doxycline-inducible SULT1E1 knockdown using a TaqMan Gene Expression Assay and by western blot analysis.

TaqMan Gene Expression assays. As described in Chapter 2.

Western blot analysis. As described in Chapter 2.

Mouse xenografts. siSULT1E1 MCF10AT1 cells were grown in T150 flasks and harvested at 70-80% confluency. Approximately 3-4 hr before harvesting, culture medium was replaced with fresh medium to remove dead and detached cells. Cells were then trypsinized and live cells were counted after trypan blue staining using a hemocytometer. Approximately $1x10^7$ cells/site in 150–200 µl of Matrigel (Collaborative Biomedical Products, Bedford, MA) were inoculated subcutaneously in female nude mice aged 55-65 days (NCRNU-M-F, Taconic Farms) at 2 sites/animal using a 21-gauge syringe. One week after injection, half of the mice were given doxycycline in the drinking water (final concentration 2 mg/ml) in water bottles that were wrapped in autoclaved aluminum foil. Fresh water containing doxycycline was prepared every other day. Twelve weeks after injection, mice were killed and xenograft lesions were formalin-fixed and provided to the Microscopy, Imaging & Cytometry Resources (MICR) Core Facility (Wayne State University, School of Medicine) for histologic and immunohistochemical analysis. These epithelial structures were graded from 0 to 5 according to the histological classification of Dawson *et al*. [319]. Xenografts were then classified according to the highest grade presented in the lesion. This experiment was

repeated 3 times and included a total of 14 mice for each group. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Wayne State University, and guidelines relating to the ethical treatment of animals and relating to tissue growth and burden were strictly followed.

Immunohistochemical staining (IHC). The following procedures were done by the MICR Core Facility. Four samples from each treatment group (control and doxycycline-treated) were used for IHC analysis. Micron sections obtained from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized, rehydrated and microwaved on high twice for five min in 1 mM sodium citrate buffer, pH 6.0. The sections were then washed three times in PBS and blocked with Super Block (Skytek Laboratories, Logan, UT) for ten min. Sequential sections were then incubated with the selected primary antibodies overnight at 4° C. The sections were then washed three times for 10 min each in PBS and linked with the appropriate host secondary antibodies (Vector Laboratories, Burlingame, Ca). The secondary antibodies were tagged with avidin-biotinylated horseradish peroxidase, colorized with 3'-3'-diaminobenzidine and counterstained with hematoxilin. Visualization and documentation were accomplished with an OLYMPUS BX40 microscope supported by a DP72 CCD Camera, and CellSens Dimension imaging software (Olympus America Inc., Center Valley, PA). The slides were reviewed by Dr. Fred Miller (Professor, Karmanos Cancer Institute, WSU) who confirmed the tumor grading. To assess SULT1E1 and pS2 protein levels, 4 brown-positive and blue-negative IHC stained slides from each group were counted and measured using CellSens digital imaging software (Olympus). Three replicates per slide were analyzed at 40×magnification. The mean percentage of brown-positive IHC stain

was determined and statistically analyzed using Prism (GraphPad Software, San Diego, CA).

Statistical analysis. As described in Chapter 2.

Results

It has been reported that SULT1E1 was robustly expressed in the MCF10AT1 cell line suggesting that estrogen inactivation through sulfonation is intact in MCF10AT1 cells [250]. The MCF10AT1 cell line will therefore provide us with a good platform to study the biological role of SULT1E1 in breast epithelial estrogen metabolism. The MCF10A lineage is a model for basal breast cancer and it has been previously reported that MCF10AT1 cells express a negligible level of ERα when compared to the ERα-positive MCF7 cell line [250]. However, ERα expression levels are sufficient in MCF10AT1 cells to produce responses to estrogen *in*

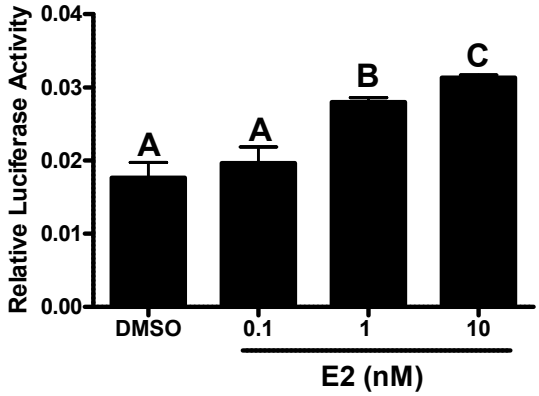


Fig. 5.1 E2-mediated activation of an estrogen-responsive reporter gene in MCF10AT1 cells. MCF10AT1 cells were transiently transfected with an estrogen-responsive luciferase reporter plasmid. After transfection, cells were treated with 0.1% DMSO, 0.1, 1 or 10 nM E2 for 48 h. After treatment, cells were harvested for the measurement of luciferase activities. Each *bar* represents the mean ± sd of normalized (firefly/*Renilla*) luciferase measurements (3 wells per treatment group) relative to the activity measured in DMSO-treated cells. Groups that do not share a capital letter are significantly different from each other ($p<0.05$)

vitro and, more importantly, in the MCF10AT1 mice xenograft model [317]. This estrogen sensitive nature of MCF10AT1 cells was confirmed using an ER-responsive reporter (Fig. 5.1). These findings make MCF10AT1 a good model to study the role of SULT1E1 as a mediator of endogenous estrogen responses.

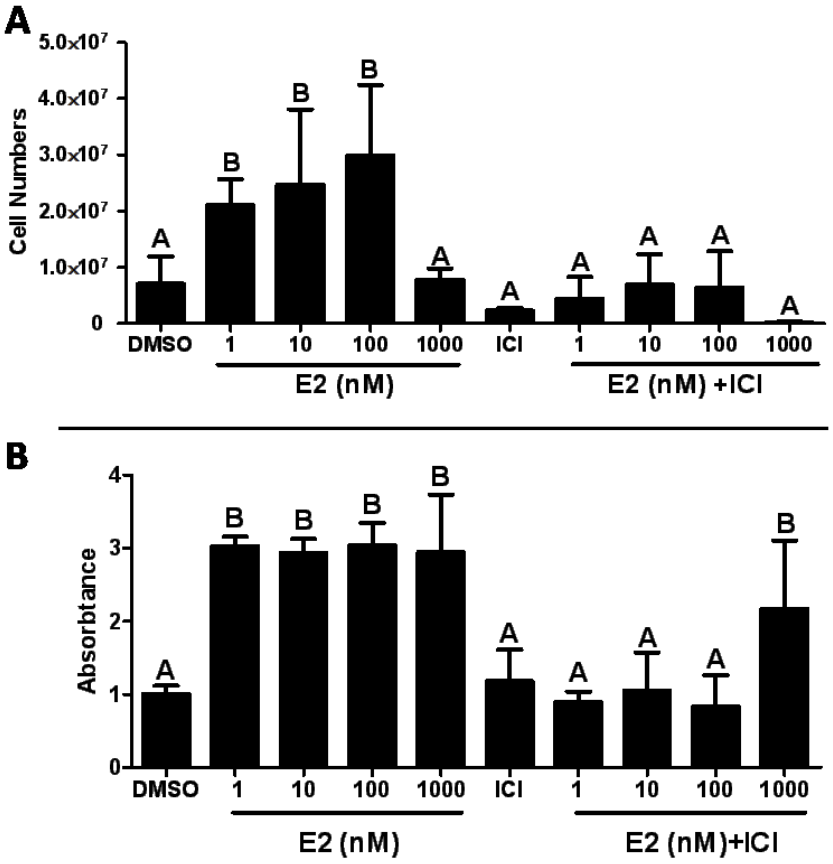


Fig. 5.2 Growth stimulation effects of estrogen on three-dimensional cultures of MCF10AT1 and HUVEC cells. The heterotypic three dimensional cultures were treated with vehicle (0.01% DMSO), the indicated concentration of E2, ICI alone, or E2 combined with a 100-fold excess of ICI for 7 days. Cell viability was quantitated by the trypan blue exclusion assay (A) and cell proliferation was quantitated by MTS assay (B) on day 7 of culture. Results are from three independent experiments performed in triplicate. Groups that do not share a capital letter are significantly different from each other ($p<0.05$). In panel A, each bar represents the mean cell number ± sd. In panel B, data are expressed relative to DMSO-treated cells as mean ± sd.

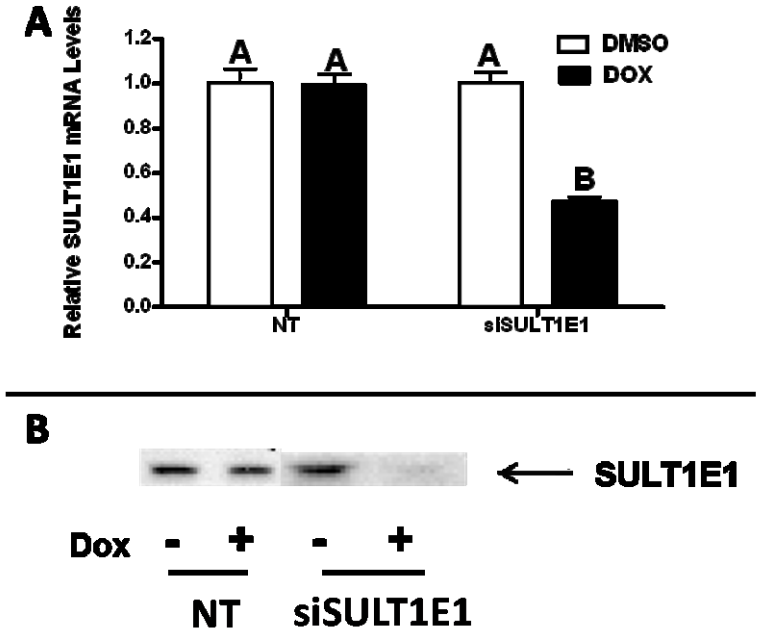


Fig. 5.3 SULT1E1 expression in MCF10AT1 cells engineered for conditional knockdown of SULT1E1. MCF10AT1 cells engineered for conditional knockdown of SULT1E1 were treated for 5 days with 0.1% DMSO or 50 ng/ml doxycycline and harvested for measurement of SULT1E1 mRNA (A) and immunoreactive protein (B) levels. MCT10AT1 cells engineered to express a short-hairpin sequence targeting no known human gene (NT) were used as control. A: SULT1E1 mRNA levels were measured in 3 independent experiments using a TaqMan Gene Expression Assay. Groups that do not share a capital letter are significantly different from each other ($p<0.001$). B: SULT1E1 immunoreactive protein levels were measured by western blot hybridization (experiments were repeated at least 3 times).

Breast epithelial cells cultured in monolayer lack interactions with other cell types that are present in whole breast, such as endothelial cells. These intercellular interactions will affect the growth as well as drug and hormone sensitivity of breast cancer cells [479]. The *in vitro* three-dimensional culture system reported by Dr. Malathy P. V. Shekhar recapitulates *in vivo* interaction between epithelial and endothelial cells [478;480]. Most importantly, this system has been shown to mimic the E2-ER induced *in vivo* effects associated with early angiogenesis and the proliferative

potential of MCF10AT xenografts [478]. Therefore, the estrogen responsiveness of MCF10AT1 cells was investigated using both MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carbo xymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and trypan blue exclusion assays in heterotypic three-dimensional cultures of MCF10AT1 and HUVEC cells (Fig.

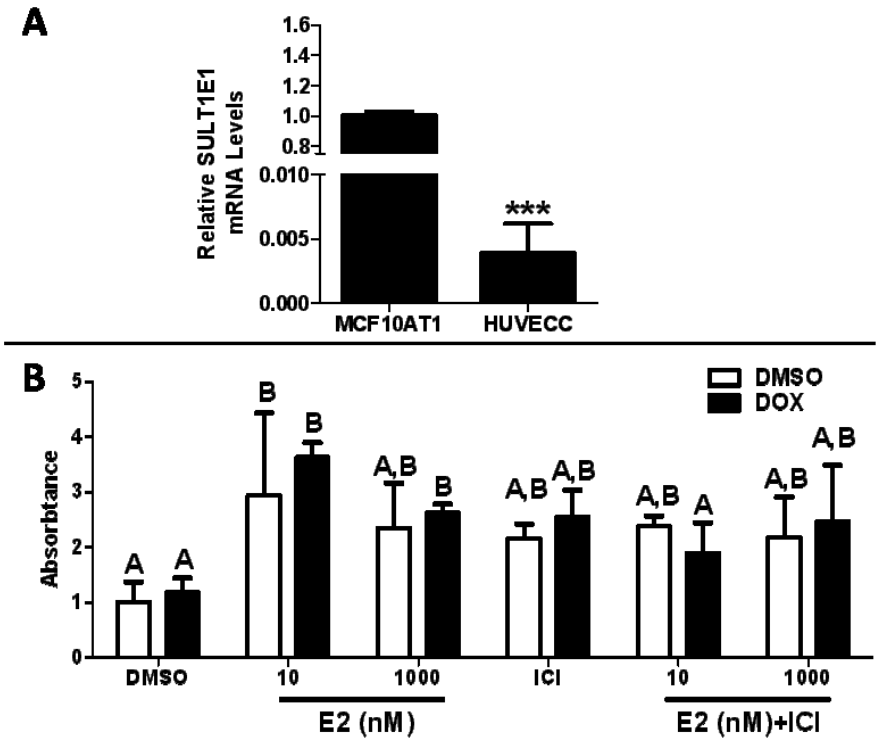


Fig. 5.4 Effects of SULT1E1 knockdown on the estrogen-regulated growth of three-dimensional cultures of MCF10AT1 and HUVEC cells. A: MCF10AT1 and HUVEC cells were harvested at approximately 70% confluency and SULT1E1 mRNA levels were measured in 3 independent experiments using a TaqMan Gene Expression Assay. Data are expressed as mean ± sd, and ***p<0.001 compared to MCF10AT1 cells. B: MCF10AT1 cells engineered for conditional knockdown of SULT1E1 were co-cultured with HUVEC cells in three dimensional cultures and treated with vehicle (0.01% DMSO), the indicated concentration of E2, ICI alone, or E2 combined with a 100-fold excess of ICI for 7 days. Growth was quantitated by the MTS assay on day 7 of culture. Data are expressed relative to DMSO-treated cells as mean ± sd. Groups that do not share a capital letter are significantly different from each other (p<0.05).

5.2). Many methods were used to measure cell proliferation. Trypan blue exclusion assay is a traditional cell counting method, which is simple and inexpensive. However,

this method is time consuming and has been reported to be inaccurate [481]. Instead, measurement of mitochondrial metabolic rate using MTS to indirectly reflect viable cell numbers was well accepted as an alternative method to measure cell proliferation. However, metabolic activity may be affected by treatments which can cause significant variation in results [482]. Therefore, both of these methods were used to accurately

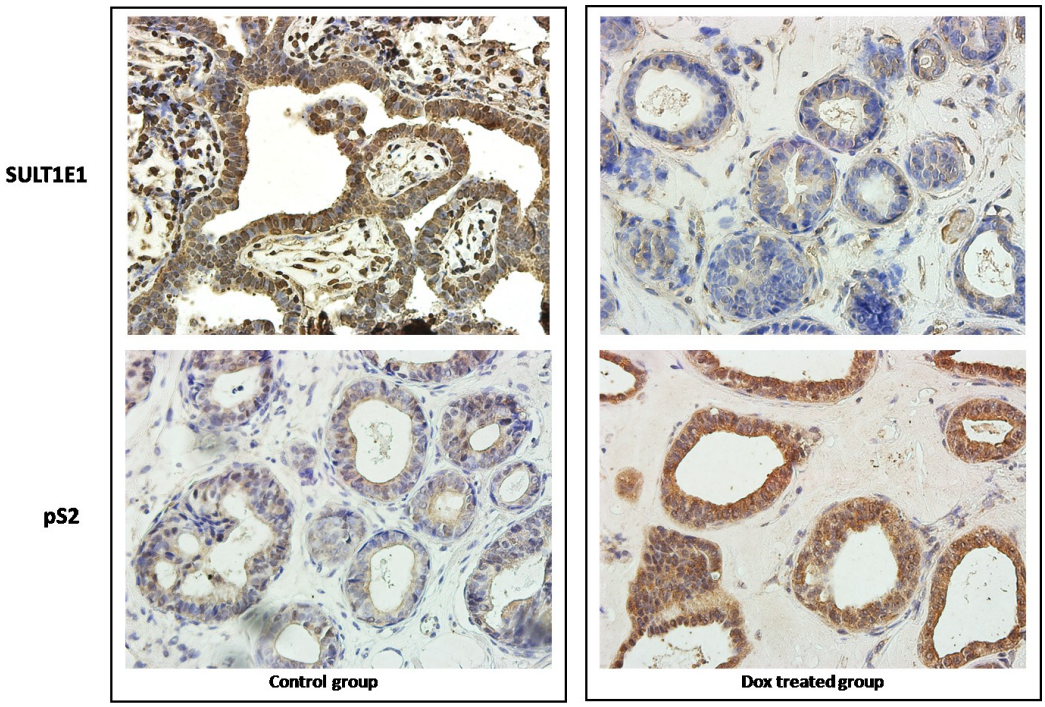


Fig. 5.5 IHC staining for SULT1E1 and pS2 in xenograft lesions. MCF10AT1 cells engineered for conditional knockdown of SULT1E1 were inoculated subcutaneously in female nude mice. Half of the mice were given doxycycline in the drinking water. Twelve weeks post injection, mice were sacrificed and 4 lesions from each group were analyzed by IHC. One representative IHC results from each group was shown. Immunostaining for human SULT1E1 (brown staining) was found in xenografts from the control group but was dramatically decreased in xenografts from the doxycycline-treated group. In contrast, immunostaining for the estrogen-responsive gene pS2 (brown staining) was higher in xenografts from the doxycycline-treated group (40×).

assess the effect of SULT1E1 knockdown on cell proliferation. Treatment with 1, 10 and 100 nM E2 significantly increased cell proliferation in a dose dependent manner by at least 3-fold (Fig. 5.2A). These effects were blocked by 100 nM, 1 μM and 10 μM of

the antiestrogen ICI, indicating that these growth stimulating effects were E2-specific (Fig. 5.2A). The results of the MTS assay were in good agreement with those obtained with the trypan blue exclusion assay, except that 1 μM E2 treatment was significantly increase cell proliferation. However, E2 treatment did not produce a dose-response, which was likely due to the saturation of the MTS assay (Fig. 5.2B).

To investigate the role of SULT1E1 in estrogen-dependent tumorigenisis, MCF10AT1 cells were engineered for knockdown of SULT1E1 expression (siSULT1E1). First, transduction-ready lentiviral particles expressing short hairpin RNA (shRNA) (MISSION™ TRC-Hs 1.0) were used to knockdown SULT1E1 gene expression in MCF10AT1 cells. SULT1E1 lentiviral transduction particles consisted of five (A-E) individual constructs targeting different regions of gene sequence. MISSION® Non-Target (NT) shRNA was used as a negative control in experiments with the MISSION shRNA target set. NT viral vector contained four base pair mismatches within the short hairpin sequence to any known human or mouse genes. Real-time RT-PCR and western blot data (data not shown) revealed that different constructs had different knockdown efficiencies. The most efficient “A” construct knocked down 92% of SULT1E1 mRNA expression (data not shown). The other four constructs knocked down 52-85% of SULT1E1 mRNA expression. Correspondingly, western blot data showed that SULT1E1 protein was detected in Non-targeted shRNA transduced cells but not in the SULT1E1 shRNA transduced cells (data not shown).

This MISSIONTM lentiviral system allowed us to identify an shRNA construct for efficient SULT1E1 knockdown quickly and easily. In this MISSION™ RNAi system, after lentiviral particles transduction, the shRNA sequence is stably integrated into the cell's

genome, and the shRNA is constantly expressed in the cell leading to the continuous knockdown of SULT1E1 expression. This should elevate the level of biologically active estrogens in the cells and increase the expression of estrogen responsive genes. However, continuous knockdown of SULT1E1 may produce undesired effects. For example, stable expression of SULT1E1 in MCF7 cells extensively inhibited cell growth [247]. Therefore, a reversible, drug-regulated lentiviral Tet-on system that permits conditional expression of SULT1E1 was employed.

The shRNA fragment of selected “A” construct was inserted into the lentiviral vector of the Tet-on system for drug inducible production of shRNA in stably transduced MCF10AT1 cells. In the presence of doxycycline, shRNA targeting SULT1E1 will be expressed and SULT1E1 gene will be down-regulated [483]. This controlled shRNA

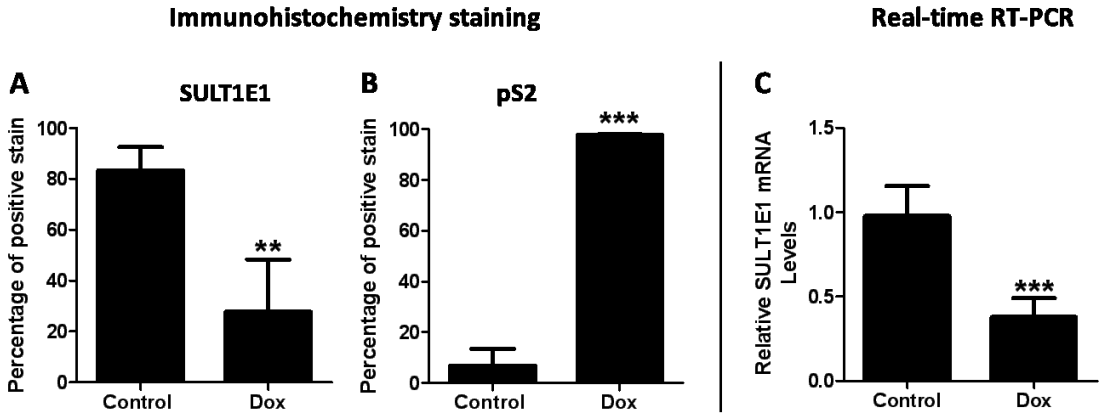


Fig. 5.6 Quantitative expression of SULT1E1 in xenograft lesions. To assess SULT1E1 (A) and pS2 (B) protein levels, immunohistochemically stained slides were analyzed for brown-positive and blue-negative content using CellSens digital imaging software (Olympus). Data represent the mean percentages of brown-positive stain. ** $p<0.01$ ***$p<0.001$ ($n=12$). C: Four lesions from each group were used to analyze the SULT1E1 mRNA level by TaqMan Gene Expression Assay. Data are expressed relative to control group as mean ± sd. ***$p<0.001$

expression system facilitates the comparison of the behavior changes of the cells

developed from the same cell colony before or after doxycycline treatment. The same genetic background of the cells make it possible to focus only on the molecular events produced by SULT1E1 knocking down.

MCF10AT1 cells that were engineered to express a short-hairpin sequence targeting no known human gene (NT) were used as a control (Fig. 5.3A). In the presence of doxycycline, the short-hairpin sequence targeting SULT1E1 produced significant knockdown of SULT1E1 mRNA content (by 53%), which was accompanied by a marked decrease in SULT1E1 immunoreactive protein levels (Fig. 5.3). In contrast, the NT control displayed neither SULT1E1 mRNA nor protein level changes after doxycycline treatment (Fig. 5.3). To characterize the effect of SULT1E1 *in vitro*, siSULT1E1 cells were used to repeat the MTS assay in the heterotypic culture system (Fig. 5.4B). In order to rule out the possibility that SULT1E1 expressed by the HUVEC cells might have contributed to estrogen metabolism, real-time RT-PCR was used to measure SULT1E1 expression in MCF10AT1 and HUVEC cells (Fig. 5.4A). HUVEC cells express an extremely low level of SULT1E1 mRNA compared to MCF10AT1 cells (Fig.5.4A). SULT1E1 knockdown after doxycycline treatment did not cause a significant change in the response to estrogen treatment, although 10 nM E2 treatment significantly induced a 3-fold and 3.6-fold increase in cell proliferation in the vehicle (DMSO) and doxycycline treated groups, respectively (Fig. 5.4B). These results indicate that interaction between epithelial and endothelial cells in three-dimensional culture system may not be enough to recapitulate the *in vivo* effect of SULT1E1 knockdown due to the differences present in the microenvironment of solid tumors compared with

cells in culture. Therefore, we analyzed the effects of SULT1E1 silencing on the progression of siSULT1E1 MCF10AT1 xenografts.

To rule out the possibility that lentiviral transfection may cause malignant transformation, parental MCF10AT1 cells and 3 siSULT1E1 MCF10AT1 clones were inoculated subcutaneously in female nude mice (4 mice/ group). According to

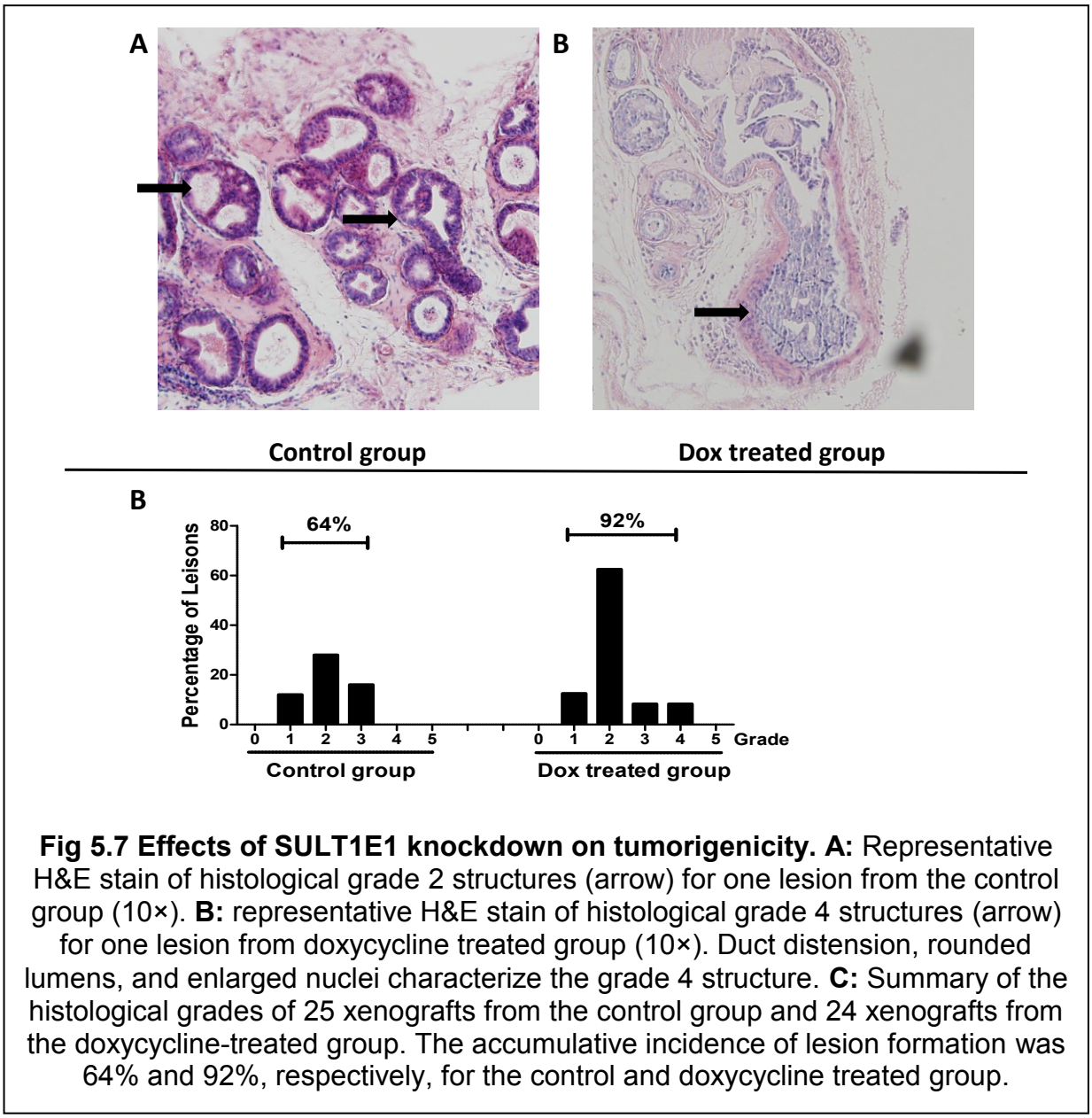


Fig 5.7 Effects of SULT1E1 knockdown on tumorigenicity. A: Representative H&E stain of histological grade 2 structures (arrow) for one lesion from the control group (10×). **B:** representative H&E stain of histological grade 4 structures (arrow) for one lesion from doxycycline treated group (10×). Duct distension, rounded lumens, and enlarged nuclei characterize the grade 4 structure. **C:** Summary of the histological grades of 25 xenografts from the control group and 24 xenografts from the doxycycline-treated group. The accumulative incidence of lesion formation was 64% and 92%, respectively, for the control and doxycycline treated group.

histological evaluation, one of the clones (S15) developed xenografts containing typical

MCF10AT1 epithelial structures and was used in the following experiments (data not shown). To determine whether doxycycline treatment was capable of suppressing SULT1E1 expression in xenograft lesions, IHC and real-time RT-PCR were performed (Fig.5.5, 5.6). In the lesions from control group, SULT1E1 was strongly stained as brown, but in lesions from doxycycline treated group, the staining was significantly suppressed (Fig. 5.5, Fig. 5.6A), indicating that SULT1E1 expression was knocked down after the induction of the short-hairpin RNA by doxycycline treatment. The IHC negative controls showed no positive staining in the lesions. The SULT1E1 down regulation was confirmed by real-time RT-PCR analysis of four lesions from each group (Fig. 5.6C). Functionality of the SULT1E1 knockdown on estrogen level changes was evaluated by induction of the estrogen-responsive gene pS2 in xenograft lesions (Fig. 5.5). There was significantly more positive brown staining of pS2 in the SULT1E1 knockdown samples than there was in the control group indicating enhanced estrogen levels in tissues with SULT1E1 knockdown (Fig. 5.5, Fig. 5.6B).Histological evaluation (Fig. 5.7) indicated that MCF10AT1 xenografts contained epithelial structures that resemble normal breast ducts and ductal epithelial lesions ranging from mild hyperplasia to carcinoma *in situ*. These epithelial structures were graded from 0 to 5 according to the histological classification of Dawson *et al*. [319]. Histologic analysis done by Dr. Fred Miller (Professor, Karmanos Cancer Institute, WSU) and MICR Core Facility (WSU) indicated that 44% of the lesions from the control group (total of 25 lesions) lacked evidence of epithelial growth, 12% were classified as grade 1, 28% were classified as grade 2, and 16% were classified as grade 3. By comparison, 92% of the lesions from the doxycycline treated group (total of 24 lesions) proliferated; 12.5%

of the lesions were classified as grade 1, 62.5% were classified as grade 2, 8.3% were classified as grade 3, and 8.3% were classified as grade 4. There was a dramatic difference in the percentage of lesions in the control group and the doxycycline-treated group xenografts containing grade 2 structures (28 versus 64%, respectively). Moreover, only lesions from the doxycycline-treated group advanced to *in situ* carcinoma (8.3% grade 4).

Discussion

The molecular changes that enable normal breast epithelial cells to transform into invasive, metastatic cancer cells are being increasingly understood [284;303], and gene expression profiles are being used to discriminate among different subtypes of breast cancer. Triple-negative breast cancers (TNBC) (ER-, PR-, and HER2-negative) have become a focus of intense research since TNBC patients have a younger age at onset, higher tumor grade, larger tumor size, increased propensity to develop metastases, and most importantly, they lack the three most significant therapeutic markers for clinical management resulting in the worst outcome when compared with other cancer subtypes [14-17;484]. The MCF10A lineage of cell lines represent a good model to study TNBC because the malignant cancer cell lines of this lineage (e.g., MCF10CA1) are triple negative [314;315].

TNBC cells respond to estrogen both in vitro and in xenografts even though they do not express ERs [326-330]. ER-negative breast cancer cell line, MDA-MB-468 and MDA-MB-231 subclone 10A, demonstrated significant E2-stimulated growth in mouse

xenograft models in two separate studies [485;486]. This E2-induced tumor growth was abrogated in ER-αKO mice, indicating that ER-α mediated the effect of estrogen on tumor growth [485]. Similarly, although MCF10AT1 cells express extremely low levels of ERα compared to MCF-7 cells, MCF10AT1 cells exhibited E2-stimulated growth *in vitro* and, more importantly, in the MCF10AT1 mice xenograft model [317]. These E2 stimulated effects may be mediated through ERβ or cross-talk between ER signaling parthway and other signal transduction routes. In the present study, E2 treatments significantly increased pre-neoplastic MCF10AT1 proliferation in three-dimensional cultures (Fig. 5.2). After E2 exposure, the luciferase activity of p2ERE-Luc reporter was significantly enhanced, indicating that ER plays an important role in MCF10AT1 estrogen responsiveness (Fig. 5.1).

Moreover, there are also clinical indications that ERα-negative tumors are influenced by hormonal factors. For instance, OVX has been shown to reduce long-term recurrence risk and mortality of both ER-positive and ER-negative pre-menopausal women [487]. Similarly, a large clinical study done on women carrying BRCA1 or BRCA2 mutations demonstrated that OVX significantly reduced cancer incidence in this high-risk population (relative risk: 80% versus 19%) [488]. Because the majority of breast tumors that develop in women carrying BRCA1 mutations are ERα-negative [489;490], this finding indicates that estrogen can affect the pathogenesis of ERα-negative breast cancers. Therefore, the level of SULT1E1 within breast tissues may play a critical role in the maintenance of estrogen balance and protection of breast tissues from the adverse effects of estrogen.

SULT1E1 expression has been associated with a decreased risk of recurrence as well as improved prognosis of breast cancer and its immunoreactivity was inversely correlated with tumor size or lymph node status [97]. The genetic polymorphisms of SULT1E1 have also been correlated with increased breast cancer risk and a disease free survival [97;491]. Moreover, it has been demonstrated SULT1E1 may also protect human breast by catalyzing the sulfonation of carcinogenic estrogen metabolites such as catecholestrogens (CEs) and methoxyestrogens (MEs) [492]. Since most breast cancer cell lines lose SULT1E1 expression [249;250], SULT1E1 silencing might be a critical event in the transition of normal breast epithelial cells to cancer cells by facilitating a breast microenvironment that favors increased estrogenic stimulation.

Knockdown of SULT1E1 did not significantly change estrogen stimulated MCF10AT1 cell proliferation in three-dimensional culture in the present study. This may be caused by the differences present in the microenvironment of solid tumors *in vivo* compared with cells in culture. It has been demonstrated that specific genes may be regulated differently *in vivo* vs *in vitro*. For example, only 11% of E2-responsive genes identified in E2 treated T47D human breast cancer cells xenografted into in nude mice overlapped with those E2-regulated genes found in the identical cells grown in cultures [493]. Nude mice are valuable research tools because they do not have rejection response. The transplanted human cells can proliferate and thereby permit *in vivo* study of the human cells. Therefore, the effect of SULT1E1 silencing in pre-neoplastic MCF10AT1 cells on tumorigenicity was studied in a nude mouse xenograft model.

SULT1E1 knockdown increased the cumulative incidence of xenograft lesion formation (from 64% in the control group to 92% in the doxycycline-treated group),

cellularity and histological grade of xenografts in immunodeficient mice. Moreover, the expression of the estrogen-responsive gene pS2 was low in the control group, which is consistent with the finding that pS2 is only expressed in ER-positive breast cancers [494;495], but was significantly enhanced in the SULT1E1 knockdown group. These findings indicate that SULT1E1 down-regulation activates the estrogen-responsive gene pS2 by increasing bioactive estrogen levels and accelerates estrogen-dependent tumorigenicity.

Most breast cancer patients are postmenopausal women who lack estrogen produced in ovary. However, the estrogen concentrations in the breast tissue of postmenopausal patients are similar to those of premenopausal women indicating *in situ* estrogen synthesis [496]. OVX mice supplemented with exogenous estrogen simulate the *in vivo* situation in the postmenopausal breast cancer patient. The effect of SULT1E1 knockdown on estrogen-dependent tumor growth can be further studied in OVX mice treated with or without estrogen. These studies will provide additional information about the interplay of SULT1E1 and estrogen during breast carcinogenesis, which might have implications for the prevention of TNBC.

REFERENCES

1. Jemal,A., Siegel,R., Ward,E., Hao,Y., Xu,J., Murray,T., and Thun,M.J. (2008) Cancer statistics, 2008. *CA Cancer J.Clin.*, 58, 71-96.

2. Jemal,A., Siegel,R., Xu,J., and Ward,E. (2010) Cancer statistics, 2010. *CA Cancer J.Clin.*, 60, 277-300.

3. (1991) NIH consensus conference. Treatment of early-stage breast cancer. *JAMA*, 265, 391-395.

4. Crown,J., Raptis,G., Hamilton,N., Hudis,C., Vahclat,L., Fennelly,D., and Norton,L. (1995) High-dose chemotherapy of breast cancer. *Can.J.Oncol.*, 5 Suppl 1, 80-82.

5. Harris,D.T. (1995) Adjuvant systemic therapy for breast cancer. *Semin.Oncol.*, 22, 553-570.

6. Jaiyesimi,I.A., Buzdar,A.U., and Hortobagyi,G. (1992) Inflammatory breast cancer: a review. *J.Clin.Oncol.*, 10, 1014-1024.

7. Morris,A.D., Morris,R.D., Wilson,J.F., White,J., Steinberg,S., Okunieff,P., Arriagada,R., Le,M.G., Blichert-Toft,M., and van Dongen,J.A. (1997) Breast-conserving therapy vs mastectomy in early-stage breast cancer: a meta-analysis of 10-year survival. *Cancer J.Sci.Am.*, 3, 6-12.

8. Zaider,M. and Hanin,L. (2011) Tumor control probability in radiation treatment. *Med.Phys.*, 38, 574-583.

9. Sabel,M.S. (2011) Surgical considerations in early-stage breast cancer: lessons learned and future directions. *Semin.Radiat.Oncol.*, 21, 10-19.

10. Ahmed,R.L., Prizment,A., Lazovich,D., Schmitz,K.H., and Folsom,A.R. (2008) Lymphedema and quality of life in breast cancer survivors: the Iowa Women's Health Study. *J.Clin.Oncol.*, 26, 5689-5696.

11. Velanovich,V. and Szymanski,W. (1999) Quality of life of breast cancer patients with lymphedema. *Am.J.Surg.*, 177, 184-187.

12. Taylor,H.S. and Manson,J.E. (2011) Update in hormone therapy use in menopause. *J.Clin.Endocrinol.Metab*, 96, 255-264.

13. Chari,R.V. (2008) Targeted cancer therapy: conferring specificity to cytotoxic drugs. *Acc.Chem.Res.*, 41, 98-107.

14. Lund,M.J., Trivers,K.F., Porter,P.L., Coates,R.J., Leyland-Jones,B., Brawley,O.W., Flagg,E.W., O'Regan,R.M., Gabram,S.G., and Eley,J.W. (2009) Race and triple negative threats to breast cancer survival: a population-based study in Atlanta, GA. *Breast Cancer Res.Treat.*, 113, 357-370.

15. Dent,R., Trudeau,M., Pritchard,K.I., Hanna,W.M., Kahn,H.K., Sawka,C.A., Lickley,L.A., Rawlinson,E., Sun,P., and Narod,S.A. (2007) Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin.Cancer Res.*, 13, 4429-4434.

16. Dawood,S. (2010) Triple-negative breast cancer: epidemiology and management options. *Drugs*, 70, 2247-2258.

17. Kaplan,H.G., Malmgren,J.A., and Atwood,M. (2009) T1N0 triple negative breast cancer: risk of recurrence and adjuvant chemotherapy. *Breast J.*, 15, 454-460.

18. Andersson,S., Geissler,W.M., Patel,S., and Wu,L. (1995) The molecular biology of androgenic 17 beta-hydroxysteroid dehydrogenases. *J.Steroid Biochem.Mol.Biol.*, 53, 37-39.

19. MacGregor,J.I. and Jordan,V.C. (1998) Basic guide to the mechanisms of antiestrogen action. *Pharmacol.Rev.*, 50, 151-196.

20. Ali,S. and Coombes,R.C. (2002) Endocrine-responsive breast cancer and strategies for combating resistance. *Nat.Rev.Cancer*, 2, 101-112.

21. (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet*, 365, 1687-1717.

22. Miller,H.W. (1973) Plan and operation of the health and nutrition examination survey. United states--1971-1973. *Vital Health Stat.1*,1-46.

23. Perera,F.P. (1996) Molecular epidemiology: insights into cancer susceptibility, risk assessment, and prevention. *J.Natl.Cancer Inst.*, 88, 496-509.

24. Perera,F.P., Blaner,W., and Mooney,L.V. (1996) Molecular epidemiologic methods in cancer chemoprevention. *Eur.J.Cancer Prev.*, 5 Suppl 2, 19-25.

25. (1997) Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer. *Lancet*, 350, 1047-1059.

26. Madigan,M.P., Ziegler,R.G., Benichou,J., Byrne,C., and Hoover,R.N. (1995) Proportion of breast cancer cases in the United States explained by well-established risk factors. *J.Natl.Cancer Inst.*, 87, 1681-1685.

27. Bernstein,L. and Ross,R.K. (1993) Endogenous hormones and breast cancer risk. *Epidemiol.Rev.*, 15, 48-65.

28. Kelsey,J.L., Gammon,M.D., and John,E.M. (1993) Reproductive factors and breast cancer. *Epidemiol.Rev.*, 15, 36-47.

29. Kreiger,N., Sloan,M., Cotterchio,M., and Kirsh,V. (1999) The risk of breast cancer following reproductive surgery. *Eur.J.Cancer*, 35, 97-101.

30. Schairer,C., Persson,I., Falkeborn,M., Naessen,T., Troisi,R., and Brinton,L.A. (1997) Breast cancer risk associated with gynecologic surgery and indications for such surgery. *Int.J.Cancer*, 70, 150-154.

31. Henderson,I.C. and Canellos,G.P. (1980) Cancer of the breast: the past decade (first of two parts). *N.Engl.J.Med.*, 302, 17-30.

32. Henderson,B.E., Ross,R., and Bernstein,L. (1988) Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation award lecture. *Cancer Res.*, 48, 246-253.

33. Tulchinsky,D., Hobel,C.J., Yeager,E., and Marshall,J.R. (1972) Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy. *Am.J.Obstet.Gynecol.*, 112, 1095-1100.

34. Buster,J.E., Sakakini,J., Jr., Killam,A.P., and Scragg,W.H. (1976) Serum unconjugated estriol levels in the third trimester and their relationship to gestational age. *Am.J.Obstet.Gynecol.*, 125, 672-676.

35. Trichopoulos,D. (1990) Hypothesis: does breast cancer originate in utero? *Lancet*, 335, 939-940.

36. Hubinette,A., Lichtenstein,P., Ekbom,A., and Cnattingius,S. (2001) Birth characteristics and breast cancer risk: a study among like-sexed twins. *Int.J.Cancer*, 91, 248-251.

37. Innes,K., Byers,T., and Schymura,M. (2000) Birth characteristics and subsequent risk for breast cancer in very young women. *Am.J.Epidemiol.*, 152, 1121-1128.

38. McCormack,V.A., dos,S.S., I, De Stavola,B.L., Mohsen,R., Leon,D.A., and Lithell,H.O. (2003) Fetal growth and subsequent risk of breast cancer: results from long term follow up of Swedish cohort. *BMJ*, 326, 248.

39. Ekbom,A., Erlandsson,G., Hsieh,C., Trichopoulos,D., Adami,H.O., and Cnattingius,S. (2000) Risk of breast cancer in prematurely born women. *J.Natl.Cancer Inst.*, 92, 840-841.

40. Ekbom,A., Hsieh,C.C., Lipworth,L., Adami,H.Q., and Trichopoulos,D. (1997) Intrauterine environment and breast cancer risk in women: a population-based study. *J.Natl.Cancer Inst.*, 89, 71-76.

41. Petridou,E., Panagiotopoulou,K., Katsouyanni,K., Spanos,E., and Trichopoulos,D. (1990) Tobacco smoking, pregnancy estrogens, and birth weight. *Epidemiology*, 1, 247-250.

42. Wang,H.S. and Chard,T. (1992) The role of insulin-like growth factor-I and insulin-like growth factor-binding protein-1 in the control of human fetal growth. *J.Endocrinol.*, 132, 11-19.

43. Mucci,L.A., Lagiou,P., Tamimi,R.M., Hsieh,C.C., Adami,H.O., and Trichopoulos,D. (2003) Pregnancy estriol, estradiol, progesterone and prolactin in relation to birth weight and other birth size variables (United States). *Cancer Causes Control*, 14, 311-318.

44. Troisi,R., Potischman,N., Roberts,J., Siiteri,P., Daftary,A., Sims,C., and Hoover,R.N. (2003) Associations of maternal and umbilical cord hormone concentrations with maternal, gestational and neonatal factors (United States). *Cancer Causes Control*, 14, 347-355.

45. Vatten,L.J., Nilsen,T.I., Tretli,S., Trichopoulos,D., and Romundstad,P.R. (2005) Size at birth and risk of breast cancer: prospective population-based study. *Int.J.Cancer*, 114, 461-464.

46. Michels,K.B., Trichopoulos,D., Robins,J.M., Rosner,B.A., Manson,J.E., Hunter,D.J., Colditz,G.A., Hankinson,S.E., Speizer,F.E., and Willett,W.C. (1996) Birthweight as a risk factor for breast cancer. *Lancet*, 348, 1542-1546.

47. Vatten,L.J., Maehle,B.O., Lund Nilsen,T.I., Tretli,S., Hsieh,C.C., Trichopoulos,D., and Stuver,S.O. (2002) Birth weight as a predictor of breast cancer: a case-control study in Norway. *Br.J.Cancer*, 86, 89-91.

48. dos,S.S., I, De Stavola,B.L., Hardy,R.J., Kuh,D.J., McCormack,V.A., and Wadsworth,M.E. (2004) Is the association of birth weight with premenopausal breast cancer risk mediated through childhood growth? *Br.J.Cancer*, 91, 519-524.

49. Hilakivi-Clarke,L., Clarke,R., and Lippman,M.E. (1994) Perinatal factors increase breast cancer risk. *Breast Cancer Res.Treat.*, 31, 273-284.

50. Hilakivi-Clarke,L., Clarke,R., Onojafe,I., Raygada,M., Cho,E., and Lippman,M. (1997) A maternal diet high in n - 6 polyunsaturated fats alters mammary gland development, puberty onset, and breast cancer risk among female rat offspring. *Proc.Natl.Acad.Sci.U.S.A*, 94, 9372-9377.

51. Long,P.A., Abell,D.A., and Beischer,N.A. (1979) Fetal growth and placental function assessed by urinary estriol excretion before the onset of pre-eclampsia. *Am.J.Obstet.Gynecol.*, 135, 344-347.

52. Garoff,L. and Seppala,M. (1976) Toxemia of pregnancy: assessment of fetal distress by urinary estriol and circulating human placental lactogen and alpha-fetoprotein levels. *Am.J.Obstet.Gynecol.*, 126, 1027-1033.

53. Ekbom,A., Trichopoulos,D., Adami,H.O., Hsieh,C.C., and Lan,S.J. (1992) Evidence of prenatal influences on breast cancer risk. *Lancet*, 340, 1015-1018.

54. Howard,B.A. and Gusterson,B.A. (2000) Human breast development. *J.Mammary.Gland.Biol.Neoplasia.*, 5, 119-137.

55. Drife,J.O. (1986) Breast development in puberty. *Ann.N.Y.Acad.Sci.*, 464, 58-65.

56. Key,T.J. and Pike,M.C. (1988) The role of oestrogens and progestagens in the epidemiology and prevention of breast cancer. *Eur.J.Cancer Clin.Oncol.*, 24, 29-43.

57. Kvale,G. and Heuch,I. (1988) Menstrual factors and breast cancer risk. *Cancer*, 62, 1625-1631.

58. Harris,J.R., Lippman,M.E., Veronesi,U., and Willett,W. (1992) Breast cancer (1). *N.Engl.J.Med.*, 327, 319-328.

59. Hunter,D.J. and Willett,W.C. (1993) Diet, body size, and breast cancer. *Epidemiol.Rev.*, 15, 110-132.

60. Ursin,G., Longnecker,M.P., Haile,R.W., and Greenland,S. (1995) A meta-analysis of body mass index and risk of premenopausal breast cancer. *Epidemiology*, 6, 137-141.

61. Hilakivi-Clarke,L., Forsen,T., Eriksson,J.G., Luoto,R., Tuomilehto,J., Osmond,C., and Barker,D.J. (2001) Tallness and overweight during childhood have opposing effects on breast cancer risk. *Br.J.Cancer*, 85, 1680-1684.

62. Herrinton,L.J. and Husson,G. (2001) Relation of childhood height and later risk of breast cancer. *Am.J.Epidemiol.*, 154, 618-623.

63. Stavola,B.L., Hardy,R., Kuh,D., Silva,I.S., Wadsworth,M., and Swerdlow,A.J. (2000) Birthweight, childhood growth and risk of breast cancer in a British cohort. *Br.J.Cancer*, 83, 964-968.

64. Le,M.L., Kolonel,L.N., Earle,M.E., and Mi,M.P. (1988) Body size at different periods of life and breast cancer risk. *Am.J.Epidemiol.*, 128, 137-152.

65. Ahlgren,M., Melbye,M., Wohlfahrt,J., and Sorensen,T.I. (2004) Growth patterns and the risk of breast cancer in women. *N.Engl.J.Med.*, 351, 1619-1626.

66. Russo,J. and Russo,I.H. (1997) Differentiation and breast cancer. *Medicina (B Aires)*, 57 Suppl 2, 81-91.

67. Albrektsen,G., Heuch,I., Hansen,S., and Kvale,G. (2005) Breast cancer risk by age at birth, time since birth and time intervals between births: exploring interaction effects. *Br.J.Cancer*, 92, 167-175.

68. Perez-Escamilla,R. and Guerrero,M.L. (2004) Epidemiology of breastfeeding: advances and multidisciplinary applications. *Adv.Exp.Med.Biol.*, 554, 45-59.

69. Althuis,M.D., Fergenbaum,J.H., Garcia-Closas,M., Brinton,L.A., Madigan,M.P., and Sherman,M.E. (2004) Etiology of hormone receptor-defined breast cancer: a systematic review of the literature. *Cancer Epidemiol.Biomarkers Prev.*, 13, 1558-1568.

70. Moon,R.C. (1969) Relationship between previous reproductive history and chemically induced mammary cancer in rats. *Int.J.Cancer*, 4, 312-317.

71. Russo,J. and Russo,I.H. (1978) DNA labeling index and structure of the rat mammary gland as determinants of its susceptibility to carcinogenesis. *J.Natl.Cancer Inst.*, 61, 1451-1459.

72. Russo,J., Wilgus,G., and Russo,I.H. (1979) Susceptibility of the mammary gland to carcinogenesis: I Differentiation of the mammary gland as determinant of tumor incidence and type of lesion. *Am.J.Pathol.*, 96, 721-736.

73. Yang,J., Yoshizawa,K., Nandi,S., and Tsubura,A. (1999) Protective effects of pregnancy and lactation against N-methyl-N-nitrosourea-induced mammary carcinomas in female Lewis rats. *Carcinogenesis*, 20, 623-628.

74. Baird,D.T. and Fraser,I.S. (1974) Blood production and ovarian secretion rates of estradiol-17 beta and estrone in women throughout the menstrual cycle. *J.Clin.Endocrinol.Metab*, 38, 1009-1017.

75. Siiteri,P.K. (1987) Adipose tissue as a source of hormones. *Am.J.Clin.Nutr.*, 45, 277-282.

76. Yue,W., Santen,R.J., Wang,J.P., Hamilton,C.J., and Demers,L.M. (1999) Aromatase within the breast. *Endocr.Relat Cancer*, 6, 157-164.

77. Simpson,E., Rubin,G., Clyne,C., Robertson,K., O'Donnell,L., Davis,S., and Jones,M. (1999) Local estrogen biosynthesis in males and females. *Endocr.Relat Cancer*, 6, 131-137.

78. Miyoshi,Y., Ando,A., Shiba,E., Taguchi,T., Tamaki,Y., and Noguchi,S. (2001) Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. *Int.J.Cancer*, 94, 685-689.

79. Sasano,H., Suzuki,T., Nakata,T., and Moriya,T. (2006) New development in intracrinology of breast carcinoma. *Breast Cancer*, 13, 129-136.

80. Labrie,F., Luu-The,V., Labrie,C., Belanger,A., Simard,J., Lin,S.X., and Pelletier,G. (2003) Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. *Endocr.Rev.*, 24, 152-182.

81. Santen,R.J., Santner,S.J., Pauley,R.J., Tait,L., Kaseta,J., Demers,L.M., Hamilton,C., Yue,W., and Wang,J.P. (1997) Estrogen production via the aromatase enzyme in breast carcinoma: which cell type is responsible? *J.Steroid Biochem.Mol.Biol.*, 61, 267-271.

82. Pasqualini,J.R., Chetrite,G., Nguyen,B.L., Maloche,C., Delalonde,L., Talbi,M., Feinstein,M.C., Blacker,C., Botella,J., and Paris,J. (1995) Estrone sulfate-sulfatase and 17 beta-hydroxysteroid dehydrogenase activities: a hypothesis for their role in the evolution of human breast cancer from hormone-dependence to hormone-independence. *J.Steroid Biochem.Mol.Biol.*, 53, 407-412.

83. Miller,W.R. and O'Neill,J. (1987) The importance of local synthesis of estrogen within the breast. *Steroids*, 50, 537-548.

84. Perel,E., Daniilescu,D., Kharlip,L., Blackstein,M., and Killinger,D.W. (1988) Steroid modulation of aromatase activity in human cultured breast carcinoma cells. *J.Steroid Biochem.*, 29, 393-399.

85. Lipton,A., Santner,S.J., Santen,R.J., Harvey,H.A., Feil,P.D., White-Hershey,D., Bartholomew,M.J., and Antle,C.E. (1987) Aromatase activity in primary and metastatic human breast cancer. *Cancer*, 59, 779-782.

86. bul-Hajj,Y.J., Iverson,R., and Kiang,D.T. (1979) Aromatization of androgens by human breast cancer. *Steroids*, 33, 205-222.

87. Vermeulen,A., Deslypere,J.P., Paridaens,R., Leclercq,G., Roy,F., and Heuson,J.C. (1986) Aromatase, 17 beta-hydroxysteroid dehydrogenase and intratissular sex hormone concentrations in cancerous and normal glandular breast tissue in postmenopausal women. *Eur.J.Cancer Clin.Oncol.*, 22, 515-525.

88. bul-Hajj,Y.J. (1979) Relationship between estrogen receptors, 17 beta-hydroxysteroid dehydrogenase and estrogen content in human breast cancer. *Steroids*, 34, 217-225.

89. Pollow,K., Boquoi,E., Baumann,J., Schmidt-Gollwitzer,M., and Pollow,B. (1977) Comparison of the in vitro conversion of estradiol-17 beta to estrone of normal and neoplastic human breast tissue. *Mol.Cell Endocrinol.*, 6, 333-348.

90. Dowsett,M. (1997) Future uses for aromatase inhibitors in breast cancer. *J.Steroid Biochem.Mol.Biol.*, 61, 261-266.

91. Utsumi,T., Harada,N., Maruta,M., and Takagi,Y. (1996) Presence of alternatively spliced transcripts of aromatase gene in human breast cancer. *J.Clin.Endocrinol.Metab*, 81, 2344-2349.

92. Martel,C., Rheaume,E., Takahashi,M., Trudel,C., Couet,J., Luu-The,V., Simard,J., and Labrie,F. (1992) Distribution of 17 beta-hydroxysteroid dehydrogenase gene expression and activity in rat and human tissues. *J.Steroid Biochem.Mol.Biol.*, 41, 597-603.

93. Suzuki,T., Moriya,T., Ariga,N., Kaneko,C., Kanazawa,M., and Sasano,H. (2000) 17Beta-hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. *Br.J.Cancer*, 82, 518-523.

94. McNeill,J.M., Reed,M.J., Beranek,P.A., Bonney,R.C., Ghilchik,M.W., Robinson,D.J., and James,V.H. (1986) A comparison of the in vivo uptake and

metabolism of 3H-oestrone and 3H-oestradiol by normal breast and breast tumour tissues in post-menopausal women. *Int.J.Cancer*, 38, 193-196.

95. Speirs,V., Green,A.R., and Atkin,S.L. (1998) Activity and gene expression of 17beta-hydroxysteroid dehydrogenase type I in primary cultures of epithelial and stromal cells derived from normal and tumourous human breast tissue: the role of IL-8. *J.Steroid Biochem.Mol.Biol.*, 67, 267-274.

96. Miyoshi,Y., Ando,A., Hasegawa,S., Ishitobi,M., Taguchi,T., Tamaki,Y., and Noguchi,S. (2003) High expression of steroid sulfatase mRNA predicts poor prognosis in patients with estrogen receptor-positive breast cancer. *Clin.Cancer Res.*, 9, 2288-2293.

97. Suzuki,T., Nakata,T., Miki,Y., Kaneko,C., Moriya,T., Ishida,T., Akinaga,S., Hirakawa,H., Kimura,M., and Sasano,H. (2003) Estrogen sulfotransferase and steroid sulfatase in human breast carcinoma. *Cancer Res.*, 63, 2762-2770.

98. Saeki,T., Takashima,S., Sasaki,H., Hanai,N., and Salomon,D.S. (1999) Localization of Estrone Sulfatase in Human Breast Carcinomas. *Breast Cancer*, 6, 331-337.

99. Utsumi,T., Yoshimura,N., Takeuchi,S., Ando,J., Maruta,M., Maeda,K., and Harada,N. (1999) Steroid sulfatase expression is an independent predictor of recurrence in human breast cancer. *Cancer Res.*, 59, 377-381.

100. McMichael,A.J. and Woodward,A. (1999) Quantitative estimation and prediction of human cancer risk: its history and role in cancer prevention. *IARC Sci.Publ.*,1-10.

101. Tubiana,M. (1989) [Carcinogenesis in humans: a multistage and multifactorial process]. *Bull.Acad.Natl.Med.*, 173, 997-1002.

102. Freedman,D.A. and Navidi,W.C. (1989) Multistage models for carcinogenesis. *Environ.Health Perspect.*, 81, 169-188.

103. Lau,K.M., Leav,I., and Ho,S.M. (1998) Rat estrogen receptor-alpha and -beta, and progesterone receptor mRNA expression in various prostatic lobes and microdissected normal and dysplastic epithelial tissues of the Noble rats. *Endocrinology*, 139, 424-427.

104. Adlercreutz,H., Gorbach,S.L., Goldin,B.R., Woods,M.N., Dwyer,J.T., and Hamalainen,E. (1994) Estrogen metabolism and excretion in Oriental and Caucasian women. *J.Natl.Cancer Inst.*, 86, 1076-1082.

105. Rosen,J.M., Humphreys,R., Krnacik,S., Juo,P., and Raught,B. (1994) The regulation of mammary gland development by hormones, growth factors, and oncogenes. *Prog.Clin.Biol.Res.*, 387, 95-111.

106. Habel,L.A. and Stanford,J.L. (1993) Hormone receptors and breast cancer. *Epidemiol.Rev.*, 15, 209-219.

107. Moolgavkar,S.H., Day,N.E., and Stevens,R.G. (1980) Two-stage model for carcinogenesis: Epidemiology of breast cancer in females. *J.Natl.Cancer Inst.*, 65, 559-569.

108. Kuiper,G.G., Lemmen,J.G., Carlsson,B., Corton,J.C., Safe,S.H., van der Saag,P.T., van der,B.B., and Gustafsson,J.A. (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology*, 139, 4252-4263.

109. Enmark,E., Pelto-Huikko,M., Grandien,K., Lagercrantz,S., Lagercrantz,J., Fried,G., Nordenskjold,M., and Gustafsson,J.A. (1997) Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J.Clin.Endocrinol.Metab*, 82, 4258-4265.

110. Kuiper,G.G., Carlsson,B., Grandien,K., Enmark,E., Haggblad,J., Nilsson,S., and Gustafsson,J.A. (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology*, 138, 863-870.

111. Pike,A.C., Brzozowski,A.M., Hubbard,R.E., Bonn,T., Thorsell,A.G., Engstrom,O., Ljunggren,J., Gustafsson,J.A., and Carlquist,M. (1999) Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO J.*, 18, 4608-4618.

112. Brzozowski,A.M., Pike,A.C., Dauter,Z., Hubbard,R.E., Bonn,T., Engstrom,O., Ohman,L., Greene,G.L., Gustafsson,J.A., and Carlquist,M. (1997) Molecular

basis of agonism and antagonism in the oestrogen receptor. *Nature*, 389, 753-758.

113. Paech,K., Webb,P., Kuiper,G.G., Nilsson,S., Gustafsson,J., Kushner,P.J., and Scanlan,T.S. (1997) Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science*, 277, 1508-1510.

114. Kininis,M., Chen,B.S., Diehl,A.G., Isaacs,G.D., Zhang,T., Siepel,A.C., Clark,A.G., and Kraus,W.L. (2007) Genomic analyses of transcription factor binding, histone acetylation, and gene expression reveal mechanistically distinct classes of estrogen-regulated promoters. *Mol.Cell Biol.*, 27, 5090-5104.

115. Safe,S. (2001) Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. *Vitam.Horm.*, 62, 231-252.

116. Kushner,P.J., Agard,D.A., Greene,G.L., Scanlan,T.S., Shiau,A.K., Uht,R.M., and Webb,P. (2000) Estrogen receptor pathways to AP-1. *J.Steroid Biochem.Mol.Biol.*, 74, 311-317.

117. Xing,W. and Archer,T.K. (1998) Upstream stimulatory factors mediate estrogen receptor activation of the cathepsin D promoter. *Mol.Endocrinol.*, 12, 1310-1321.

118. Altucci,L., Addeo,R., Cicatiello,L., Dauvois,S., Parker,M.G., Truss,M., Beato,M., Sica,V., Bresciani,F., and Weisz,A. (1996) 17beta-Estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells. *Oncogene*, 12, 2315-2324.

119. Geum,D., Sun,W., Paik,S.K., Lee,C.C., and Kim,K. (1997) Estrogen-induced cyclin D1 and D3 gene expressions during mouse uterine cell proliferation in vivo: differential induction mechanism of cyclin D1 and D3. *Mol.Reprod.Dev.*, 46, 450-458.

120. Dong,L., Wang,W., Wang,F., Stoner,M., Reed,J.C., Harigai,M., Samudio,I., Kladde,M.P., Vyhlidal,C., and Safe,S. (1999) Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells. *J.Biol.Chem.*, 274, 32099-32107.

121. Liu,Y., Ludes-Meyers,J., Zhang,Y., Munoz-Medellin,D., Kim,H.T., Lu,C., Ge,G., Schiff,R., Hilsenbeck,S.G., Osborne,C.K., and Brown,P.H. (2002) Inhibition of AP-1 transcription factor causes blockade of multiple signal transduction pathways and inhibits breast cancer growth. *Oncogene*, 21, 7680-7689.

122. Osborne,C.K., Schiff,R., Fuqua,S.A., and Shou,J. (2001) Estrogen receptor: current understanding of its activation and modulation. *Clin.Cancer Res.*, 7, 4338s-4342s.

123. Sisci,D. and Surmacz,E. (2007) Crosstalk between IGF signaling and steroid hormone receptors in breast cancer. *Curr.Pharm.Des*, 13, 705-717.

124. Joel,P.B., Smith,J., Sturgill,T.W., Fisher,T.L., Blenis,J., and Lannigan,D.A. (1998) pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Mol.Cell Biol.*, 18, 1978-1984.

125. Arnold,S.F., Obourn,J.D., Jaffe,H., and Notides,A.C. (1994) Serine 167 is the major estradiol-induced phosphorylation site on the human estrogen receptor. *Mol.Endocrinol.*, 8, 1208-1214.

126. Martin,M.B., Franke,T.F., Stoica,G.E., Chambon,P., Katzenellenbogen,B.S., Stoica,B.A., McLemore,M.S., Olivo,S.E., and Stoica,A. (2000) A role for Akt in mediating the estrogenic functions of epidermal growth factor and insulin-like growth factor I. *Endocrinology*, 141, 4503-4511.

127. Reddy,K.B., Yee,D., Hilsenbeck,S.G., Coffey,R.J., and Osborne,C.K. (1994) Inhibition of estrogen-induced breast cancer cell proliferation by reduction in autocrine transforming growth factor alpha expression. *Cell Growth Differ.*, 5, 1275-1282.

128. Kato,S., Endoh,H., Masuhiro,Y., Kitamoto,T., Uchiyama,S., Sasaki,H., Masushige,S., Gotoh,Y., Nishida,E., Kawashima,H., Metzger,D., and Chambon,P. (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science*, 270, 1491-1494.

129. Bunone,G., Briand,P.A., Miksicek,R.J., and Picard,D. (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.*, 15, 2174-2183.

130. Kato,S., Masuhiro,Y., Watanabe,M., Kobayashi,Y., Takeyama,K.I., Endoh,H., and Yanagisawa,J. (2000) Molecular mechanism of a cross-talk between oestrogen and growth factor signalling pathways. *Genes Cells*, 5, 593-601.

131. Rowan,B.G., Weigel,N.L., and O'Malley,B.W. (2000) Phosphorylation of steroid receptor coactivator-1. Identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway. *J.Biol.Chem.*, 275, 4475-4483.

132. Font de,M.J. and Brown,M. (2000) AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Mol.Cell Biol.*, 20, 5041-5047.

133. Liao,J.K. (2003) Cross-coupling between the oestrogen receptor and phosphoinositide 3-kinase. *Biochem.Soc.Trans.*, 31, 66-70.

134. Levin,E.R. (2002) Cellular functions of plasma membrane estrogen receptors. *Steroids*, 67, 471-475.

135. Santen,R.J., Song,R.X., McPherson,R., Kumar,R., Adam,L., Jeng,M.H., and Yue,W. (2002) The role of mitogen-activated protein (MAP) kinase in breast cancer. *J.Steroid Biochem.Mol.Biol.*, 80, 239-256.

136. Song,R.X., McPherson,R.A., Adam,L., Bao,Y., Shupnik,M., Kumar,R., and Santen,R.J. (2002) Linkage of rapid estrogen action to MAPK activation by ERalpha-Shc association and Shc pathway activation. *Mol.Endocrinol.*, 16, 116-127.

137. Razandi,M., Pedram,A., Greene,G.L., and Levin,E.R. (1999) Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Mol.Endocrinol.*, 13, 307-319.

138. Migliaccio,A., Di,D.M., Castoria,G., de,F.A., Bontempo,P., Nola,E., and Auricchio,F. (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J.*, 15, 1292-1300.

139. Aronica,S.M., Kraus,W.L., and Katzenellenbogen,B.S. (1994) Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc.Natl.Acad.Sci.U.S.A*, 91, 8517-8521.

140. Pollak,M.N., Schernhammer,E.S., and Hankinson,S.E. (2004) Insulin-like growth factors and neoplasia. *Nat.Rev.Cancer*, 4, 505-518.

141. Govind,A.P. and Thampan,R.V. (2003) Membrane associated estrogen receptors and related proteins: localization at the plasma membrane and the endoplasmic reticulum. *Mol.Cell Biochem.*, 253, 233-240.

142. Stoica,G.E., Franke,T.F., Moroni,M., Mueller,S., Morgan,E., Iann,M.C., Winder,A.D., Reiter,R., Wellstein,A., Martin,M.B., and Stoica,A. (2003) Effect of estradiol on estrogen receptor-alpha gene expression and activity can be modulated by the ErbB2/PI 3-K/Akt pathway. *Oncogene*, 22, 7998-8011.

143. Levin,E.R. (2003) Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. *Mol.Endocrinol.*, 17, 309-317.

144. Levin,E.R. (2001) Cell localization, physiology, and nongenomic actions of estrogen receptors. *J.Appl.Physiol*, 91, 1860-1867.

145. Chen,X., Danes,C., Lowe,M., Herliczek,T.W., and Keyomarsi,K. (2000) Activation of the estrogen-signaling pathway by p21(WAF1/CIP1) in estrogen receptor-negative breast cancer cells. *J.Natl.Cancer Inst.*, 92, 1403-1413.

146. Carmeci,C., Thompson,D.A., Ring,H.Z., Francke,U., and Weigel,R.J. (1997) Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. *Genomics*, 45, 607-617.

147. Filardo,E.J., Quinn,J.A., Bland,K.I., and Frackelton,A.R., Jr. (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol.Endocrinol.*, 14, 1649-1660.

148. Thomas,P., Pang,Y., Filardo,E.J., and Dong,J. (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology*, 146, 624-632.

149. Maggiolini,M., Vivacqua,A., Fasanella,G., Recchia,A.G., Sisci,D., Pezzi,V., Montanaro,D., Musti,A.M., Picard,D., and Ando,S. (2004) The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-estradiol and phytoestrogens in breast cancer cells. *J.Biol.Chem.*, 279, 27008-27016.

150. Prossnitz,E.R., Oprea,T.I., Sklar,L.A., and Arterburn,J.B. (2008) The ins and outs of GPR30: a transmembrane estrogen receptor. *J.Steroid Biochem.Mol.Biol.*, 109, 350-353.

151. Prossnitz,E.R., Arterburn,J.B., Smith,H.O., Oprea,T.I., Sklar,L.A., and Hathaway,H.J. (2008) Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annu.Rev.Physiol*, 70, 165-190.

152. Prossnitz,E.R., Sklar,L.A., Oprea,T.I., and Arterburn,J.B. (2008) GPR30: a novel therapeutic target in estrogen-related disease. *Trends Pharmacol.Sci.*, 29, 116-123.

153. Posern,G. and Treisman,R. (2006) Actin' together: serum response factor, its cofactors and the link to signal transduction. *Trends Cell Biol.*, 16, 588-596.

154. Gutierrez-Hartmann,A., Duval,D.L., and Bradford,A.P. (2007) ETS transcription factors in endocrine systems. *Trends Endocrinol.Metab*, 18, 150-158.

155. Liu,Z., Yu,X., and Shaikh,Z.A. (2008) Rapid activation of ERK1/2 and AKT in human breast cancer cells by cadmium. *Toxicol.Appl.Pharmacol.*, 228, 286-294.

156. Kleuser,B., Malek,D., Gust,R., Pertz,H.H., and Potteck,H. (2008) 17-Beta-estradiol inhibits transforming growth factor-beta signaling and function in breast cancer cells via activation of extracellular signal-regulated kinase through the G protein-coupled receptor 30. *Mol.Pharmacol.*, 74, 1533-1543.

157. Albanito,L., Lappano,R., Madeo,A., Chimento,A., Prossnitz,E.R., Cappello,A.R., Dolce,V., Abonante,S., Pezzi,V., and Maggiolini,M. (2008) G-protein-coupled receptor 30 and estrogen receptor-alpha are involved in the proliferative effects induced by atrazine in ovarian cancer cells. *Environ.Health Perspect.*, 116, 1648-1655.

158. Lapensee,E.W., Tuttle,T.R., Fox,S.R., and Ben-Jonathan,N. (2009) Bisphenol A at low nanomolar doses confers chemoresistance in estrogen receptor-alpha-positive and -negative breast cancer cells. *Environ.Health Perspect.*, 117, 175-180.

159. Kang,K., Lee,S.B., Jung,S.H., Cha,K.H., Park,W.D., Sohn,Y.C., and Nho,C.W. (2009) Tectoridin, a poor ligand of estrogen receptor alpha, exerts its estrogenic effects via an ERK-dependent pathway. *Mol.Cells*, 27, 351-357.

160. Henic,E., Noskova,V., Hoyer-Hansen,G., Hansson,S., and Casslen,B. (2009) Estradiol attenuates EGF-induced rapid uPAR mobilization and cell migration via the G-protein-coupled receptor 30 in ovarian cancer cells. *Int.J.Gynecol.Cancer*, 19, 214-222.

161. Filardo,E.J., Graeber,C.T., Quinn,J.A., Resnick,M.B., Giri,D., DeLellis,R.A., Steinhoff,M.M., and Sabo,E. (2006) Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. *Clin.Cancer Res.*, 12, 6359-6366.

162. Albanito,L., Madeo,A., Lappano,R., Vivacqua,A., Rago,V., Carpino,A., Oprea,T.I., Prossnitz,E.R., Musti,A.M., Ando,S., and Maggiolini,M. (2007) G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17beta-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. *Cancer Res.*, 67, 1859-1866.

163. Kuo,W.H., Chang,L.Y., Liu,D.L., Hwa,H.L., Lin,J.J., Lee,P.H., Chen,C.N., Lien,H.C., Yuan,R.H., Shun,C.T., Chang,K.J., and Hsieh,F.J. (2007) The interactions between GPR30 and the major biomarkers in infiltrating ductal carcinoma of the breast in an Asian population. *Taiwan.J.Obstet.Gynecol.*, 46, 135-145.

164. Filardo,E.J., Quinn,J.A., and Sabo,E. (2008) Association of the membrane estrogen receptor, GPR30, with breast tumor metastasis and transactivation of the epidermal growth factor receptor. *Steroids*, 73, 870-873.

165. Pandey,D.P., Lappano,R., Albanito,L., Madeo,A., Maggiolini,M., and Picard,D. (2009) Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF. *EMBO J.*, 28, 523-532.

166. He,Y.Y., Cai,B., Yang,Y.X., Liu,X.L., and Wan,X.P. (2009) Estrogenic G protein-coupled receptor 30 signaling is involved in regulation of endometrial carcinoma by promoting proliferation, invasion potential, and interleukin-6 secretion via the MEK/ERK mitogen-activated protein kinase pathway. *Cancer Sci.*, 100, 1051-1061.

167. Zhang,Y., Gaikwad,N.W., Olson,K., Zahid,M., Cavalieri,E.L., and Rogan,E.G. (2007) Cytochrome P450 isoforms catalyze formation of catechol estrogen quinones that react with DNA. *Metabolism*, 56, 887-894.

168. Zhu,B.T., Bui,Q.D., Weisz,J., and Liehr,J.G. (1994) Conversion of estrone to 2- and 4-hydroxyestrone by hamster kidney and liver microsomes: implications for

the mechanism of estrogen-induced carcinogenesis. *Endocrinology*, 135, 1772-1779.

169. Cavalieri,E., Frenkel,K., Liehr,J.G., Rogan,E., and Roy,D. (2000) Estrogens as endogenous genotoxic agents--DNA adducts and mutations. *J.Natl.Cancer Inst.Monogr*,75-93.

170. Roy,D. and Liehr,J.G. (1988) Temporary decrease in renal quinone reductase activity induced by chronic administration of estradiol to male Syrian hamsters. Increased superoxide formation by redox cycling of estrogen. *J.Biol.Chem.*, 263, 3646-3651.

171. Ashburn,S.P., Han,X., and Liehr,J.G. (1993) Microsomal hydroxylation of 2- and 4-fluoroestradiol to catechol metabolites and their conversion to methyl ethers: catechol estrogens as possible mediators of hormonal carcinogenesis. *Mol.Pharmacol.*, 43, 534-541.

172. Malins,D.C., Holmes,E.H., Polissar,N.L., and Gunselman,S.J. (1993) The etiology of breast cancer. Characteristic alteration in hydroxyl radical-induced DNA base lesions during oncogenesis with potential for evaluating incidence risk. *Cancer*, 71, 3036-3043.

173. Osborne,M.P., Bradlow,H.L., Wong,G.Y., and Telang,N.T. (1993) Upregulation of estradiol C16 alpha-hydroxylation in human breast tissue: a potential biomarker of breast cancer risk. *J.Natl.Cancer Inst.*, 85, 1917-1920.

174. Sipe,H.J., Jr., Jordan,S.J., Hanna,P.M., and Mason,R.P. (1994) The metabolism of 17 beta-estradiol by lactoperoxidase: a possible source of oxidative stress in breast cancer. *Carcinogenesis*, 15, 2637-2643.

175. Cavalieri,E.L., Stack,D.E., Devanesan,P.D., Todorovic,R., Dwivedy,I., Higginbotham,S., Johansson,S.L., Patil,K.D., Gross,M.L., Gooden,J.K., Ramanathan,R., Cerny,R.L., and Rogan,E.G. (1997) Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. *Proc.Natl.Acad.Sci.U.S.A*, 94, 10937-10942.

176. Martucci,C.P. and Fishman,J. (1993) P450 enzymes of estrogen metabolism. *Pharmacol.Ther.*, 57, 237-257.

177. Yager,J.D. and Liehr,J.G. (1996) Molecular mechanisms of estrogen carcinogenesis. *Annu.Rev.Pharmacol.Toxicol.*, 36, 203-232.

178. Zhu,B.T. and Conney,A.H. (1998) Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis*, 19, 1-27.

179. Huang,Z., Fasco,M.J., Figge,H.L., Keyomarsi,K., and Kaminsky,L.S. (1996) Expression of cytochromes P450 in human breast tissue and tumors. *Drug Metab Dispos.*, 24, 899-905.

180. Hellmold,H., Rylander,T., Magnusson,M., Reihner,E., Warner,M., and Gustafsson,J.A. (1998) Characterization of cytochrome P450 enzymes in human breast tissue from reduction mammaplasties. *J.Clin.Endocrinol.Metab*, 83, 886-895.

181. Forrester,L.M., Hayes,J.D., Millis,R., Barnes,D., Harris,A.L., Schlager,J.J., Powis,G., and Wolf,C.R. (1990) Expression of glutathione S-transferases and cytochrome P450 in normal and tumor breast tissue. *Carcinogenesis*, 11, 2163-2170.

182. Ball,P. and Knuppen,R. (1978) Formation of 2- and 4-hydroxyestrogens by brain, pituitary, and liver of the human fetus. *J.Clin.Endocrinol.Metab*, 47, 732-737.

183. Shimada,T., Hayes,C.L., Yamazaki,H., Amin,S., Hecht,S.S., Guengerich,F.P., and Sutter,T.R. (1996) Activation of chemically diverse procarcinogens by human cytochrome P-450 1B1. *Cancer Res.*, 56, 2979-2984.

184. Muskhelishvili,L., Thompson,P.A., Kusewitt,D.F., Wang,C., and Kadlubar,F.F. (2001) In situ hybridization and immunohistochemical analysis of cytochrome P450 1B1 expression in human normal tissues. *J.Histochem.Cytochem.*, 49, 229-236.

185. Gibson,P., Gill,J.H., Khan,P.A., Seargent,J.M., Martin,S.W., Batman,P.A., Griffith,J., Bradley,C., Double,J.A., Bibby,M.C., and Loadman,P.M. (2003) Cytochrome P450 1B1 (CYP1B1) is overexpressed in human colon adenocarcinomas relative to normal colon: implications for drug development. *Mol.Cancer Ther.*, 2, 527-534.

186. Tokizane,T., Shiina,H., Igawa,M., Enokida,H., Urakami,S., Kawakami,T., Ogishima,T., Okino,S.T., Li,L.C., Tanaka,Y., Nonomura,N., Okuyama,A., and

Dahiya,R. (2005) Cytochrome P450 1B1 is overexpressed and regulated by hypomethylation in prostate cancer. *Clin.Cancer Res.*, 11, 5793-5801.

187. Lee,A.J., Cai,M.X., Thomas,P.E., Conney,A.H., and Zhu,B.T. (2003) Characterization of the oxidative metabolites of 17beta-estradiol and estrone formed by 15 selectively expressed human cytochrome p450 isoforms. *Endocrinology*, 144, 3382-3398.

188. Liehr,J.G., Ricci,M.J., Jefcoate,C.R., Hannigan,E.V., Hokanson,J.A., and Zhu,B.T. (1995) 4-Hydroxylation of estradiol by human uterine myometrium and myoma microsomes: implications for the mechanism of uterine tumorigenesis. *Proc.Natl.Acad.Sci.U.S.A*, 92, 9220-9224.

189. Hayes,C.L., Spink,D.C., Spink,B.C., Cao,J.Q., Walker,N.J., and Sutter,T.R. (1996) 17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *Proc.Natl.Acad.Sci.U.S.A*, 93, 9776-9781.

190. Stack,D.E., Byun,J., Gross,M.L., Rogan,E.G., and Cavalieri,E.L. (1996) Molecular characteristics of catechol estrogen quinones in reactions with deoxyribonucleosides. *Chem.Res.Toxicol.*, 9, 851-859.

191. Akanni,A. and bul-Hajj,Y.J. (1997) Estrogen-nucleic acid adducts: reaction of 3,4-estrone-o-quinone radical anion with deoxyribonucleosides. *Chem.Res.Toxicol.*, 10, 760-766.

192. Bolton,J.L., Pisha,E., Zhang,F., and Qiu,S. (1998) Role of quinoids in estrogen carcinogenesis. *Chem.Res.Toxicol.*, 11, 1113-1127.

193. Liehr,J.G., Fang,W.F., Sirbasku,D.A., and ri-Ulubelen,A. (1986) Carcinogenicity of catechol estrogens in Syrian hamsters. *J.Steroid Biochem.*, 24, 353-356.

194. Liehr,J.G. and Roy,D. (1990) Free radical generation by redox cycling of estrogens. *Free Radic.Biol.Med.*, 8, 415-423.

195. Nutter,L.M., Wu,Y.Y., Ngo,E.O., Sierra,E.E., Gutierrez,P.L., and bul-Hajj,Y.J. (1994) An o-quinone form of estrogen produces free radicals in human breast cancer cells: correlation with DNA damage. *Chem.Res.Toxicol.*, 7, 23-28.

196. Fotsis,T., Zhang,Y., Pepper,M.S., Adlercreutz,H., Montesano,R., Nawroth,P.P., and Schweigerer,L. (1994) The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumour growth. *Nature*, 368, 237-239.

197. Klauber,N., Parangi,S., Flynn,E., Hamel,E., and D'Amato,R.J. (1997) Inhibition of angiogenesis and breast cancer in mice by the microtubule inhibitors 2-methoxyestradiol and taxol. *Cancer Res.*, 57, 81-86.

198. Lemon,H.M., Heidel,J.W., and Rodriguez-Sierra,J.F. (1992) Increased catechol estrogen metabolism as a risk factor for nonfamilial breast cancer. *Cancer*, 69, 457-465.

199. Liehr,J.G. and Ricci,M.J. (1996) 4-Hydroxylation of estrogens as marker of human mammary tumors. *Proc.Natl.Acad.Sci.U.S.A*, 93, 3294-3296.

200. Emons,G., Merriam,G.R., Pfeiffer,D., Loriaux,D.L., Ball,P., and Knuppen,R. (1987) Metabolism of exogenous 4- and 2-hydroxyestradiol in the human male. *J.Steroid Biochem.*, 28, 499-504.

201. Han,X. and Liehr,J.G. (1995) Microsome-mediated 8-hydroxylation of guanine bases of DNA by steroid estrogens: correlation of DNA damage by free radicals with metabolic activation to quinones. *Carcinogenesis*, 16, 2571-2574.

202. Newbold,R.R. and Liehr,J.G. (2000) Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. *Cancer Res.*, 60, 235-237.

203. Van Aswegen,C.H., Purdy,R.H., and Wittliff,J.L. (1989) Binding of 2-hydroxyestradiol and 4-hydroxyestradiol to estrogen receptors from human breast cancers. *J.Steroid Biochem.*, 32, 485-492.

204. Zhu,B.T. and Liehr,J.G. (1996) Inhibition of catechol O-methyltransferase-catalyzed O-methylation of 2- and 4-hydroxyestradiol by quercetin. Possible role in estradiol-induced tumorigenesis. *J.Biol.Chem.*, 271, 1357-1363.

205. Kono,S. and Fujino,T. (1983) [The mechanism of central regulation and metabolic clearance of catechol estrogens]. *Horumon To Rinsho*, 31, 111-118.

206. Schneider,J., Huh,M.M., Bradlow,H.L., and Fishman,J. (1984) Antiestrogen action of 2-hydroxyestrone on MCF-7 human breast cancer cells. *J.Biol.Chem.*, 259, 4840-4845.

207. Li,S.A., Purdy,R.H., and Li,J.J. (1989) Variations in catechol O-methyltransferase activity in rodent tissues: possible role in estrogen carcinogenicity. *Carcinogenesis*, 10, 63-67.

208. Vandewalle,B. and Lefebvre,J. (1989) Opposite effects of estrogen and catecholestrogen on hormone-sensitive breast cancer cell growth and differentiation. *Mol.Cell Endocrinol.*, 61, 239-246.

209. Schutze,N., Vollmer,G., Tiemann,I., Geiger,M., and Knuppen,R. (1993) Catecholestrogens are MCF-7 cell estrogen receptor agonists. *J.Steroid Biochem.Mol.Biol.*, 46, 781-789.

210. Schutze,N., Vollmer,G., and Knuppen,R. (1994) Catecholestrogens are agonists of estrogen receptor dependent gene expression in MCF-7 cells. *J.Steroid Biochem.Mol.Biol.*, 48, 453-461.

211. D'Amato,R.J., Lin,C.M., Flynn,E., Folkman,J., and Hamel,E. (1994) 2-Methoxyestradiol, an endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site. *Proc.Natl.Acad.Sci.U.S.A*, 91, 3964-3968.

212. Hamel,E., Lin,C.M., Flynn,E., and D'Amato,R.J. (1996) Interactions of 2-methoxyestradiol, an endogenous mammalian metabolite, with unpolymerized tubulin and with tubulin polymers. *Biochemistry*, 35, 1304-1310.

213. Guldberg,H.C. and Marsden,C.A. (1975) Catechol-O-methyl transferase: pharmacological aspects and physiological role. *Pharmacol.Rev.*, 27, 135-206.

214. Bertocci,B., Miggiano,V., Da,P.M., Dembic,Z., Lahm,H.W., and Malherbe,P. (1991) Human catechol-O-methyltransferase: cloning and expression of the membrane-associated form. *Proc.Natl.Acad.Sci.U.S.A*, 88, 1416-1420.

215. Merriam,G.R., MacLusky,N.J., Picard,M.K., and Naftolin,F. (1980) Comparative properties of the catechol estrogens, I: methylation by catechol-O-methyltransferase and binding to cytosol estrogen receptors. *Steroids*, 36, 1-11.

216. Ball,P. and Knuppen,R. (1980) Catecholoestrogens (2-and 4-hydroxyoestrogens): chemistry, biogenesis, metabolism, occurrence and physiological significance. *Acta Endocrinol.Suppl (Copenh)*, 232, 1-127.

217. Seegers,J.C., Aveling,M.L., Van Aswegen,C.H., Cross,M., Koch,F., and Joubert,W.S. (1989) The cytotoxic effects of estradiol-17 beta, catecholestradiols and methoxyestradiols on dividing MCF-7 and HeLa cells. *J.Steroid Biochem.*, 32, 797-809.

218. Lottering,M.L., Haag,M., and Seegers,J.C. (1992) Effects of 17 beta-estradiol metabolites on cell cycle events in MCF-7 cells. *Cancer Res.*, 52, 5926-5932.

219. Brierley,C.H. and Burchell,B. (1993) Human UDP-glucuronosyl transferases: chemical defence, jaundice and gene therapy. *Bioessays*, 15, 749-754.

220. Ebner,T., Remmel,R.P., and Burchell,B. (1993) Human bilirubin UDP-glucuronosyltransferase catalyzes the glucuronidation of ethinylestradiol. *Mol.Pharmacol.*, 43, 649-654.

221. Albert,C., Vallee,M., Beaudry,G., Belanger,A., and Hum,D.W. (1999) The monkey and human uridine diphosphate-glucuronosyltransferase UGT1A9, expressed in steroid target tissues, are estrogen-conjugating enzymes. *Endocrinology*, 140, 3292-3302.

222. Weisburger,J.H. (1977) Social and ethical implications of claims for cancer hazards. *Med.Pediatr.Oncol.*, 3, 137-140.

223. Weinshilboum,R.M. (1986) Phenol sulfotransferase in humans: properties, regulation, and function. *Fed.Proc.*, 45, 2223-2228.

224. Falany,C.N. (1991) Molecular enzymology of human liver cytosolic sulfotransferases. *Trends Pharmacol.Sci.*, 12, 255-259.

225. Aksoy,I.A., Wood,T.C., and Weinshilboum,R. (1994) Human liver estrogen sulfotransferase: identification by cDNA cloning and expression. *Biochem.Biophys.Res.Commun.*, 200, 1621-1629.

226. Blanchard,R.L., Freimuth,R.R., Buck,J., Weinshilboum,R.M., and Coughtrie,M.W. (2004) A proposed nomenclature system for the cytosolic sulfotransferase (SULT) superfamily. *Pharmacogenetics*, 14, 199-211.

227. Huttner,W.B. (1982) Sulphation of tyrosine residues-a widespread modification of proteins. *Nature*, 299, 273-276.

228. Falany,C.N. (1997) Enzymology of human cytosolic sulfotransferases. *FASEB J.*, 11, 206-216.

229. Habuchi,O. (2000) Diversity and functions of glycosaminoglycan sulfotransferases. *Biochim.Biophys.Acta*, 1474, 115-127.

230. Negishi,M., Pedersen,L.G., Petrotchenko,E., Shevtsov,S., Gorokhov,A., Kakuta,Y., and Pedersen,L.C. (2001) Structure and function of sulfotransferases. *Arch.Biochem.Biophys.*, 390, 149-157.

231. Farooqui,A.A. (1980) 3'-phosphoadenosine 5'-phosphosulphate metabolism in mammalian tissues. *Int.J.Biochem.*, 12, 529-536.

232. Guengerich,F.P. (1990) Metabolism of 17 alpha-ethynylestradiol in humans. *Life Sci.*, 47, 1981-1988.

233. Hobkirk,R. (1985) Steroid sulfotransferases and steroid sulfate sulfatases: characteristics and biological roles. *Can.J.Biochem.Cell Biol.*, 63, 1127-1144.

234. Vermeulen,A. (1986) Human mammary cancer as a site of sex steroid metabolism. *Cancer Surv.*, 5, 585-595.

235. Roy,A.K. (1992) Regulation of steroid hormone action in target cells by specific hormone-inactivating enzymes. *Proc.Soc.Exp.Biol.Med.*, 199, 265-272.

236. Adams,J.B. and Phillips,N.S. (1990) Properties of estrogen and hydroxysteroid sulphotransferases in human mammary cancer. *J.Steroid Biochem.*, 36, 695-701.

237. Hernandez,J.S., Watson,R.W., Wood,T.C., and Weinshilboum,R.M. (1992) Sulfation of estrone and 17 beta-estradiol in human liver. Catalysis by

thermostable phenol sulfotransferase and by dehydroepiandrosterone sulfotransferase. *Drug Metab Dispos.*, 20, 413-422.

238. Her,C., Szumlanski,C., Aksoy,I.A., and Weinshilboum,R.M. (1996) Human jejunal estrogen sulfotransferase and dehydroepiandrosterone sulfotransferase: immunochemical characterization of individual variation. *Drug Metab Dispos.*, 24, 1328-1335.

239. Coughtrie,M.W., Sharp,S., Maxwell,K., and Innes,N.P. (1998) Biology and function of the reversible sulfation pathway catalysed by human sulfotransferases and sulfatases. *Chem.Biol.Interact.*, 109, 3-27.

240. Adams,J.B., Pewnim,T., Chandra,D.P., Archibald,L., and Foo,M.S. (1979) A correlation between estrogen sulfotransferase levels and estrogen receptor status in human primary breast carcinoma. *Cancer Res.*, 39, 5124-5126.

241. Pewnim,T., Adams,J.B., and Ho,K.P. (1980) A relationship between estrogen sulfurylation and estrogen and progesterone receptor status in human mammary carcinoma. *Cancer Res.*, 40, 1360-1362.

242. Raju,U., Blaustein,A., and Levitz,M. (1980) Conjugation of androgens and estrogens by human breast tumors in vitro. *Steroids*, 35, 685-695.

243. Price,R.A., Spielman,R.S., Lucena,A.L., Van Loon,J.A., Maidak,B.L., and Weinshilboum,R.M. (1989) Genetic polymorphism for human platelet thermostable phenol sulfotransferase (TS PST) activity. *Genetics*, 122, 905-914.

244. Adams,J.B., Phillips,N.S., and Pewnim,T. (1989) Expression of hydroxysteroid sulphotransferase is related to estrogen receptor status in human mammary cancer. *J.Steroid Biochem.*, 33, 637-642.

245. Weinshilboum,R.M., Otterness,D.M., Aksoy,I.A., Wood,T.C., Her,C., and Raftogianis,R.B. (1997) Sulfation and sulfotransferases 1: Sulfotransferase molecular biology: cDNAs and genes. *FASEB J.*, 11, 3-14.

246. Adjei,A.A. and Weinshilboum,R.M. (2002) Catecholestrogen sulfation: possible role in carcinogenesis. *Biochem.Biophys.Res.Commun.*, 292, 402-408.

247. Falany,J.L., Macrina,N., and Falany,C.N. (2002) Regulation of MCF-7 breast cancer cell growth by beta-estradiol sulfation. *Breast Cancer Res.Treat.*, 74, 167-176.

248. Schrag,M.L., Cui,D., Rushmore,T.H., Shou,M., Ma,B., and Rodrigues,A.D. (2004) Sulfotransferase 1E1 is a low km isoform mediating the 3-O-sulfation of ethinyl estradiol. *Drug Metab Dispos.*, 32, 1299-1303.

249. Falany,J.L. and Falany,C.N. (1996) Expression of cytosolic sulfotransferases in normal mammary epithelial cells and breast cancer cell lines. *Cancer Res.*, 56, 1551-1555.

250. Fu,J., Weise,A.M., Falany,J.L., Falany,C.N., Thibodeau,B.J., Miller,F.R., Kocarek,T.A., and Runge-Morris,M. (2010) Expression of estrogenicity genes in a lineage cell culture model of human breast cancer progression. *Breast Cancer Res.Treat.*, 120, 35-45.

251. Wild,M.J., Rudland,P.S., and Back,D.J. (1991) Metabolism of the oral contraceptive steroids ethynylestradiol and norgestimate by normal (Huma 7) and malignant (MCF-7 and ZR-75-1) human breast cells in culture. *J.Steroid Biochem.Mol.Biol.*, 39, 535-543.

252. Falany,J.L., Lawing,L., and Falany,C.N. (1993) Identification and characterization of cytosolic sulfotransferase activities in MCF-7 human breast carcinoma cells. *J.Steroid Biochem.Mol.Biol.*, 46, 481-487.

253. Sharp,S., Anderson,J.M., and Coughtrie,M.W. (1994) Immunohistochemical localisation of hydroxysteroid sulphotransferase in human breast carcinoma tissue: a preliminary study. *Eur.J.Cancer*, 30A, 1654-1659.

254. Falany,C.N., Wheeler,J., Oh,T.S., and Falany,J.L. (1994) Steroid sulfation by expressed human cytosolic sulfotransferases. *J.Steroid Biochem.Mol.Biol.*, 48, 369-375.

255. Qian,Y., Deng,C., and Song,W.C. (1998) Expression of estrogen sulfotransferase in MCF-7 cells by cDNA transfection suppresses the estrogen response: potential role of the enzyme in regulating estrogen-dependent growth of breast epithelial cells. *J.Pharmacol.Exp.Ther.*, 286, 555-560.

256. Dupont,W.D. and Page,D.L. (1985) Risk factors for breast cancer in women with proliferative breast disease. *N.Engl.J.Med.*, 312, 146-151.

257. Wang,J., Costantino,J.P., Tan-Chiu,E., Wickerham,D.L., Paik,S., and Wolmark,N. (2004) Lower-category benign breast disease and the risk of invasive breast cancer. *J.Natl.Cancer Inst.*, 96, 616-620.

258. London,S.J., Connolly,J.L., Schnitt,S.J., and Colditz,G.A. (1992) A prospective study of benign breast disease and the risk of breast cancer. *JAMA*, 267, 941-944.

259. Dupont,W.D., Parl,F.F., Hartmann,W.H., Brinton,L.A., Winfield,A.C., Worrell,J.A., Schuyler,P.A., and Plummer,W.D. (1993) Breast cancer risk associated with proliferative breast disease and atypical hyperplasia. *Cancer*, 71, 1258-1265.

260. Mommers,E.C., van Diest,P.J., Leonhart,A.M., Meijer,C.J., and Baak,J.P. (1998) Expression of proliferation and apoptosis-related proteins in usual ductal hyperplasia of the breast. *Hum.Pathol.*, 29, 1539-1545.

261. Mommers,E.C., van Diest,P.J., Leonhart,A.M., Meijer,C.J., and Baak,J.P. (1999) Balance of cell proliferation and apoptosis in breast carcinogenesis. *Breast Cancer Res.Treat.*, 58, 163-169.

262. Mommers,E.C., Poulin,N., Sangulin,J., Meijer,C.J., Baak,J.P., and van Diest,P.J. (2001) Nuclear cytometric changes in breast carcinogenesis. *J.Pathol.*, 193, 33-39.

263. Wellings,S.R., Jensen,H.M., and Marcum,R.G. (1975) An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions. *J.Natl.Cancer Inst.*, 55, 231-273.

264. Ellis,I.O. (2010) Intraductal proliferative lesions of the breast: morphology, associated risk and molecular biology. *Mod.Pathol.*, 23 Suppl 2, S1-S7.

265. Page,D.L. and Dupont,W.D. (1990) Anatomic markers of human premalignancy and risk of breast cancer. *Cancer*, 66, 1326-1335.

266. Shaaban,A.M., Sloane,J.P., West,C.R., and Foster,C.S. (2002) Breast cancer risk in usual ductal hyperplasia is defined by estrogen receptor-alpha and Ki-67 expression. *Am.J.Pathol.*, 160, 597-604.

267. Schmitt,F.C. (1995) Multistep progression from an oestrogen-dependent growth towards an autonomous growth in breast carcinogenesis. *Eur.J.Cancer*, 31A, 2049-2052.

268. Barnes,R. and Masood,S. (1990) Potential value of hormone receptor assay in carcinoma in situ of breast. *Am.J.Clin.Pathol.*, 94, 533-537.

269. Page,D.L., Vander,Z.R., Rogers,L.W., Williams,L.T., Walker,W.E., and Hartmann,W.H. (1978) Relation between component parts of fibrocystic disease complex and breast cancer. *J.Natl.Cancer Inst.*, 61, 1055-1063.

270. Page,D.L., Dupont,W.D., Rogers,L.W., and Rados,M.S. (1985) Atypical hyperplastic lesions of the female breast. A long-term follow-up study. *Cancer*, 55, 2698-2708.

271. Tavassoli,F.A. and Norris,H.J. (1990) A comparison of the results of long-term follow-up for atypical intraductal hyperplasia and intraductal hyperplasia of the breast. *Cancer*, 65, 518-529.

272. McDivitt,R.W., Stevens,J.A., Lee,N.C., Wingo,P.A., Rubin,G.L., and Gersell,D. (1992) Histologic types of benign breast disease and the risk for breast cancer. The Cancer and Steroid Hormone Study Group. *Cancer*, 69, 1408-1414.

273. BLOOM,H.J. and RICHARDSON,W.W. (1957) Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years. *Br.J.Cancer*, 11, 359-377.

274. Sarnelli,R. and Squartini,F. (1989) Cancerous versus noncancerous breasts. A comparative morphological analysis of the entire glandular tree of the breast. *Virchows Arch.A Pathol.Anat.Histopathol.*, 414, 257-262.

275. Fuqua,S.A., Wiltschke,C., Zhang,Q.X., Borg,A., Castles,C.G., Friedrichs,W.E., Hopp,T., Hilsenbeck,S., Mohsin,S., O'Connell,P., and Allred,D.C. (2000) A hypersensitive estrogen receptor-alpha mutation in premalignant breast lesions. *Cancer Res.*, 60, 4026-4029.

276. Page,D.L. and Dupont,W.D. (1993) Anatomic indicators (histologic and cytologic) of increased breast cancer risk. *Breast Cancer Res.Treat.*, 28, 157-166.

277. Hartmann,L.C., Sellers,T.A., Frost,M.H., Lingle,W.L., Degnim,A.C., Ghosh,K., Vierkant,R.A., Maloney,S.D., Pankratz,V.S., Hillman,D.W., Suman,V.J., Johnson,J., Blake,C., Tlsty,T., Vachon,C.M., Melton,L.J., III, and Visscher,D.W.

(2005) Benign breast disease and the risk of breast cancer. *N.Engl.J.Med.*, 353, 229-237.

278. Ernster,V.L., Ballard-Barbash,R., Barlow,W.E., Zheng,Y., Weaver,D.L., Cutter,G., Yankaskas,B.C., Rosenberg,R., Carney,P.A., Kerlikowske,K., Taplin,S.H., Urban,N., and Geller,B.M. (2002) Detection of ductal carcinoma in situ in women undergoing screening mammography. *J.Natl.Cancer Inst.*, 94, 1546-1554.

279. Olivotto,I.A., Bancej,C., Goel,V., Snider,J., McAuley,R.G., Irvine,B., Kan,L., Mirsky,D., Sabine,M.J., McGilly,R., and Caines,J.S. (2001) Waiting times from abnormal breast screen to diagnosis in 7 Canadian provinces. *CMAJ.*, 165, 277-283.

280. Radford,D.M., Phillips,N.J., Fair,K.L., Ritter,J.H., Holt,M., and Donis-Keller,H. (1995) Allelic loss and the progression of breast cancer. *Cancer Res.*, 55, 5180-5183.

281. Stratton,M.R., Collins,N., Lakhani,S.R., and Sloane,J.P. (1995) Loss of heterozygosity in ductal carcinoma in situ of the breast. *J.Pathol.*, 175, 195-201.

282. Bocker,W., Bier,B., Freytag,G., Brommelkamp,B., Jarasch,E.D., Edel,G., Dockhorn-Dworniczak,B., and Schmid,K.W. (1992) An immunohistochemical study of the breast using antibodies to basal and luminal keratins, alpha-smooth muscle actin, vimentin, collagen IV and laminin. Part II: Epitheliosis and ductal carcinoma in situ. *Virchows Arch.A Pathol.Anat.Histopathol.*, 421, 323-330.

283. Damiani,S., Ludvikova,M., Tomasic,G., Bianchi,S., Gown,A.M., and Eusebi,V. (1999) Myoepithelial cells and basal lamina in poorly differentiated in situ duct carcinoma of the breast. An immunocytochemical study. *Virchows Arch.*, 434, 227-234.

284. Porter,D.A., Krop,I.E., Nasser,S., Sgroi,D., Kaelin,C.M., Marks,J.R., Riggins,G., and Polyak,K. (2001) A SAGE (serial analysis of gene expression) view of breast tumor progression. *Cancer Res.*, 61, 5697-5702.

285. Luzzi,V., Holtschlag,V., and Watson,M.A. (2001) Expression profiling of ductal carcinoma in situ by laser capture microdissection and high-density oligonucleotide arrays. *Am.J.Pathol.*, 158, 2005-2010.

286. Bryan,B.B., Schnitt,S.J., and Collins,L.C. (2006) Ductal carcinoma in situ with basal-like phenotype: a possible precursor to invasive basal-like breast cancer. *Mod.Pathol.*, 19, 617-621.

287. Bellamy,C.O., McDonald,C., Salter,D.M., Chetty,U., and Anderson,T.J. (1993) Noninvasive ductal carcinoma of the breast: the relevance of histologic categorization. *Hum.Pathol.*, 24, 16-23.

288. (1997) Consensus conference on the classification of ductal carcinoma in situ. *Hum.Pathol.*, 28, 1221-1225.

289. Silverstein,M.J., Poller,D.N., Waisman,J.R., Colburn,W.J., Barth,A., Gierson,E.D., Lewinsky,B., Gamagami,P., and Slamon,D.J. (1995) Prognostic classification of breast ductal carcinoma-in-situ. *Lancet*, 345, 1154-1157.

290. Badve,S., A'Hern,R.P., Ward,A.M., Millis,R.R., Pinder,S.E., Ellis,I.O., Gusterson,B.A., and Sloane,J.P. (1998) Prediction of local recurrence of ductal carcinoma in situ of the breast using five histological classifications: a comparative study with long follow-up. *Hum.Pathol.*, 29, 915-923.

291. Bethwaite,P., Smith,N., Delahunt,B., and Kenwright,D. (1998) Reproducibility of new classification schemes for the pathology of ductal carcinoma in situ of the breast. *J.Clin.Pathol.*, 51, 450-454.

292. Douglas-Jones,A.G., Morgan,J.M., Appleton,M.A., Attanoos,R.L., Caslin,A., Champ,C.S., Cotter,M., Dallimore,N.S., Dawson,A., Fortt,R.W., Griffiths,A.P., Hughes,M., Kitching,P.A., O'Brien,C., Rashid,A.M., Stock,D., Verghese,A., Williams,D.W., Williams,N.W., and Williams,S. (2000) Consistency in the observation of features used to classify duct carcinoma in situ (DCIS) of the breast. *J.Clin.Pathol.*, 53, 596-602.

293. Sloane,J.P., Amendoeira,I., Apostolikas,N., Bellocq,J.P., Bianchi,S., Boecker,W., Bussolati,G., Coleman,D., Connolly,C.E., Dervan,P., Eusebi,V., De,M.C., Drijkoningen,M., Elston,C.W., Faverley,D., Gad,A., Jacquemier,J., Lacerda,M., Martinez-Penuela,J., Munt,C., Peterse,J.L., Rank,F., Sylvan,M., Tsakraklides,V., and Zafrani,B. (1998) Consistency achieved by 23 European pathologists in categorizing ductal carcinoma in situ of the breast using five classifications. European Commission Working Group on Breast Screening Pathology. *Hum.Pathol.*, 29, 1056-1062.

294. Silverstein,M.J., Lagios,M.D., Craig,P.H., Waisman,J.R., Lewinsky,B.S., Colburn,W.J., and Poller,D.N. (1996) A prognostic index for ductal carcinoma in situ of the breast. *Cancer*, 77, 2267-2274.

295. Kanthan,R., Xiang,J., and Magliocco,A.M. (2000) p53, ErbB2, and TAG-72 expression in the spectrum of ductal carcinoma in situ of the breast classified by the Van Nuys system. *Arch.Pathol.Lab Med.*, 124, 234-239.

296. Bobrow,L.G., Happerfield,L.C., Gregory,W.M., Springall,R.D., and Millis,R.R. (1994) The classification of ductal carcinoma in situ and its association with biological markers. *Semin.Diagn.Pathol.*, 11, 199-207.

297. Poller,D.N., Silverstein,M.J., Galea,M., Locker,A.P., Elston,C.W., Blamey,R.W., and Ellis,I.O. (1994) Ideas in pathology. Ductal carcinoma in situ of the breast: a proposal for a new simplified histological classification association between cellular proliferation and c-erbB-2 protein expression. *Mod.Pathol.*, 7, 257-262.

298. Poller,D.N., Roberts,E.C., Bell,J.A., Elston,C.W., Blamey,R.W., and Ellis,I.O. (1993) p53 protein expression in mammary ductal carcinoma in situ: relationship to immunohistochemical expression of estrogen receptor and c-erbB-2 protein. *Hum.Pathol.*, 24, 463-468.

299. Provenzano,E., Hopper,J.L., Giles,G.G., Marr,G., Venter,D.J., and Armes,J.E. (2003) Biological markers that predict clinical recurrence in ductal carcinoma in situ of the breast. *Eur.J.Cancer*, 39, 622-630.

300. Sagiv,S.K., Gaudet,M.M., Eng,S.M., Abrahamson,P.E., Shantakumar,S., Teitelbaum,S.L., Britton,J.A., Bell,P., Thomas,J.A., Neugut,A.I., Santella,R.M., and Gammon,M.D. (2007) Active and passive cigarette smoke and breast cancer survival. *Ann.Epidemiol.*, 17, 385-393.

301. Ellis,I.O., Galea,M., Broughton,N., Locker,A., Blamey,R.W., and Elston,C.W. (1992) Pathological prognostic factors in breast cancer. II. Histological type. Relationship with survival in a large study with long-term follow-up. *Histopathology*, 20, 479-489.

302. Bratthauer,G.L. and Tavassoli,F.A. (2002) Lobular intraepithelial neoplasia: previously unexplored aspects assessed in 775 cases and their clinical implications. *Virchows Arch.*, 440, 134-138.

303. van,d., V (1993) Molecular genetic changes in human breast cancer. *Adv.Cancer Res.*, 61, 25-56.

304. Black,D.M. (1994) The genetics of breast cancer. *Eur.J.Cancer*, 30A, 1957-1961.

305. Nandi,S., Guzman,R.C., and Yang,J. (1995) Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. *Proc.Natl.Acad.Sci.U.S.A*, 92, 3650-3657.

306. Perou,C.M., Sorlie,T., Eisen,M.B., van de,R.M., Jeffrey,S.S., Rees,C.A., Pollack,J.R., Ross,D.T., Johnsen,H., Akslen,L.A., Fluge,O., Pergamenschikov,A., Williams,C., Zhu,S.X., Lonning,P.E., Borresen-Dale,A.L.,

Brown,P.O., and Botstein,D. (2000) Molecular portraits of human breast tumours. *Nature*, 406, 747-752.

307. Weigelt,B. and Reis-Filho,J.S. (2009) Histological and molecular types of breast cancer: is there a unifying taxonomy? *Nat.Rev.Clin.Oncol.*, 6, 718-730.

308. Melchor,L., Honrado,E., Garcia,M.J., Alvarez,S., Palacios,J., Osorio,A., Nathanson,K.L., and Benitez,J. (2008) Distinct genomic aberration patterns are found in familial breast cancer associated with different immunohistochemical subtypes. *Oncogene*, 27, 3165-3175.

309. Loo,L.W., Grove,D.I., Williams,E.M., Neal,C.L., Cousens,L.A., Schubert,E.L., Holcomb,I.N., Massa,H.F., Glogovac,J., Li,C.I., Malone,K.E., Daling,J.R., Delrow,J.J., Trask,B.J., Hsu,L., and Porter,P.L. (2004) Array comparative genomic hybridization analysis of genomic alterations in breast cancer subtypes. *Cancer Res.*, 64, 8541-8549.

310. Lacroix,M., Toillon,R.A., and Leclercq,G. (2004) Stable 'portrait' of breast tumors during progression: data from biology, pathology and genetics. *Endocr.Relat Cancer*, 11, 497-522.

311. Rennstam,K., hlstedt-Soini,M., Baldetorp,B., Bendahl,P.O., Borg,A., Karhu,R., Tanner,M., Tirkkonen,M., and Isola,J. (2003) Patterns of chromosomal imbalances defines subgroups of breast cancer with distinct clinical features and prognosis. A study of 305 tumors by comparative genomic hybridization. *Cancer Res.*, 63, 8861-8868.

312. Sorlie,T., Perou,C.M., Tibshirani,R., Aas,T., Geisler,S., Johnsen,H., Hastie,T., Eisen,M.B., van de,R.M., Jeffrey,S.S., Thorsen,T., Quist,H., Matese,J.C., Brown,P.O., Botstein,D., Eystein,L.P., and Borresen-Dale,A.L. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc.Natl.Acad.Sci.U.S.A*, 98, 10869-10874.

313. Marquis,S.T., Rajan,J.V., Wynshaw-Boris,A., Xu,J., Yin,G.Y., Abel,K.J., Weber,B.L., and Chodosh,L.A. (1995) The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues. *Nat.Genet.*, 11, 17-26.

314. Shekhar,P.V., Chen,M.L., Werdell,J., Heppner,G.H., Miller,F.R., and Christman,J.K. (1998) Transcriptional activation of functional endogenous estrogen receptor gene expression in MCF10AT cells: a model for early breast cancer. *Int.J.Oncol.*, 13, 907-915.

315. Soule,H.D., Maloney,T.M., Wolman,S.R., Peterson,W.D., Jr., Brenz,R., McGrath,C.M., Russo,J., Pauley,R.J., Jones,R.F., and Brooks,S.C. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res.*, 50, 6075-6086.

316. Pauley,R.J., Soule,H.D., Tait,L., Miller,F.R., Wolman,S.R., Dawson,P.J., and Heppner,G.H. (1993) The MCF10 family of spontaneously immortalized human breast epithelial cell lines: models of neoplastic progression. *Eur.J.Cancer Prev.*, 2 Suppl 3, 67-76.

317. Miller,F.R. (2000) Xenograft models of premalignant breast disease. *J.Mammary.Gland.Biol.Neoplasia.*, 5, 379-391.

318. Santner,S.J., Dawson,P.J., Tait,L., Soule,H.D., Eliason,J., Mohamed,A.N., Wolman,S.R., Heppner,G.H., and Miller,F.R. (2001) Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. *Breast Cancer Res.Treat.*, 65, 101-110.

319. Dawson,P.J., Wolman,S.R., Tait,L., Heppner,G.H., and Miller,F.R. (1996) MCF10AT: a model for the evolution of cancer from proliferative breast disease. *Am.J.Pathol.*, 148, 313-319.

320. Miller,F.R., Santner,S.J., Tait,L., and Dawson,P.J. (2000) MCF10DCIS.com xenograft model of human comedo ductal carcinoma in situ. *J.Natl.Cancer Inst.*, 92, 1185-1186.

321. Baumann,C.K. and Castiglione-Gertsch,M. (2007) Estrogen receptor modulators and down regulators: optimal use in postmenopausal women with breast cancer. *Drugs*, 67, 2335-2353.

322. Hulka,B.S. and Moorman,P.G. (2001) Breast cancer: hormones and other risk factors. *Maturitas*, 38, 103-113.

323. Sasano,H., Suzuki,T., Miki,Y., and Moriya,T. (2008) Intracrinology of estrogens and androgens in breast carcinoma. *J.Steroid Biochem.Mol.Biol.*, 108, 181-185.

324. Dawling,S., Roodi,N., and Parl,F.F. (2003) Methoxyestrogens exert feedback inhibition on cytochrome P450 1A1 and 1B1. *Cancer Res.*, 63, 3127-3132.

325. Subramanian,A., Salhab,M., and Mokbel,K. (2008) Oestrogen producing enzymes and mammary carcinogenesis: a review. *Breast Cancer Res.Treat.*, 111, 191-202.

326. Miller,F.R., Soule,H.D., Tait,L., Pauley,R.J., Wolman,S.R., Dawson,P.J., and Heppner,G.H. (1993) Xenograft model of progressive human proliferative breast disease. *J.Natl.Cancer Inst.*, 85, 1725-1732.

327. Shekhar,M.P., Nangia-Makker,P., Wolman,S.R., Tait,L., Heppner,G.H., and Visscher,D.W. (1998) Direct action of estrogen on sequence of progression of human preneoplastic breast disease. *Am.J.Pathol.*, 152, 1129-1132.

328. Strickland,L.B., Dawson,P.J., Santner,S.J., and Miller,F.R. (2000) Progression of premalignant MCF10AT generates heterogeneous malignant variants with characteristic histologic types and immunohistochemical markers. *Breast Cancer Res.Treat.*, 64, 235-240.

329. Lim,K.T., Cosgrave,N., Hill,A.D., and Young,L.S. (2006) Nongenomic oestrogen signalling in oestrogen receptor negative breast cancer cells: a role for the angiotensin II receptor AT1. *Breast Cancer Res.*, 8, R33.

330. Yoo,J.Y., Lessor,T., and Hamburger,A.W. (1998) Inhibition of cell proliferation by 17beta-estradiol and heregulin beta1 in estrogen receptor negative human breast carcinoma cell lines. *Breast Cancer Res.Treat.*, 51, 71-81.

331. Laidlaw,I.J., Clarke,R.B., Howell,A., Owen,A.W., Potten,C.S., and Anderson,E. (1995) The proliferation of normal human breast tissue implanted into athymic nude mice is stimulated by estrogen but not progesterone. *Endocrinology*, 136, 164-171.

332. Schafer,J.M., Lee,E.S., O'Regan,R.M., Yao,K., and Jordan,V.C. (2000) Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice. *Clin.Cancer Res.*, 6, 4373-4380.

333. Visscher,D.W., Nanjia-Makker,P., Heppner,G., and Shekhar,P.V. (2001) Tamoxifen suppresses histologic progression to atypia and DCIS in MCFIOAT xenografts, a model of early human breast cancer. *Breast Cancer Res.Treat.*, 65, 41-47.

334. Nkhata,K.J., Ray,A., Dogan,S., Grande,J.P., and Cleary,M.P. (2009) Mammary tumor development from T47-D human breast cancer cells in obese ovariectomized mice with and without estradiol supplements. *Breast Cancer Res.Treat.*, 114, 71-83.

335. Kawai,H., Li,H., Avraham,S., Jiang,S., and Avraham,H.K. (2003) Overexpression of histone deacetylase HDAC1 modulates breast cancer progression by negative regulation of estrogen receptor alpha. *Int.J.Cancer*, 107, 353-358.

336. Munster,P.N., Thurn,K.T., Thomas,S., Raha,P., Lacevic,M., Miller,A., Melisko,M., Ismail-Khan,R., Rugo,H., Moasser,M., and Minton,S.E. (2011) A phase II study of the histone deacetylase inhibitor vorinostat combined with tamoxifen for the

treatment of patients with hormone therapy-resistant breast cancer. *Br.J.Cancer*, 104, 1828-1835.

337. Linares,A., Dalenc,F., Balaguer,P., Boulle,N., and Cavailles,V. (2011) Manipulating protein acetylation in breast cancer: a promising approach in combination with hormonal therapies? *J.Biomed.Biotechnol.*, 2011, 856985.

338. Munster,P., Marchion,D., Bicaku,E., Lacevic,M., Kim,J., Centeno,B., Daud,A., Neuger,A., Minton,S., and Sullivan,D. (2009) Clinical and biological effects of valproic acid as a histone deacetylase inhibitor on tumor and surrogate tissues: phase I/II trial of valproic acid and epirubicin/FEC. *Clin.Cancer Res.*, 15, 2488-2496.

339. Vansteenkiste,J., Van,C.E., Dumez,H., Chen,C., Ricker,J.L., Randolph,S.S., and Schoffski,P. (2008) Early phase II trial of oral vorinostat in relapsed or refractory breast, colorectal, or non-small cell lung cancer. *Invest New Drugs*, 26, 483-488.

340. Duanmu,Z., Weckle,A., Koukouritaki,S.B., Hines,R.N., Falany,J.L., Falany,C.N., Kocarek,T.A., and Runge-Morris,M. (2006) Developmental expression of aryl, estrogen, and hydroxysteroid sulfotransferases in pre- and postnatal human liver. *J.Pharmacol.Exp.Ther.*, 316, 1310-1317.

341. Falany,C.N., Krasnykh,V., and Falany,J.L. (1995) Bacterial expression and characterization of a cDNA for human liver estrogen sulfotransferase. *J.Steroid Biochem.Mol.Biol.*, 52, 529-539.

342. Comer,K.A. and Falany,C.N. (1992) Immunological characterization of dehydroepiandrosterone sulfotransferase from human liver and adrenal. *Mol.Pharmacol.*, 41, 645-651.

343. Falany,C.N., He,D., Dumas,N., Frost,A.R., and Falany,J.L. (2006) Human cytosolic sulfotransferase 2B1: isoform expression, tissue specificity and subcellular localization. *J.Steroid Biochem.Mol.Biol.*, 102, 214-221.

344. Miettinen,M.M., Mustonen,M.V., Poutanen,M.H., Isomaa,V.V., and Vihko,R.K. (1996) Human 17 beta-hydroxysteroid dehydrogenase type 1 and type 2 isoenzymes have opposite activities in cultured cells and characteristic cell- and tissue-specific expression. *Biochem.J.*, 314 (Pt 3), 839-845.

345. Suzuki,T., Miki,Y., Akahira,J., Moriya,T., Ohuchi,N., and Sasano,H. (2008) Aromatase in human breast carcinoma as a key regulator of intratumoral sex steroid concentrations. *Endocr.J.*, 55, 455-463.

346. Chun,Y.J. and Kim,S. (2003) Discovery of cytochrome P450 1B1 inhibitors as new promising anti-cancer agents. *Med.Res.Rev.*, 23, 657-668.

347. De los,S.M., Martinez-Iglesias,O., and Aranda,A. (2007) Anti-estrogenic actions of histone deacetylase inhibitors in MCF-7 breast cancer cells. *Endocr.Relat Cancer*, 14, 1021-1028.

348. Brueggemeier,R.W. (2006) Update on the use of aromatase inhibitors in breast cancer. *Expert.Opin.Pharmacother.*, 7, 1919-1930.

349. Day,J.M., Foster,P.A., Tutill,H.J., Parsons,M.F., Newman,S.P., Chander,S.K., Allan,G.M., Lawrence,H.R., Vicker,N., Potter,B.V., Reed,M.J., and Purohit,A. (2008) 17beta-hydroxysteroid dehydrogenase Type 1, and not Type 12, is a target for endocrine therapy of hormone-dependent breast cancer. *Int.J.Cancer*, 122, 1931-1940.

350. Saji,S. and Kuroi,K. (2008) Application of selective estrogen receptor modulators for breast cancer treatment according to their intrinsic nature. *Breast Cancer*, 15, 262-269.

351. Stanway,S.J., Purohit,A., Woo,L.W., Sufi,S., Vigushin,D., Ward,R., Wilson,R.H., Stanczyk,F.Z., Dobbs,N., Kulinskaya,E., Elliott,M., Potter,B.V., Reed,M.J., and Coombes,R.C. (2006) Phase I study of STX 64 (667 Coumate) in breast cancer patients: the first study of a steroid sulfatase inhibitor. *Clin.Cancer Res.*, 12, 1585-1592.

352. Wittmann,B.M., Sherk,A., and McDonnell,D.P. (2007) Definition of functionally important mechanistic differences among selective estrogen receptor down-regulators. *Cancer Res.*, 67, 9549-9560.

353. Barkhem,T., Carlsson,B., Nilsson,Y., Enmark,E., Gustafsson,J., and Nilsson,S. (1998) Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. *Mol.Pharmacol.*, 54, 105-112.

354. Klinge,C.M. (2001) Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res.*, 29, 2905-2919.

355. Shaaban,A.M., Green,A.R., Karthik,S., Alizadeh,Y., Hughes,T.A., Harkins,L., Ellis,I.O., Robertson,J.F., Paish,E.C., Saunders,P.T., Groome,N.P., and Speirs,V. (2008) Nuclear and cytoplasmic expression of ERbeta1, ERbeta2, and ERbeta5 identifies distinct prognostic outcome for breast cancer patients. *Clin.Cancer Res.*, 14, 5228-5235.

356. Novelli,F., Milella,M., Melucci,E., Di,B.A., Sperduti,I., Perrone-Donnorso,R., Perracchio,L., Venturo,I., Nistico,C., Fabi,A., Buglioni,S., Natali,P.G., and Mottolese,M. (2008) A divergent role for estrogen receptor-beta in node-positive and node-negative breast cancer classified according to molecular subtypes: an observational prospective study. *Breast Cancer Res.*, 10, R74.

357. Widschwendter,M., Lichtenberg-Frate,H., Hasenbrink,G., Schwarzer,S., Dawnay,A., Lam,A., Menon,U., Apostolidou,S., Raum,E., Stegmaier,C., Jacobs,I.J., and Brenner,H. (2009) Serum oestrogen receptor alpha and beta bioactivity are independently associated with breast cancer: a proof of principle study. *Br.J.Cancer*, 101, 160-165.

358. Markey,G.C., Cullen,R., Diggin,P., Hill,A.D., Mc Dermott,E.W., O'Higgins,N.J., and Duffy,M.J. (2009) Estrogen receptor-beta mRNA is associated with adverse outcome in patients with breast cancer. *Tumour.Biol.*, 30, 171-175.

359. Suzuki,T., Miki,Y., Moriya,T., Akahira,J., Hirakawa,H., Ohuchi,N., and Sasano,H. (2007) In situ production of sex steroids in human breast carcinoma. *Med.Mol.Morphol.*, 40, 121-127.

360. Suzuki,T., Miki,Y., Ohuchi,N., and Sasano,H. (2008) Intratumoral estrogen production in breast carcinoma: significance of aromatase. *Breast Cancer*, 15, 270-277.

361. Luczak,M.W. and Jagodzinski,P.P. (2009) Trichostatin A down-regulates CYP19 transcript and protein levels in MCF-7 breast cancer cells. *Biomed.Pharmacother.*, 63, 262-266.

362. Day,J.M., Tutill,H.J., Purohit,A., and Reed,M.J. (2008) Design and validation of specific inhibitors of 17beta-hydroxysteroid dehydrogenases for therapeutic application in breast and prostate cancer, and in endometriosis. *Endocr.Relat Cancer*, 15, 665-692.

363. Salhab,M., Reed,M.J., Al,S.W., Jiang,W.G., and Mokbel,K. (2006) The role of aromatase and 17-beta-hydroxysteroid dehydrogenase type 1 mRNA expression in predicting the clinical outcome of human breast cancer. *Breast Cancer Res.Treat.*, 99, 155-162.

364. Gunnarsson,C., Olsson,B.M., and Stal,O. (2001) Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. *Cancer Res.*, 61, 8448-8451.

365. Thomas,R.D., Green,M.R., Wilson,C., Weckle,A.L., Duanmu,Z., Kocarek,T.A., and Runge-Morris,M. (2006) Cytochrome P450 expression and metabolic activation of cooked food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-

b]pyridine (PhIP) in MCF10A breast epithelial cells. *Chem.Biol.Interact.*, 160, 204-216.

366. Wen,W., Ren,Z., Shu,X.O., Cai,Q., Ye,C., Gao,Y.T., and Zheng,W. (2007) Expression of cytochrome P450 1B1 and catechol-O-methyltransferase in breast tissue and their associations with breast cancer risk. *Cancer Epidemiol.Biomarkers Prev.*, 16, 917-920.

367. McKay,J.A., Melvin,W.T., Ah-See,A.K., Ewen,S.W., Greenlee,W.F., Marcus,C.B., Burke,M.D., and Murray,G.I. (1995) Expression of cytochrome P450 CYP1B1 in breast cancer. *FEBS Lett.*, 374, 270-272.

368. Nakajima,M., Iwanari,M., and Yokoi,T. (2003) Effects of histone deacetylation and DNA methylation on the constitutive and TCDD-inducible expressions of the human CYP1 family in MCF-7 and HeLa cells. *Toxicol.Lett.*, 144, 247-256.

369. Hooven,L.A., Mahadevan,B., Keshava,C., Johns,C., Pereira,C., Desai,D., Amin,S., Weston,A., and Baird,W.M. (2005) Effects of suberoylanilide hydroxamic acid and trichostatin A on induction of cytochrome P450 enzymes and benzo[a]pyrene DNA adduct formation in human cells. *Bioorg.Med.Chem.Lett.*, 15, 1283-1287.

370. Glatt,H. (2000) Sulfotransferases in the bioactivation of xenobiotics. *Chem.Biol.Interact.*, 129, 141-170.

371. Qian,Y.M., Sun,X.J., Tong,M.H., Li,X.P., Richa,J., and Song,W.C. (2001) Targeted disruption of the mouse estrogen sulfotransferase gene reveals a role

of estrogen metabolism in intracrine and paracrine estrogen regulation. *Endocrinology*, 142, 5342-5350.

372. Rivera,S.P., Saarikoski,S.T., and Hankinson,O. (2002) Identification of a novel dioxin-inducible cytochrome P450. *Mol.Pharmacol.*, 61, 255-259.

373. Runge-Morris,M. and Wilusz,J. (1994) Suppression of hydroxysteroid sulfotransferase-a gene expression by 3-methylcholanthrene. *Toxicol.Appl.Pharmacol.*, 125, 133-141.

374. Runge-Morris,M. (1998) Regulation of sulfotransferase gene expression by glucocorticoid hormones and xenobiotics in primary rat hepatocyte culture. *Chem.Biol.Interact.*, 109, 315-327.

375. Puga,A., Maier,A., and Medvedovic,M. (2000) The transcriptional signature of dioxin in human hepatoma HepG2 cells. *Biochem.Pharmacol.*, 60, 1129-1142.

376. Alnouti,Y. and Klaassen,C.D. (2008) Regulation of sulfotransferase enzymes by prototypical microsomal enzyme inducers in mice. *J.Pharmacol.Exp.Ther.*, 324, 612-621.

377. Cartharius,K., Frech,K., Grote,K., Klocke,B., Haltmeier,M., Klingenhoff,A., Frisch,M., Bayerlein,M., and Werner,T. (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics.*, 21, 2933-2942.

378. Gillett,C.E., Smith,P., Peters,G., Lu,X., and Barnes,D.M. (1999) Cyclin-dependent kinase inhibitor p27Kip1 expression and interaction with other cell cycle-associated proteins in mammary carcinoma. *J.Pathol.*, 187, 200-206.

379. Sadek,C.M. and len-Hoffmann,B.L. (1994) Cytochrome P450IA1 is rapidly induced in normal human keratinocytes in the absence of xenobiotics. *J.Biol.Chem.*, 269, 16067-16074.

380. Sadek,C.M. and len-Hoffmann,B.L. (1994) Suspension-mediated induction of Hepa 1c1c7 Cyp1a-1 expression is dependent on the Ah receptor signal transduction pathway. *J.Biol.Chem.*, 269, 31505-31509.

381. Monk,S.A., Denison,M.S., and Rice,R.H. (2001) Transient expression of CYP1A1 in rat epithelial cells cultured in suspension. *Arch.Biochem.Biophys.*, 393, 154-162.

382. Cho,Y.C., Zheng,W., and Jefcoate,C.R. (2004) Disruption of cell-cell contact maximally but transiently activates AhR-mediated transcription in 10T1/2 fibroblasts. *Toxicol.Appl.Pharmacol.*, 199, 220-238.

383. Ikuta,T., Kobayashi,Y., and Kawajiri,K. (2004) Cell density regulates intracellular localization of aryl hydrocarbon receptor. *J.Biol.Chem.*, 279, 19209-19216.

384. Santini,R.P., Myrand,S., Elferink,C., and Reiners,J.J., Jr. (2001) Regulation of Cyp1a1 induction by dioxin as a function of cell cycle phase. *J.Pharmacol.Exp.Ther.*, 299, 718-728.

385. Safe,S. and Wormke,M. (2003) Inhibitory aryl hydrocarbon receptor-estrogen receptor alpha cross-talk and mechanisms of action. *Chem.Res.Toxicol.*, 16, 807-816.

386. Klinge,C.M., Kaur,K., and Swanson,H.I. (2000) The aryl hydrocarbon receptor interacts with estrogen receptor alpha and orphan receptors COUP-TFI and ERRalpha1. *Arch.Biochem.Biophys.*, 373, 163-174.

387. Tian,Y., Ke,S., Denison,M.S., Rabson,A.B., and Gallo,M.A. (1999) Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity. *J.Biol.Chem.*, 274, 510-515.

388. Vogel,C.F., Sciullo,E., and Matsumura,F. (2007) Involvement of RelB in aryl hydrocarbon receptor-mediated induction of chemokines. *Biochem.Biophys.Res.Commun.*, 363, 722-726.

389. Ge,N.L. and Elferink,C.J. (1998) A direct interaction between the aryl hydrocarbon receptor and retinoblastoma protein. Linking dioxin signaling to the cell cycle. *J.Biol.Chem.*, 273, 22708-22713.

390. Puga,A., Ma,C., and Marlowe,J.L. (2009) The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. *Biochem.Pharmacol.*, 77, 713-722.

391. Takemoto,K., Nakajima,M., Fujiki,Y., Katoh,M., Gonzalez,F.J., and Yokoi,T. (2004) Role of the aryl hydrocarbon receptor and Cyp1b1 in the antiestrogenic activity of 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Arch.Toxicol.*, 78, 309-315.

392. Rogers,J.M. and Denison,M.S. (2002) Analysis of the antiestrogenic activity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in human ovarian carcinoma BG-1 cells. *Mol.Pharmacol.*, 61, 1393-1403.

393. Krishnan,V., Porter,W., Santostefano,M., Wang,X., and Safe,S. (1995) Molecular mechanism of inhibition of estrogen-induced cathepsin D gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in MCF-7 cells. *Mol.Cell Biol.*, 15, 6710-6719.

394. Khan,S., Barhoumi,R., Burghardt,R., Liu,S., Kim,K., and Safe,S. (2006) Molecular mechanism of inhibitory aryl hydrocarbon receptor-estrogen receptor/Sp1 cross talk in breast cancer cells. *Mol.Endocrinol.*, 20, 2199-2214.

395. Tian,Y., Ke,S., Thomas,T., Meeker,R.J., and Gallo,M.A. (1998) Transcriptional suppression of estrogen receptor gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *J.Steroid Biochem.Mol.Biol.*, 67, 17-24.

396. Wormke,M., Stoner,M., Saville,B., Walker,K., Abdelrahim,M., Burghardt,R., and Safe,S. (2003) The aryl hydrocarbon receptor mediates degradation of estrogen receptor alpha through activation of proteasomes. *Mol.Cell Biol.*, 23, 1843-1855.

397. Ruegg,J., Swedenborg,E., Wahlstrom,D., Escande,A., Balaguer,P., Pettersson,K., and Pongratz,I. (2008) The transcription factor aryl hydrocarbon receptor nuclear translocator functions as an estrogen receptor beta-selective coactivator, and its recruitment to alternative pathways mediates antiestrogenic effects of dioxin. *Mol.Endocrinol.*, 22, 304-316.

398. Ohtake,F., Takeyama,K., Matsumoto,T., Kitagawa,H., Yamamoto,Y., Nohara,K., Tohyama,C., Krust,A., Mimura,J., Chambon,P., Yanagisawa,J., Fujii-Kuriyama,Y., and Kato,S. (2003) Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature*, 423, 545-550.

399. Brosens,J.J. and Parker,M.G. (2003) Gene expression: Oestrogen receptor hijacked. *Nature*, 423, 487-488.

400. Shipley,J.M. and Waxman,D.J. (2006) Aryl hydrocarbon receptor-independent activation of estrogen receptor-dependent transcription by 3-methylcholanthrene. *Toxicol.Appl.Pharmacol.*, 213, 87-97.

401. Abdelrahim,M., Ariazi,E., Kim,K., Khan,S., Barhoumi,R., Burghardt,R., Liu,S., Hill,D., Finnell,R., Wlodarczyk,B., Jordan,V.C., and Safe,S. (2006) 3-Methylcholanthrene and other aryl hydrocarbon receptor agonists directly activate estrogen receptor alpha. *Cancer Res.*, 66, 2459-2467.

402. Boverhof,D.R., Kwekel,J.C., Humes,D.G., Burgoon,L.D., and Zacharewski,T.R. (2006) Dioxin induces an estrogen-like, estrogen receptor-dependent gene expression response in the murine uterus. *Mol.Pharmacol.*, 69, 1599-1606.

403. Baba,T., Mimura,J., Nakamura,N., Harada,N., Yamamoto,M., Morohashi,K., and Fujii-Kuriyama,Y. (2005) Intrinsic function of the aryl hydrocarbon (dioxin) receptor as a key factor in female reproduction. *Mol.Cell Biol.*, 25, 10040-10051.

404. (1986) Tobacco smoking. *IARC Monogr Eval.Carcinog.Risk Chem.Hum.*, 38, 35-394.

405. Fuchs,C.S., Colditz,G.A., Stampfer,M.J., Giovannucci,E.L., Hunter,D.J., Rimm,E.B., Willett,W.C., and Speizer,F.E. (1996) A prospective study of cigarette smoking and the risk of pancreatic cancer. *Arch.Intern.Med.*, 156, 2255-2260.

406. Brennan,P., Bogillot,O., Cordier,S., Greiser,E., Schill,W., Vineis,P., Lopez-Abente,G., Tzonou,A., Chang-Claude,J., Bolm-Audorff,U., Jockel,K.H., Donato,F., Serra,C., Wahrendorf,J., Hours,M., T'Mannetje,A., Kogevinas,M., and Boffetta,P. (2000) Cigarette smoking and bladder cancer in men: a pooled analysis of 11 case-control studies. *Int.J.Cancer*, 86, 289-294.

407. Nakamura,K., Nagata,C., Wada,K., Tamai,Y., Tsuji,M., Takatsuka,N., and Shimizu,H. (2011) Cigarette smoking and other lifestyle factors in relation to the risk of pancreatic cancer death: a prospective cohort study in Japan. *Jpn.J.Clin.Oncol.*, 41, 225-231.

408. Peto,R., Lopez,A.D., Boreham,J., Thun,M., Heath,C., Jr., and Doll,R. (1996) Mortality from smoking worldwide. *Br.Med.Bull.*, 52, 12-21.

409. Doll,R. (1998) Epidemiological evidence of the effects of behaviour and the environment on the risk of human cancer. *Recent Results Cancer Res.*, 154, 3-21.

410. Burns,D.M. (2003) Epidemiology of smoking-induced cardiovascular disease. *Prog.Cardiovasc.Dis.*, 46, 11-29.

411. el-Bayoumy,K. (1992) Environmental carcinogens that may be involved in human breast cancer etiology. *Chem.Res.Toxicol.*, 5, 585-590.

412. Vainio,H., Weiderpass,E., and Kleihues,P. (2001) Smoking cessation in cancer prevention. *Toxicology*, 166, 47-52.

413. Smith,C.J., Perfetti,T.A., Garg,R., and Hansch,C. (2003) IARC carcinogens reported in cigarette mainstream smoke and their calculated log P values. *Food Chem.Toxicol.*, 41, 807-817.

414. Shu,H.P. and Bymun,E.N. (1983) Systemic excretion of benzo(a)pyrene in the control and microsomally induced rat: the influence of plasma lipoproteins and albumin as carrier molecules. *Cancer Res.*, 43, 485-490.

415. Plant,A.L., Benson,D.M., and Smith,L.C. (1985) Cellular uptake and intracellular localization of benzo(a)pyrene by digital fluorescence imaging microscopy. *J.Cell Biol.*, 100, 1295-1308.

416. Petrakis,N.L., Gruenke,L.D., Beelen,T.C., Castagnoli,N., Jr., and Craig,J.C. (1978) Nicotine in breast fluid of nonlactating women. *Science*, 199, 303-305.

417. Petrakis,N.L., Maack,C.A., Lee,R.E., and Lyon,M. (1980) Mutagenic activity in nipple aspirates of human breast fluid. *Cancer Res.*, 40, 188-189.

418. Bartsch,H. (1996) DNA adducts in human carcinogenesis: etiological relevance and structure-activity relationship. *Mutat.Res.*, 340, 67-79.

419. La,D.K. and Swenberg,J.A. (1996) DNA adducts: biological markers of exposure and potential applications to risk assessment. *Mutat.Res.*, 365, 129-146.

420. Zhao,C., Tyndyk,M., Eide,I., and Hemminki,K. (1999) Endogenous and background DNA adducts by methylating and 2-hydroxyethylating agents. *Mutat.Res.*, 424, 117-125.

421. Seo,K.Y., Jelinsky,S.A., and Loechler,E.L. (2000) Factors that influence the mutagenic patterns of DNA adducts from chemical carcinogens. *Mutat.Res.*, 463, 215-246.

422. Hecht,S.S., McIntee,E.J., and Wang,M. (2001) New DNA adducts of crotonaldehyde and acetaldehyde. *Toxicology*, 166, 31-36.

423. Siriwardhana,N. and Wang,H.C. (2008) Precancerous carcinogenesis of human breast epithelial cells by chronic exposure to benzo[a]pyrene. *Mol.Carcinog.*, 47, 338-348.

424. Millikan,R.C., Pittman,G.S., Newman,B., Tse,C.K., Selmin,O., Rockhill,B., Savitz,D., Moorman,P.G., and Bell,D.A. (1998) Cigarette smoking, N-acetyltransferases 1 and 2, and breast cancer risk. *Cancer Epidemiol.Biomarkers Prev.*, 7, 371-378.

425. Conway,K., Edmiston,S.N., Cui,L., Drouin,S.S., Pang,J., He,M., Tse,C.K., Geradts,J., Dressler,L., Liu,E.T., Millikan,R., and Newman,B. (2002) Prevalence and spectrum of p53 mutations associated with smoking in breast cancer. *Cancer Res.*, 62, 1987-1995.

426. Coles,B.F. and Kadlubar,F.F. (2005) Human alpha class glutathione S-transferases: genetic polymorphism, expression, and susceptibility to disease. *Methods Enzymol.*, 401, 9-42.

427. Bradbury,B.D., Wilk,J.B., Aschengrau,A., and Lash,T.L. (2006) Departure from multiplicative interaction for catechol-O-methyltransferase genotype and active/passive exposure to tobacco smoke among women with breast cancer. *J.Carcinog.*, 5, 3.

428. Roos,P.H. and Bolt,H.M. (2005) Cytochrome P450 interactions in human cancers: new aspects considering CYP1B1. *Expert.Opin.Drug Metab Toxicol.*, 1, 187-202.

429. Alexandrov,K., Rojas,M., and Rolando,C. (2006) DNA damage by benzo(a)pyrene in human cells is increased by cigarette smoke and decreased by a filter containing rosemary extract, which lowers free radicals. *Cancer Res.*, 66, 11938-11945.

430. Kelsey,J.L., LiVolsi,V.A., Holford,T.R., Fischer,D.B., Mostow,E.D., Schwartz,P.E., O'Connor,T., and White,C. (1982) A case-control study of cancer of the endometrium. *Am.J.Epidemiol.*, 116, 333-342.

431. MacMahon,B., Trichopoulos,D., Cole,P., and Brown,J. (1982) Cigarette smoking and urinary estrogens. *N.Engl.J.Med.*, 307, 1062-1065.

432. Baron,J.A. (1984) Smoking and estrogen-related disease. *Am.J.Epidemiol.*, 119, 9-22.

433. Smith,E.M., Sowers,M.F., and Burns,T.L. (1984) Effects of smoking on the development of female reproductive cancers. *J.Natl.Cancer Inst.*, 73, 371-376.

434. Martin,M.B., Reiter,R., Johnson,M., Shah,M.S., Iann,M.C., Singh,B., Richards,J.K., Wang,A., and Stoica,A. (2007) Effects of tobacco smoke condensate on estrogen receptor-alpha gene expression and activity. *Endocrinology*, 148, 4676-4686.

435. Meek,M.D. and Finch,G.L. (1999) Diluted mainstream cigarette smoke condensates activate estrogen receptor and aryl hydrocarbon receptor-mediated gene transcription. *Environ.Res.*, 80, 9-17.

436. Garcia-Morales,P., Saceda,M., Kenney,N., Kim,N., Salomon,D.S., Gottardis,M.M., Solomon,H.B., Sholler,P.F., Jordan,V.C., and Martin,M.B. (1994) Effect of cadmium on estrogen receptor levels and estrogen-induced responses in human breast cancer cells. *J.Biol.Chem.*, 269, 16896-16901.

437. Lipworth,L. (1995) Epidemiology of breast cancer. *Eur.J.Cancer Prev.*, 4, 7-30.

438. Key,T.J., Verkasalo,P.K., and Banks,E. (2001) Epidemiology of breast cancer. *Lancet Oncol.*, 2, 133-140.

439. Whitlock,J.P., Jr. (1999) Induction of cytochrome P4501A1. *Annu.Rev.Pharmacol.Toxicol.*, 39, 103-125.

440. Shimada,T., Yun,C.H., Yamazaki,H., Gautier,J.C., Beaune,P.H., and Guengerich,F.P. (1992) Characterization of human lung microsomal cytochrome

P-450 1A1 and its role in the oxidation of chemical carcinogens. *Mol.Pharmacol.*, 41, 856-864.

441. Lu,Y.F., Santostefano,M., Cunningham,B.D., Threadgill,M.D., and Safe,S. (1995) Identification of 3'-methoxy-4'-nitroflavone as a pure aryl hydrocarbon (Ah) receptor antagonist and evidence for more than one form of the nuclear Ah receptor in MCF-7 human breast cancer cells. *Arch.Biochem.Biophys.*, 316, 470-477.

442. Lichtenstein,P., Holm,N.V., Verkasalo,P.K., Iliadou,A., Kaprio,J., Koskenvuo,M., Pukkala,E., Skytthe,A., and Hemminki,K. (2000) Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *N.Engl.J.Med.*, 343, 78-85.

443. O'Brien,J.M. (2000) Environmental and heritable factors in the causation of cancer: analyses of cohorts of twins from Sweden, Denmark, and Finland, by P. Lichtenstein, N.V. Holm, P.K. Verkasalo, A. Iliadou, J. Kaprio, M. Koskenvuo, E. Pukkala, A. Skytthe, and K. Hemminki. N Engl J Med 343:78-84, 2000. *Surv.Ophthalmol.*, 45, 167-168.

444. Stratton,M.R. and Rahman,N. (2008) The emerging landscape of breast cancer susceptibility. *Nat.Genet.*, 40, 17-22.

445. Bieche,I. and Lidereau,R. (2011) Genome-based and transcriptome-based molecular classification of breast cancer. *Curr.Opin.Oncol.*, 23, 93-99.

446. Laden,F. and Hunter,D.J. (1998) Environmental risk factors and female breast cancer. *Annu.Rev.Public Health*, 19, 101-123.

447. John,E.M. and Kelsey,J.L. (1993) Radiation and other environmental exposures and breast cancer. *Epidemiol.Rev.*, 15, 157-162.

448. Lewis-Michl,E.L., Melius,J.M., Kallenbach,L.R., Ju,C.L., Talbot,T.O., Orr,M.F., and Lauridsen,P.E. (1996) Breast cancer risk and residence near industry or traffic in Nassau and Suffolk Counties, Long Island, New York. *Arch.Environ.Health*, 51, 255-265.

449. Hoffmann,D., Hoffmann,I., and el-Bayoumy,K. (2001) The less harmful cigarette: a controversial issue. a tribute to Ernst L. Wynder. *Chem.Res.Toxicol.*, 14, 767-790.

450. Russo,I.H. (2002) Cigarette smoking and risk of breast cancer in women. *Lancet*, 360, 1033-1034.

451. Terry,P.D., Miller,A.B., and Rohan,T.E. (2002) Cigarette smoking and breast cancer risk: a long latency period? *Int.J.Cancer*, 100, 723-728.

452. Innes,K.E. and Byers,T.E. (2001) Smoking during pregnancy and breast cancer risk in very young women (United States). *Cancer Causes Control*, 12, 179-185.

453. Murin,S. and Inciardi,J. (2001) Cigarette smoking and the risk of pulmonary metastasis from breast cancer. *Chest*, 119, 1635-1640.

454. Band,P.R., Le,N.D., Fang,R., and Deschamps,M. (2002) Carcinogenic and endocrine disrupting effects of cigarette smoke and risk of breast cancer. *Lancet*, 360, 1044-1049.

455. Lash,T.L. and Aschengrau,A. (1999) Active and passive cigarette smoking and the occurrence of breast cancer. *Am.J.Epidemiol.*, 149, 5-12.

456. Nagata,C., Mizoue,T., Tanaka,K., Tsuji,I., Wakai,K., Inoue,M., and Tsugane,S. (2006) Tobacco smoking and breast cancer risk: an evaluation based on a systematic review of epidemiological evidence among the Japanese population. *Jpn.J.Clin.Oncol.*, 36, 387-394.

457. Palmer,J.R. and Rosenberg,L. (1993) Cigarette smoking and the risk of breast cancer. *Epidemiol.Rev.*, 15, 145-156.

458. Lash,T.L. and Aschengrau,A. (2002) A null association between active or passive cigarette smoking and breast cancer risk. *Breast Cancer Res.Treat.*, 75, 181-184.

459. Holden,W.E., Maier,J.M., and Malinow,M.R. (1989) Cigarette smoke extract increases albumin flux across pulmonary endothelium in vitro. *J.Appl.Physiol*, 66, 443-449.

460. Rowlands,J.C. and Gustafsson,J.A. (1997) Aryl hydrocarbon receptor-mediated signal transduction. *Crit Rev.Toxicol.*, 27, 109-134.

461. Gebremichael,A., Tullis,K., Denison,M.S., Cheek,J.M., and Pinkerton,K.E. (1996) Ah-receptor-dependent modulation of gene expression by aged and diluted sidestream cigarette smoke. *Toxicol.Appl.Pharmacol.*, 141, 76-83.

462. Lofroth,G. and Rannug,A. (1988) Ah receptor ligands in tobacco smoke. *Toxicol.Lett.*, 42, 131-136.

463. Graziano,M.J. and Dorough,H.W. (1984) Effect of cigarette smoking on hepatic biotransformations in rats. *Toxicol.Appl.Pharmacol.*, 75, 229-239.

464. Shimizu,Y., Nakatsuru,Y., Ichinose,M., Takahashi,Y., Kume,H., Mimura,J., Fujii-Kuriyama,Y., and Ishikawa,T. (2000) Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. *Proc.Natl.Acad.Sci.U.S.A*, 97, 779-782.

465. Marshall,S.F., Clarke,C.A., Deapen,D., Henderson,K., Largent,J., Neuhausen,S.L., Reynolds,P., Ursin,G., Horn-Ross,P.L., Stram,D.O., Templeman,C., and Bernstein,L. (2010) Recent breast cancer incidence trends according to hormone therapy use: the California Teachers Study cohort. *Breast Cancer Res.*, 12, R4.

466. Chlebowski,R.T., Kuller,L.H., Prentice,R.L., Stefanick,M.L., Manson,J.E., Gass,M., Aragaki,A.K., Ockene,J.K., Lane,D.S., Sarto,G.E., Rajkovic,A., Schenken,R., Hendrix,S.L., Ravdin,P.M., Rohan,T.E., Yasmeen,S., and Anderson,G. (2009) Breast cancer after use of estrogen plus progestin in postmenopausal women. *N.Engl.J.Med.*, 360, 573-587.

467. Glass,A.G., Lacey,J.V., Jr., Carreon,J.D., and Hoover,R.N. (2007) Breast cancer incidence, 1980-2006: combined roles of menopausal hormone therapy, screening mammography, and estrogen receptor status. *J.Natl.Cancer Inst.*, 99, 1152-1161.

468. Robbins,A.S. and Clarke,C.A. (2007) Regional changes in hormone therapy use and breast cancer incidence in California from 2001 to 2004. *J.Clin.Oncol.*, 25, 3437-3439.

469. Breen,N., Cronin,A., Meissner,H.I., Taplin,S.H., Tangka,F.K., Tiro,J.A., and McNeel,T.S. (2007) Reported drop in mammography : is this cause for concern? *Cancer*, 109, 2405-2409.

470. Ravdin,P.M., Cronin,K.A., Howlader,N., Berg,C.D., Chlebowski,R.T., Feuer,E.J., Edwards,B.K., and Berry,D.A. (2007) The decrease in breast-cancer incidence in 2003 in the United States. *N.Engl.J.Med.*, 356, 1670-1674.

471. Chalbos,D., Vignon,F., Keydar,I., and Rochefort,H. (1982) Estrogens stimulate cell proliferation and induce secretory proteins in a human breast cancer cell line (T47D). *J.Clin.Endocrinol.Metab*, 55, 276-283.

472. Welshons,W.V., Rottinghaus,G.E., Nonneman,D.J., Dolan-Timpe,M., and Ross,P.F. (1990) A sensitive bioassay for detection of dietary estrogens in animal feeds. *J.Vet.Diagn.Invest*, 2, 268-273.

473. Hayashi,S. and Yamaguchi,Y. (2008) Estrogen signaling in cancer microenvironment and prediction of response to hormonal therapy. *J.Steroid Biochem.Mol.Biol.*, 109, 201-206.

474. Prausova,J., Eckschlager,T., Mrhalova,M., Slobodnik,A., Kubackova,K., Vinakurau,S., and Kodet,R. (2007) Clinical, pathological and molecular characteristics of newly diagnosed breast cancers. *Neoplasma*, 54, 407-412.

475. Lonning,P.E. (1990) New endocrine drugs for treatment of advanced breast cancer. *Acta Oncol.*, 29, 379-386.

476. Lonning,P.E. (2004) Oestrogen suppression--lessons from clinical studies. *Best.Pract.Res.Clin.Endocrinol.Metab*, 18, 33-45.

477. Liehr,J.G. (2000) Is estradiol a genotoxic mutagenic carcinogen? *Endocr.Rev.*, 21, 40-54.

478. Shekhar,M.P., Werdell,J., and Tait,L. (2000) Interaction with endothelial cells is a prerequisite for branching ductal-alveolar morphogenesis and hyperplasia of preneoplastic human breast epithelial cells: regulation by estrogen. *Cancer Res.*, 60, 439-449.

479. Miller,B.E., McInerney,D., Jackson,D., and Miller,F.R. (1986) Metabolic cooperation between mouse mammary tumor subpopulations in three-dimensional collagen gel cultures. *Cancer Res.*, 46, 89-93.

480. Shekhar,M.P., Werdell,J., Santner,S.J., Pauley,R.J., and Tait,L. (2001) Breast stroma plays a dominant regulatory role in breast epithelial growth and differentiation: implications for tumor development and progression. *Cancer Res.*, 61, 1320-1326.

481. Kanemura,Y., Mori,H., Kobayashi,S., Islam,O., Kodama,E., Yamamoto,A., Nakanishi,Y., Arita,N., Yamasaki,M., Okano,H., Hara,M., and Miyake,J. (2002) Evaluation of in vitro proliferative activity of human fetal neural stem/progenitor cells using indirect measurements of viable cells based on cellular metabolic activity. *J.Neurosci.Res.*, 69, 869-879.

482. Plumb,J.A., Milroy,R., and Kaye,S.B. (1989) Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. *Cancer Res.*, 49, 4435-4440.

483. Deuschle,U., Meyer,W.K., and Thiesen,H.J. (1995) Tetracycline-reversible silencing of eukaryotic promoters. *Mol.Cell Biol.*, 15, 1907-1914.

484. Anders,C. and Carey,L.A. (2008) Understanding and treating triple-negative breast cancer. *Oncology (Williston.Park)*, 22, 1233-1239.

485. Curran,E.M., Judy,B.M., Duru,N.A., Wang,H.Q., Vergara,L.A., Lubahn,D.B., and Estes,D.M. (2006) Estrogenic regulation of host immunity against an estrogen receptor-negative human breast cancer. *Clin.Cancer Res.*, 12, 5641-5647.

486. Friedl,A. and Jordan,V.C. (1994) Oestradiol stimulates growth of oestrogen receptor-negative MDA-MB-231 breast cancer cells in immunodeficient mice by reducing cell loss. *Eur.J.Cancer*, 30A, 1559-1564.

487. (1992) Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Early Breast Cancer Trialists' Collaborative Group. *Lancet*, 339, 71-85.

488. Rebbeck,T.R. (2000) Prophylactic oophorectomy in BRCA1 and BRCA2 mutation carriers. *J.Clin.Oncol.*, 18, 100S-103S.

489. Chappuis,P.O., Nethercot,V., and Foulkes,W.D. (2000) Clinico-pathological characteristics of B. *Semin.Surg.Oncol.*, 18, 287-295.

490. Putti,T.C., El-Rehim,D.M., Rakha,E.A., Paish,C.E., Lee,A.H., Pinder,S.E., and Ellis,I.O. (2005) Estrogen receptor-negative breast carcinomas: a review of morphology and immunophenotypical analysis. *Mod.Pathol.*, 18, 26-35.

491. Choi,J.Y., Lee,K.M., Park,S.K., Noh,D.Y., Ahn,S.H., Chung,H.W., Han,W., Kim,J.S., Shin,S.G., Jang,I.J., Yoo,K.Y., Hirvonen,A., and Kang,D. (2005) Genetic polymorphisms of SULT1A1 and SULT1E1 and the risk and survival of breast cancer. *Cancer Epidemiol.Biomarkers Prev.*, 14, 1090-1095.

492. Hui,Y., Yasuda,S., Liu,M.Y., Wu,Y.Y., and Liu,M.C. (2008) On the sulfation and methylation of catecholestrogens in human mammary epithelial cells and breast cancer cells. *Biol.Pharm.Bull.*, 31, 769-773.

493. Harvell,D.M., Richer,J.K., Allred,D.C., Sartorius,C.A., and Horwitz,K.B. (2006) Estradiol regulates different genes in human breast tumor xenografts compared with the identical cells in culture. *Endocrinology*, 147, 700-713.

494. Rio,M.C., Bellocq,J.P., Gairard,B., Rasmussen,U.B., Krust,A., Koehl,C., Calderoli,H., Schiff,V., Renaud,R., and Chambon,P. (1987) Specific expression of the pS2 gene in subclasses of breast cancers in comparison with expression of the estrogen and progesterone receptors and the oncogene ERBB2. *Proc.Natl.Acad.Sci.U.S.A*, 84, 9243-9247.

495. Zajchowski,D., Band,V., Pauzie,N., Tager,A., Stampfer,M., and Sager,R. (1988) Expression of growth factors and oncogenes in normal and tumor-derived human mammary epithelial cells. *Cancer Res.*, 48, 7041-7047.

496. Russo,J., Fernandez,S.V., Russo,P.A., Fernbaugh,R., Sheriff,F.S., Lareef,H.M., Garber,J., and Russo,I.H. (2006) 17-Beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells. *FASEB J.*, 20, 1622-1634.

ABSTRACT

ESTROGEN SULFOTRANSFERASE (SULT1E1) EXPRESSION AND FUNCTION IN MCF10A-SERIES BREAST EPITHELIAL CELLS: ROLE AS A MODIFIER OF BREAST CARCINOGENESIS AND REGULATION BY PROLIFERATION STATE

by

JIAQI FU

August 2011

Advisor: Dr. Melissa Runge-Morris

Major: Molecular and Cellular Toxicology

Degree: Doctor of Philosophy

Estrogen sulfotransferase (SULT1E1) catalyzes the sulfonation of estrogens, which limits estrogen mitogenicity. TaqMan Gene Expression assays were used to profile the mRNA expression of estrogen receptor (ERα and ERβ) and estrogen metabolism enzymes including cytosolic sulfotransferases (SULT1E1, SULT1A1, SULT2A1, and SULT2B1), steroid sulfatase (STS), aromatase (CYP19), 17β-hydroxysteroid dehydrogenases (17βHSD1 and 2), CYP1B1, and COMT in an MCF10A-derived lineage cell culture model for basal-like human breast cancer progression and in ERα-positive luminal MCF7 breast cancer cells. Low levels of ERα and ERβ mRNA were present in MCF10A-derived cell lines. SULT1E1 mRNA was more abundant in confluent relative to pre-confluent MCF10A cells, a non-tumorigenic proliferative breast disease cell line. SULT1E1 was also expressed in preneoplastic MCF10AT1 and MCF10AT1K.cl2 cells, but was markedly repressed in neoplastic MCF10A-derived cell lines as well as in MCF7 cells. Steroid-metabolizing enzymes

SULT1A1 and SULT2B1 were only expressed in MCF7 cells. STS and COMT were widely detected across cell lines. Pro-estrogenic 17βHSD1 mRNA was most abundant in neoplastic MCF10CA1a and MCF10DCIS.com cells, while 17βHSD2 mRNA was more prominent in parental MCF10A cells. CYP1B1 mRNA was most abundant in MCF7 cells. Treatment with the histone deacetylase inhibitor TSA induced SULT1E1 and CYP19 mRNA but suppressed CYP1B1, STS, COMT, 17βHSD1, and 17βHSD2 mRNA in MCF10A lineage cell lines. In MCF7 cells, TSA treatment suppressed ERα, CYP1B1, STS, COMT, SULT1A1, and SULT2B1 but induced ERβ, CYP19 and SULT2A1 mRNA expression. The results indicate that relative to the MCF7 breast cancer cell line, key determinants of breast estrogen metabolism are differentially regulated in the MCF10A-derived lineage model for breast cancer progression.

We recently reported that SULT1E1 expression is low in pre-confluent MCF10A breast epithelial cells but increases when the cells become confluent. Pulse-chase labeling experiments with bromouridine demonstrated that the confluence-mediated increase in SULT1E1 expression was due to increased mRNA synthesis. Because AhR activation has been shown to suppress SULT1E1 expression and loss of cell-cell contact has been shown to activate AhR in other cell types, we tested whether the confluency-associated changes in SULT1E1 expression were mediated by the AhR. Relative to confluent MCF10A cells, pre-confluent cells had higher levels of CYP1A1 mRNA and greater activation of an AhR-responsive luciferase reporter, demonstrating that AhR was active in the pre-confluent cells. AhR and ARNT mRNA and protein levels were also higher in pre-confluent than in confluent cultures. Treatment of pre-confluent cells with the AhR antagonist, MNF, or AhR knockdown significantly increased

SULT1E1 expression. MCF10A cells stably transfected with a luciferase reporter containing ~7 kb of the SULT1E1 5'-flanking region showed both MNF- and confluence-inducible luciferase expression. Pre-confluent cells transiently transfected with the reporter showed both MNF treatment- and AhR knockdown-mediated luciferase induction, but mutation of a computationally predicted dioxin response element (DRE) at nt -3476 did not attenuate these effects. These results demonstrate that SULT1E1 expression in MCF10A cells is transcriptionally regulated by confluency through a suppressive action of the AhR, which is not mediated through a DRE at nt -3476.

Tobacco smoke has been implicated as a risk factor for breast cancer. We evaluated the effect of TSC on expression of the estrogen-inactivating enzyme SULT1E1 in the MCF10A human breast epithelial cell line. Because TSC contains components that are known AhR agonists, effects of TSC treatment were compared to those of TCDD, and effects on SULT1E1 expression were compared to those on CYP1A1. Treatment for 24-72 h with 0.05-10 μg/ml TSC produced concentration-dependent increases in CYP1A1 mRNA content, decreases in SULT1E1 mRNA content, and increases in expression from a transfected AhR-responsive reporter plasmid. Treatment with 10 μg/ml TSC and 30 nM TCDD produced comparable increases in CYP1A1 mRNA levels (~300-fold) and decreases in SULT1E1 mRNA levels (~90%). Treatment with the AhR antagonist 3'-methyl-4'-nitroflavone (MNF, at 1 uM) completely inhibited TCDD-inducible CYP1A1 expression and partially reversed TCDD-mediated SULT1E1 suppression. MNF also inhibited TSC-mediated CYP1A1 induction and SULT1E1 suppression as indicated by rightward shifts of the TSC concentration-response curves. These findings

support a role for the AhR in TSC-mediated regulation of CYP1A1 and SULT1E1 expression in human breast epithelial cells.

Despite a growing understanding of SULT1E1's function in steroid hormone and drug metabolism, the role of SULT1E1 in the malignant progression of breast epithelial cells is still unknown. In this research, MCF10AT1 cells that have preserved SULT1E1 expression and E2-ER regulative growth kinetics was used to determine the impact of SULT1E1 expression on tumorigenicity. The effect of SULT1E1 silencing in pre-neoplastic MCF10AT1 cells on tumorigenicity was studied in nude mice xenografts model. The *in vivo* and *in vitro* SULT1E1 silencing was confirmed by real-time RT-PCR, western blot hybridization and immunohistochemistry staining. This SULT1E1 knockdown increases the cumulative incidence of xenograft lesions formation (from 64% in the control group to 92% in the doxycycline treated group), cellularity and histological grades of xenografts in immunodeficient mice. Moreover, the expression of estrogen responsive gene pS2 was low in control group but was significantly increased in SULT1E1 knockdown group. These findings indicate that SULT1E1 down regulation activate estrogen responsive gene pS2 by increasing bioactive estrogen level.

AUTOBIOGRAPHICAL STATEMENT

JIAQI FU

EDUCATION: **Master of Science in Beijing Normal University (August, 2004)**

AWARDS:

Experimental Biology Student Travel Award, Experimental Biology 2008

University Dissertation Fellowship, Wayne State University, winter 2011

PUBLICATIONS:

- **Regulation of SULT1E1 Expression by Confluency of MCF10A Breast Epithelial Cells: Role of the Aryl Hydrocarbon Receptor.** **Fu, J.**, Fang, H., Paulsen, M., Ljungman, M., Kocarek, T.A. and Runge-Morris, M. (Accepted)
- **Expression of estrogenicity genes in a lineage cell culture model of human breast cancer progression.** **Fu, J.**, Weise, A.M., Falany, J.L., Falany, C.N., Thibodeau, B.J., Miller, F.R., Kocarek, T.A. and Runge-Morris, M. *Breast Cancer Res.Treat*., 120: 35-45, 2010.
- **Positive and negative regulation of human hepatic hydroxysteroid sulfotransferase (SULT2A1) gene transcription by rifampicin: Roles of hepatocyte nuclear factor 4α and pregnane X receptor.** Fang HL, Strom SC, Ellis E, Duanmu Z, **Fu J.**, Duniec-Dmuchowski Z, Falany CN, Falany JL, Kocarek TA and Runge-Morris M. *J Pharmacol Exper Ther* 323:586-598, 2007.
- **Characterization of a Novel Human gene Acbp5 cDNA.** Wang Luan, **Fu Jia-qi**, Chen Wei, Ci Hongliang, Li Yi-ping. *Chinese J Biochem & Mol Bio* 21, 2005.
- **Cloning and characterization of a novel Human Ceg1 gene cDNA.** **Fu J.**, Wang L, Chen W, Ci HL and Li YP. *Shi Yan Sheng Wu Xue Bao*, *37:* 409-417, 2004.