AD_____

Award Number: DAMD17-03-1-0229

TITLE: Estrogen-Induced Depurination of DNA: A Novel Target for Breast Cancer Prevention

PRINCIPAL INVESTIGATOR: Ercole L. Cavalieri

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, NE 68198-6805

REPORT DATE: May 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-05-2007	Annual	7 Apr 2006 – 6 Apr 2007

4. TITLE AND SUBTITLE

Estrogen-Induced Depurination of DNA: A Novel Target for Breast Cancer Prevention

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0229

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Ercole L. Cavalieri

Email: ecavalie@unmc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Nebraska Medical Center
Omaha, NE 68198-6805

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

The research conducted in this COE grant is based on the paradigm that estrogens can become endogenous carcinogens when their metabolism is unbalanced, favoring formation of catechol estrogen quinones and their reaction with DNA. Compelling evidence obtained in the various specific aims of this COE will be decisive for determining the risk of breast cancer by using the depurinating estrogen-DNA adducts as biomarkers. These biomarkers will also be used for evaluating the ability of specific antioxidants to prevent breast cancer initiation. Much of the research accomplished by this COE was published in a review article in BBA-Reviews in Cancer, which was co-authored by all the participants of the COE.

15. SUBJECT TERMS Estrogens, estrogen-initiated cancer, depurinating estrogen-DNA adducts, analysis of estrogen metabolites and conjugates, estrogen-induced mutation and cell transformation, overexpression of aromatase, gene expression by DNA microarray, human breast ductal lavage, breast cancer prevention, biomarkers for breast cancer susceptibility

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	119	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

SUMMARY OF OVERALL DISCOVERIES

The research conducted in this COE grant was based on the paradigm that estrogens can become endogenous carcinogens when their metabolism is unbalanced, favoring formation of catechol estrogen quinones and their reaction with DNA [1]. The further evidence obtained in the various specific aims of this COE will be decisive for determining the risk of breast cancer by using the depurinating estrogen-DNA adducts as biomarkers. These biomarkers will also be used for evaluating the ability of specific antioxidants to prevent breast cancer initiation.

Much of the research accomplished by this COE was published in a review article in *BBA-Reviews in Cancer* [1], which was authored by all of the participants in the COE.

In Specific Aim 1, the major discovery has been to introduce a new methodology for analyzing 40 estrogen metabolites, estrogen conjugates and depurinating estrogen-DNA adducts. This new methodology has enabled us to determine that healthy women have relatively low levels of estrogen-DNA adducts, compared to women at high risk of breast cancer and women with breast cancer, who have relatively high levels of these adducts ($p<0.001$). This new finding will lead to studies of prevention with specific antioxidants that decrease the level of depurinating estrogen-DNA adducts, which represent the first critical step in the initiation of breast cancer.

In Specific Aim 2, we demonstrated that the human breast epithelial cells (MCF-10F cells), which are estrogen receptor-negative, are transformed at physiological doses of estradiol and 4-hydroxyestradiol and to a much lesser extent by 2-hydroxyestradiol. These transformations also occur in the presence of antiestrogens, such as ICI-182,780. Furthermore, selected cells from these transformed populations form solid tumors in SCID mice. These results support the hypothesis that initiation of breast cancer occurs through the genotoxicity of estrogens.

In Specific Aim 3, the major goal has been to demonstrate that 4-hydroxyestradiol is mutagenic, in both cultured BB rat2 embryonic cells and the mammary gland of female Big Blue rats. Once again, the mutations were observed after treatment with 4-hydroxyestradiol, but not 2-hydroxyestradiol, suggesting that the major culprit in initiation of cancer by estrogens derives from 4-catechol estrogens and not 2-catechol estrogens. These results are analogous to those found in the cell transformation studies in Specific Aim 2.

The studies in Specific Aim 4 have been conducted with female ERKO/Wnt-1 mice, which develop mammary tumors, but do not express estrogen receptor-alpha. Using ovariectomized ERKO/Wnt-1 mice, in a dose-response experiment it was demonstrated that the development of mammary tumors was proportional to the dose of estradiol implanted in the mice. These studies suggest that genotoxic pathways of estrogen carcinogenesis play the critical role in the initiation of these mammary tumors and that aromatase inhibitors should be superior to antiestrogens for the prevention of breast cancer.

The work conducted in Specific Aim 5 contributed to the clinical study in Specific 1, in which analysis of estrogen compounds in urine samples from 12 women at high risk for breast

cancer and 17 women with a personal history of breast cancer was compared to the analysis of urine samples from 46 healthy women. The results of these analyses demonstrated that the relative levels of depurinating estrogen-DNA adducts were significantly higher in urine samples from women with breast cancer ($p<0.001$) or at high risk ($p<0.001$), compared to control subjects. The high-risk and breast cancer groups were not significantly different ($p=0.62$). These results support the hypothesis that depurinating estrogen-DNA adducts can serve as potential biomarkers of risk of developing breast cancer.

The Molecular Biology Core continued to study the role of cytochrome P450 1B1 in estrogen hydroxylation and risk of developing breast cancer. This Core contributed to the areas of comparative metabolism, aromatase inhibition, genetic predisposition and tumor classification. In genomic analysis of the cell transformation model established by the Russo laboratory, the Core identified important molecular events leading to the expression of tumorigenic markers and epithelial-mesenchymal transition, which is an important cellular determinant of invasiveness and metastasis. By identifying these characteristics occurring during malignant cell transformation by estrogens in the estrogen receptor-negative cell line MCF-10F, it identifies a new and essential cell model for understanding the especially aggressive characteristics of estrogen receptor-negative tumors.

The Analytical Core supported the studies in Specific Aims 1-5 by analyzing samples from human urine, mouse mammary tissue, rat mammary tissue and culture medium from MCF-10F cells. Ultraperformance liquid chromatography/tandem mass spectrometry was used to identify and quantify estrogen metabolites, conjugates and/or depurinating DNA adducts in these samples.

The Advocacy Core served as an integral part of the COE, providing input into the specific aims of the grant, as well as specific advocacy issues related to consent documents, pilot study design and implementation and funding opportunities. The Core published a consumer guide to involvement in basic research, entitled “Partners in Research, Advocates & Scientists: Advocates’ Guide”. This guide is available for upload to organizational websites.

Reference

1. Cavalieri, E., Chakravarti, D., Guttenplan, J., Hart, E., Ingle, J., Jankowiak, R., Muti, P., Rogan, E., Russo, J., Santen, R. and Sutter, T. Catechol estrogen quinones as initiators of breast and other human cancers. Implications for biomarkers of susceptibility and cancer prevention. BBA-Reviews on Cancer, 1766:63-78, 2006.

Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1766 (2006) 63–78
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.elsevier.com/locate/bbacan

Review

Catechol estrogen quinones as initiators of breast and other human cancers: Implications for biomarkers of susceptibility and cancer prevention ☆

Ercole Cavalieri [a,*], Dhubajyoti Chakravarti [a], Joseph Guttenplan [b], Elizabeth Hart [c], James Ingle [d], Ryszard Jankowiak [e], Paola Muti [f], Eleanor Rogan [a], Jose Russo [g], Richard Santen [h], Thomas Sutter [i]

[a] *Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE 68198-6805, USA*
[b] *New York University Dental and Medical Schools, New York, NY 10032, USA*
[c] *Hart International, Dallas, TX, USA*
[d] *Mayo Clinic, Rochester, MN, USA*
[e] *Department of Chemistry, Kansas State University, Manhattan, KS, USA*
[f] *Italian National Cancer Institute, Rome, Italy*
[g] *Fox Chase Cancer Center, Philadelphia, PA, USA*
[h] *University of Virginia Health System, Charlottesville, VA, USA*
[i] *Center for Genomic Research, University of Memphis, Memphis, TN, USA*

Received 11 January 2006; received in revised form 14 March 2006; accepted 19 March 2006
Available online 19 April 2006

Abstract

Exposure to estrogens is associated with increased risk of breast and other types of human cancer. Estrogens are converted to metabolites, particularly the catechol estrogen-3,4-quinones (CE-3,4-Q), that can react with DNA to form depurinating adducts. These adducts are released from DNA to generate apurinic sites. Error-prone base excision repair of this damage may lead to the mutations that can initiate breast, prostate and other types of cancer.

The reaction of CE-3,4-Q with DNA forms the depurinating adducts 4-hydroxyestrone(estradiol) [4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua. These two adducts constitute more than 99% of the total DNA adducts formed. Increased levels of these quinones and their reaction with DNA occur when estrogen metabolism is unbalanced. Such an imbalance is the result of overexpression of estrogen activating enzymes and/or deficient expression of the deactivating (protective) enzymes. This unbalanced metabolism has been observed in breast biopsy tissue from women with breast cancer, compared to control women. Recently, the depurinating adduct 4-$OHE_1(E_2)$-1-N3Ade has been detected in the urine of prostate cancer patients, but not in urine from healthy men.

Mutagenesis by CE-3,4-Q has been approached from two different perspectives: one is mutagenic activity in the lacI reporter gene in Fisher 344 rats and the other is study of the reporter Harvey-*ras* gene in mouse skin and rat mammary gland. A→G and G→A mutations have been observed in the mammary tissue of rats implanted with the CE-3,4-Q precursor, 4-OHE_2. Mutations have also been observed in the Harvey-*ras* gene in mouse skin and rat mammary gland within 6–12 h after treatment with E_2-3,4-Q, suggesting that these mutations arise by error-prone base excision repair of the apurinic sites generated by the depurinating adducts.

Treatment of MCF-10F cells, which are estrogen receptor-α-negative immortalized human breast epithelial cells, with E_2, 4-OHE_2 or 2-OHE_2 induces their neoplastic transformation in vitro, even in the presence of the antiestrogen ICI-182,780. This suggests that transformation is independent of the estrogen receptor. The transformed cells exhibit specific mutations in several genes. Poorly differentiated adenocarcinomas

Abbreviations: AP, apurinic; BB®, Big Blue; BER, base excision repair; BP, benzo[*a*]pyrene; CE, catechol estrogen; CE-3,4-Q, catechol estrogen-3,4-quinone; COMT, catechol-*O*-methyltransferase; CYP, cytochrome P450; CYP19, aromatase; E_1, estrone; E_2, estradiol; E_2-3,4-Q, estradiol-3,4-quinone; ER, estrogen receptor; ERKO, estrogen receptor α-knock out; FASS, field amplified sample stacking; GSH, glutathione; H, Harvey; HBEC, human breast epithelial cells; LC/MS/MS, ultraperformance liquid chromatography/tandem mass spectrometry; LOD, limit of detection; LOH, loss of heterozygosity; MAb, monoclonal antibody; OHE_2, hydroxyestradiol; 4-$OHE_1(E_2)$-1-N3Ade, 4-hydroxyestrone(estradiol)-1-N3Adenine; 4-$OHE_1(E_2)$-1-N7Gua, 4-hydroxyestrone(estradiol)1-N7Guanine; SCID, severe combined immune depressed; TAM, tamoxifen

☆ Dedicated to Joachim G. Liehr (1942–2003), our colleague, collaborator and friend.

* Corresponding author. Tel.: +1 402 559 7237; fax: +1 402 559 8068.
E-mail address: ecavalie@unmc.edu (E. Cavalieri).

doi:10.1016/j.bbcan.2006.03.001

develop when aggressively transformed MCF-10F cells are selected and injected into severe combined immune depressed (SCID) mice. These results represent the first in vitro/in vivo model of estrogen-induced carcinogenesis in human breast epithelial cells.

In other studies, the development of mammary tumors in estrogen receptor-α knockout mice expressing the Wnt-1 oncogene (ERKO/Wnt-1) provides direct evidence that estrogens may cause breast cancer through a genotoxic, non-estrogen receptor-α-mediated mechanism.

In summary, this evidence strongly indicates that estrogens can become endogenous tumor initiators when CE-3,4-Q react with DNA to form specific depurinating adducts. Initiated cells may be promoted by a number of processes, including hormone receptor stimulated proliferation. These results lay the groundwork for assessing risk and preventing disease.

Keywords: Cancer initiation; Carcinogenicity; Cell transformation; Depurinating estrogen-DNA adduct; Estrogens; Mutations

Contents

1. Introduction

In this review article, we present major scientific advancements supporting the hypothesis that specific estrogen metabolites, namely, catechol estrogen-3,4-quinones (CE-3,4-Q), can initiate breast, prostate and other human cancers. The lines of evidence include epidemiological studies, reaction of the catechol estrogen quinone metabolites with DNA to form specific depurinating adducts, imbalance of estrogen metabolism in the breast of women with breast carcinoma, induction of mammary tumors in estrogen receptor α-knock out mice, in vitro and in vivo mutagenicity induced by CE-Q, malignant transformation of human breast epithelial cells by catechol estrogen metabolites with resulting genetic instability, and biomarkers of cancer risk in men and women.

2. Estrogens, androgens and breast cancer development—the epidemiological evidence

Until the last decade, epidemiological evidence of an association between sex steroid hormones and breast cancer risk, based on a retrospective study design such as case-control studies, was generally inconsistent. In spite of the lack of evidence, prospective cohort studies conducted in the last 10 years consistently observed that elevated levels of serum estrogens and androgens preceded the occurrence of breast cancer. Nine research groups have published results from prospective studies of endogenous hormones and breast cancer in different populations in the world. These studies, based on recruitment of thousands of healthy women and their epidemiological follow-up, are characterized by very different methodological approaches in terms of population sampling, collecting, processing and storing biological specimens [1–13]. In the pooled analysis, both estrogens and androgens were strongly associated with an increase in breast cancer risk, with evidence of a dose–response relationship [14].

This evidence, mainly found in postmenopausal women, has been corroborated in premenopausal women. Recently, a large cohort study, developed within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, has also definitively provided evidence for an etiological link between sex steroids and breast cancer development in premenopausal women [15].

Etiological research conducted in experimental settings and in population and clinical studies shows a remarkable coherence and consistency of evidence. On-going investigations now need to focus on the origin of cancer, and how and why sex steroid hormones are so closely related to breast cancer development. These investigations need to develop beyond the stochastic model based on the paradigm that estrogens bind to estrogen receptors and stimulate the transcription of genes involved in cell proliferation, creating potential errors in DNA replication and potential mutations [16,17]. To initiate cancer, these random mutations must occur in specific sites in DNA; this is an extremely unlikely outcome. In contrast, initiation of cancer by genotoxic estrogen metabolites that generate specific mutations correlates with the strong coherence of the results of experimental and epidemiological studies, and the consistency of their associations across different studies and different populations. The evidence for estrogen genotoxicity and mutagenesis is summarized in this article.

3. Estrogens as tumor initiators

The initial failure to demonstrate that estrogens induce mutations in bacterial and mammalian test systems [18–23] resulted in the classification of estrone (E_1) and estradiol (E_2) as epigenetic carcinogens that function by stimulating abnormal cell proliferation via estrogen receptor-mediated processes [16,17,20,24–26]. The stimulated cell proliferation could result in increased accumulation of genetic damage, leading to carcinogenesis [16,26,27].

Compelling evidence has led to a new paradigm of cancer initiation by estrogens. Discovery that specific oxidative metabolites of estrogens can react with DNA [28–31] led to and has supported the hypothesis that these metabolites can become endogenous chemical carcinogens. Some of the mutations generated by the specific DNA damage can result in the initiation of cancer in hormone-dependent and -independent tissues [32–35].

Chemical carcinogens covalently bind to DNA to form two types of adducts: stable ones that remain in the DNA, unless removed by repair, and depurinating ones that are lost from the DNA by destabilization of the glycosyl bond [29,30,36,37]. Evidence that depurinating polycyclic aromatic hydrocarbon- and estrogen-DNA adducts play a major role in tumor initiation derives from a correlation between depurinating adducts and oncogenic Harvey (H)-*ras* mutations in mouse skin papillomas, preneoplastic mouse skin and preneoplastic rat mammary gland (see Section 5 below) [32,33,38,39]. These observations have provided the impetus for discovering the estrogen metabolites that form depurinating DNA adducts and lead to the mutations that can eventually initiate cancer [28–30]. Experiments on estrogen metabolism [40–42], formation of DNA adducts [28–31], carcinogenicity [18,43,44], mutagenicity [32–35] and cell transformation [45–48] have led to and supported the hypothesis that reaction of specific estrogen metabolites, namely, CE-3,4-Q and to a much lesser extent, CE-2,3-Q, with DNA can generate the critical mutations that initiate breast, prostate and other cancers [30,35].

3.1. Formation, metabolism and DNA adducts of estrogens

Catechol estrogens (CE) are among the major metabolites of E_1 and E_2 [49–51]. If these metabolites are oxidized to the electrophilic CE-Q, they may react with DNA. Specifically, the carcinogenic 4-OHE_1(E_2) [18,43,44] are oxidized to E_1(E_2)-3,4-Q, which can react with DNA to form predominantly depurinating adducts [28–30]. These adducts generate apurinic sites that may lead to cancer-initiating mutations (see Section 5 below) [32–35], which transform cells (see Section 6 below), thereby initiating cancer [45–48]. The extremely weak carcinogen 2-OHE_1(E_2) [44] also forms depurinating adducts, but to a much lesser extent [52]. The depurinating N3Ade and N7Gua adducts are released from DNA at different rates, the former instantaneously and the latter with a half-life of 3 h [52,53].

E_1 and E_2 are formed by aromatization of androstenedione and testosterone, respectively, catalyzed by cytochrome P450 (CYP) 19, aromatase (Fig. 1). E_1 and E_2 are interconverted by the enzyme 17β-estradiol dehydrogenase. These estrogens are metabolized by two major pathways: formation of CE and, to a lesser extent, 16α-hydroxylation (not shown in Fig. 1). The CE formed are the 2-OHE_1(E_2) and 4-OHE_1(E_2). The 2-OHE_1(E_2) are generally the major CE formed. Increases in the level of CYP1B1 and other 4-hydroxylases could render the minor CE metabolites, 4-OHE_1(E_2), as the major ones. The CE is generally inactivated by conjugating reactions such as glucuronidation and sulfation, especially in the liver (not shown in Fig. 1). The most common pathway of conjugation in extrahepatic tissues occurs, however, by *O*-methylation catalyzed by the ubiquitous catechol-*O*-methyltransferase (COMT) [54]. If conjugation of CE via methylation becomes insufficient, the competitive catalytic oxidation of CE to CE-Q can occur.

Redox cycling via reduction of CE-Q to semiquinones, catalyzed by CYP reductase, and subsequent oxidation back to CE-Q by O_2 forms super-anion radicals and then H_2O_2. In the presence of Fe^{2+}, H_2O_2 forms hydroxyl radicals (Fig. 1).

The 4-OHE_1(E_2) exhibit greater carcinogenic potency than the 2-OHE_1(E_2), which are borderline carcinogens [18,43,44]. It is difficult to attribute the greater potency of 4-OHE_1(E_2) to the redox cycling of the 2-OHE_1(E_2) and 4-OHE_1(E_2), because they have similar redox potentials [55,56]. Instead, one can relate the greater carcinogenic potency of the 4-OHE_1(E_2) to the much higher level of depurinating DNA adducts formed by E_1(E_2)-3,4-Q compared to E_1(E_2)-2,3-Q [52]. Thus, we think the role of CE-Q in initiating cancer is through formation of depurinating DNA adducts.

The reactivity of CE-Q with DNA can be prevented by conjugation with glutathione (GSH, Fig. 1). A second inactivating pathway for CE-Q is their reduction to CE by quinone reductase and/or CYP reductase [57,58]. If these inactivating processes are insufficient, CE-Q may react with DNA to form predominantly depurinating adducts (Fig. 1) [28–31]. When mouse skin [32] or rat mammary gland [33] was treated with E_2-3,4-Q, the 4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua adducts were formed. In these tissues, E_2-3,4-Q induced mainly A to G mutations in the reporter H-*ras* gene, presumably because the N3Ade adducts depurinate rapidly, leading to premutagenic

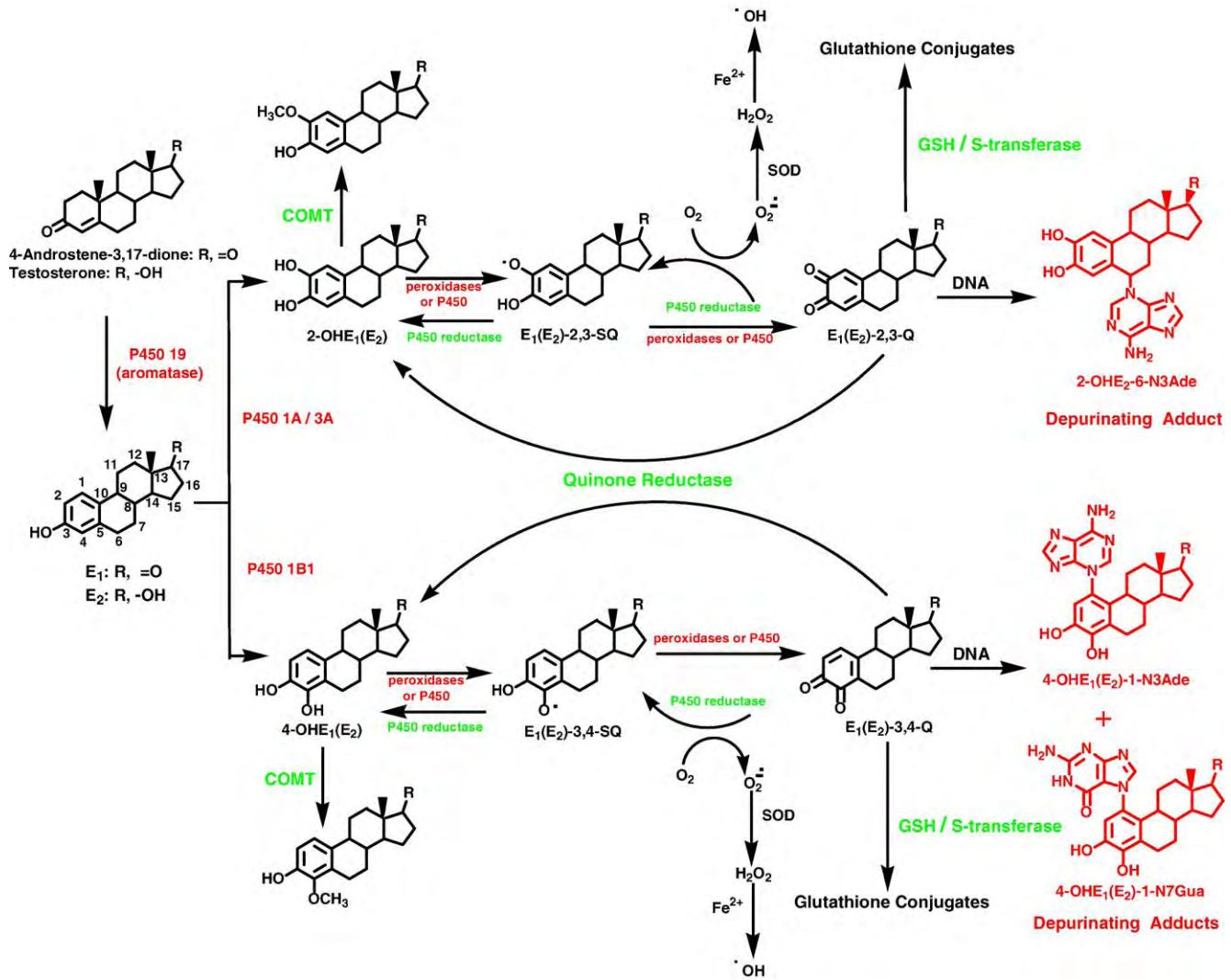


Fig. 1. Formation, metabolism and DNA adducts of estrogens. Activating enzymes and depurinating adducts are in red, and protective enzymes are in green.

apurinic (AP) sites, but the N7Gua adducts depurinate relatively slowly, allowing accurate DNA repair [32,33,52,53]. These mutagenicity results suggest that E_2-3,4-Q may be the major carcinogenic metabolite of estrogens.

3.2. Imbalance of estrogen homeostasis

The above paradigm of cancer initiation by estrogens hinges on estrogen metabolism that involves a disrupted homeostatic balance between activating and deactivating pathways (Fig. 1). Several factors can unbalance estrogen homeostasis, namely, the equilibrium between estrogen activating and deactivating pathways to avert oxidative stress, in particular the formation of endogenous carcinogenic CE-Q and their reaction with DNA (Fig. 1).

Critical factors in elevating estrogen levels are excessive synthesis of estrogens by overexpression of CYP19 in target tissues [59–61] and/or the presence of unregulated sulfatase that converts excess stored E_1-sulfate to E_1 [62,63]. The observation that breast tissue can synthesize E_2 in situ suggests that much more E_2 is present in target tissues than would be predicted from plasma concentrations [61]. A striking result of in situ E_2 production is that the E_2 levels in human breast tissue are similar in pre- and post-menopausal women, even though plasma levels are 50- to 100-fold lower in postmenopausal women compared to premenopausal [63,64].

Another critical factor unbalancing estrogen homeostasis may be higher levels of 4-$OHE_1(E_2)$ due to overexpression of CYP1B1, which converts E_2 predominantly to 4-OHE_2 (Fig. 1) [65,66]. This could result in relatively large amounts of 4-$OHE_1(E_2)$ and subsequently more oxidation to $E_1(E_2)$-3,4-Q. An additional factor could be a lack or low level of COMT activity, because of polymorphic variation [67]. If this enzyme activity is insufficient, 4-$OHE_1(E_2)$ would not be effectively methylated, but could be oxidized to the ultimate metabolites $E_1(E_2)$-3,4-Q. Finally, a low level of GSH, and/or low levels of quinone reductase and/or CYP reductase, could result in a higher level of $E_1(E_2)$-3,4-Q that may react with DNA.

We postulate that unbalanced estrogen homeostasis is a condition that precedes the initiation of breast and prostate cancer. The effects of some of the above factors have already

been observed in several animal models for estrogen carcinogenesis and in human breast. Imbalances in estrogen homeostasis leading to substantial formation of CE-GSH conjugates and depurinating CE-DNA adducts have been observed in the kidney of male Syrian golden hamsters [40], the prostate of Noble rats [41] and the mammary gland of female estrogen receptor-α knockout (ERKO/Wnt-1) mice (see Section 4 below) [68]. A study of breast tissue from women with and without breast cancer provides key evidence in support of unbalanced estrogen homeostasis. In fact, this imbalance was observed in women with breast cancer (Fig. 2) [42]. Levels of $E_1(E_2)$ in women with carcinoma were higher than in controls, and the levels of 4-$OHE_1(E_2)$ were nearly four times higher in women with breast carcinoma than in women without cancer. In women with breast carcinoma, 4-$OHE_1(E_2)$ were three-times more abundant than 2-$OHE_1(E_2)$. Levels of CE-Q conjugates in women with breast cancer were three-times those in the controls, suggesting a greater probability of CE-Q reacting with DNA in the breast tissue of women with breast carcinoma. Levels of 4-$OHE_1(E_2)$ ($P<0.01$) and CE-Q conjugates ($P<0.003$) appeared to be significantly associated with breast cancer [42]. One established example of this imbalance is the overexpression of the estrogen 4-hydroxylase, CYP1B1, in tumors of the breast [69–71]. Therefore, the oxidative pathway that leads to formation of CE-Q is the result of unbalancing one or more factors involved in estrogen homeostasis.

3.3. Unifying mechanism of tumor initiation by synthetic estrogens

We have proposed that oxidation of CE to CE-Q is a pathway to initiate cancer by endogenous estrogens, as well as synthetic estrogens such as the human carcinogen diethylstilbestrol [72] and its hydrogenated derivative hexestrol. These two compounds, similarly to the endogenous estrogens, are carcinogenic in the kidney of Syrian golden hamsters [73,74], and the major metabolites are their catechols [74–77]. These catechols can be easily oxidized to catechol quinones. Their chemical and biochemical properties are similar to those of CE-3,4-Q, namely, they specifically form the N7Gua and N3Ade adducts after reaction with DNA [78–80]. Therefore, the catechol quinones of hexestrol and diethylstilbestrol appear to be the critical initiators of cancer by these synthetic estrogens. In turn, these results support the hypothesis that CE-3,4-Q may be endogenous tumor initiators because they react with DNA to form N7Gua and N3Ade adducts. In summary, catechol quinones of natural and synthetic estrogens can be initiators of a variety of human cancers, including breast and prostrate.

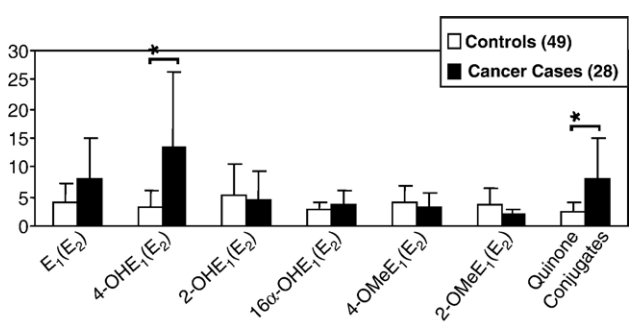


Fig. 2. Relative imbalance of estrogen metabolism in non-tumor breast tissue of women with breast cancer vs. controls. The level of 4-$OHE_1(E_2)$ was significantly higher in cases compared to controls ($P<0.01$). Quinone conjugates were 4-$OHE_1(E_2)$-2-NAcCys, 4-$OHE_1(E_2)$-2-Cys, 2-$OHE_1(E_2)$-(1+4)-NAcCys, and 2-$OHE_1(E_2)$-(1+4)-Cys. The levels of quinone conjugates were significantly higher in cases than in controls ($P<0.003$). *Statistically significant differences were determined using the Wilcoxon rank sum test.

3.4. Unifying mechanism of initiation of cancer and other diseases by catechol quinones

Oxidation of catechols to quinones and semiquinones is not only a mechanism of tumor initiation by natural and synthetic estrogens, but could be the mechanism of tumor initiation by the leukemogen benzene. In fact, reaction of the benzene catechol quinone with DNA specifically produces N3Ade and N7Gua adducts [81]. The same DNA adducts can be obtained by enzymatic oxidation of the benzene catechol in the presence of DNA [81]. The catecholamine neurotransmitter dopamine can undergo an oxidative process that is analogous to the one described for the catechol of benzene and the natural and synthetic estrogens. In fact, reaction of dopamine quinone with DNA forms specific depurinating N3Ade and N7Gua adducts [81]. In conclusion, the catechol quinones of natural and synthetic estrogens, benzene and dopamine can react with DNA to form specific depurinating adducts bonded at the N-7 of Gua or the N-3 of Ade. The apurinic sites formed by these adducts may be converted by error-prone repair into mutations that can initiate cancer and neurodegenerative diseases. This hypothesized unifying mechanism in the induction of these diseases supports the mechanism described for natural and synthetic estrogens.

4. Further evidence for the genotoxicity of estrogen metabolites in the induction of cancer

Experiments using transgenic mice with estrogen receptor-α (ER-α) knocked-out (ERKO/Wnt-1 mice) and metabolism in aromatase (CYP19) overexpressing MCF-7 human breast cancer cells have provided further important evidence for genotoxic effects of estrogen metabolites in cancer initiation.

4.1. Tumor incidence in ERKO/Wnt-1 mice

Bocchinfuso and his associates showed that ERKO/Wnt-1 mice exhibit a delayed onset of tumor development compared to mice expressing the wild type ER-α. Nonetheless, they observed a nearly 100% incidence of mammary tumors in the absence of ER-α and β [82,83]. To directly determine the effect of E_2 in the absence of ER, mice were castrated at 15 days of age and half were treated with silastic implants containing E_2 and the other half with implants of cholesterol. After 100 weeks of observation, the E_2-treated mice developed more tumors (12/15 vs. 4/10), which appeared earlier than those in the mice receiving cholesterol implants (50% of tumors at 50 weeks versus 25% of tumors at 100 weeks, $P<0.004$) (Fig. 3) [84,85].

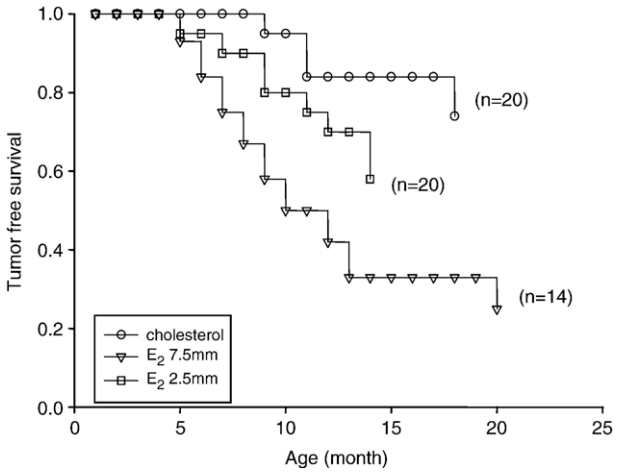


Fig. 3. Tumor-free survival in ERKO/Wnt-1 transgenic knock-out mice, which were oophorectomized before 15 days of age. Animals were treated with silastic implants containing cholesterol alone (control group), a 7.5 mm silastic implant containing E_2 and producing plasma E_2 levels of ~300 pg/ml and a 2.5 mm silastic implant that results in plasma levels of E_2 of ~75 pg/ml.

Mammary tumors developed even when the mice were treated with both E_2 and the pure antiestrogen ICI-182,780 [86]. Overall, these experiments provide evidence that E_2 exerts effects through both an ER-α-independent pathway, as well as an ER-dependent pathway, to produce breast tumors. Presumably, the tumors are initiated by estrogen genotoxicity in an ER-α-independent pathway, followed by proliferation of the initiated cells mediated by an ER-dependent pathway.

4.2. Aromatase-transfected MCF-7 breast cancer cell model

A model system was used to determine whether the enzymes responsible for E_2 metabolism to GSH conjugates and depurinating adducts were present in MCF-7 breast cancer cells. These cells formed large amounts of 4-methoxyE_2 when cultured with 4-OHE_2 (data not shown), indicating the presence of the COMT enzyme. Substantial amounts of the GSH-quinone conjugates were detected, providing evidence of the enzymatic oxidation of 4-OHE_2 to CE-Q. The CE-Q bound to DNA, with formation of depurinating adducts, detected as 4-$OHE_1(E_2)$-1-N7Gua. The next question was whether these cells could aromatize a sufficient amount of testosterone to E_2 to result in formation of the depurinating adducts. Detection of 131 pg estrogen/ml of medium in testosterone-treated cells indicated the production of estrogens by aromatization. The 4-$OHE_1(E_2)$-1-N7Gua adducts were also present at a total concentration of 0.17 pg/ml, as were the GSH, cysteine, and *N*-acetylcysteine conjugates of E_2-3,4-Q. Finally, as further evidence of the presence of the aromatase enzyme, the aromatase-inhibitor letrozole reduced estrogen formation from a total of 131 pg/ml of E_1 and E_2 to 2.8 pg/ml and the GSH conjugates and DNA adducts to undetectable levels [87].

4.3. Implications for estrogen genotoxicity

The above findings provide evidence in a model system that E_2 can influence the incidence of mammary tumors, as well as their rate of development. In these studies, the first aim was to demonstrate that human breast cancer cells convert testosterone or 4-OHE_2 to genotoxic products. This was clearly demonstrated in the MCF-7 cell model system by using a highly sensitive and specific assay for estrogen metabolites. A commonly expressed criticism of the genotoxic hypothesis is that supraphysiologic amounts of estrogen are needed to form genotoxic metabolites of E_2 [88]. Our in vitro experiments can be criticized on the same basis. However, the in vivo model allows assessment of effects in response to E_2 levels in the animal. Nonetheless, the ERKO/Wnt-1 animals have circulating E_2 levels in the range of 325 pg/ml (K. Korach, personal communication, 2002). This is approximately 30- to 50-fold higher than normal as a consequence of the absence of E_2 negative feedback on the pituitary and the resultant rise in luteinizing hormone levels. In addition, the breast tissue from these animals appears to convert little 4-OHE_2 to 4-methoxyE_2, a metabolite which is inactive and cannot be converted to genotoxic metabolites [68]. On the basis of these two effects, namely minimal detoxification through the CE pathway and high E_2 levels, the ERKO/Wnt-1 model develops mammary tumors with a 100% incidence in the absence of ER-α. Accordingly, this model is ideal for providing proof of the principle that E_2, in the absence of a functioning ER-α, can induce breast tumors.

5. Estrogens as mutagens

There is evidence that estrogens contribute to the induction of mutations in breast cancer in humans. A survey of the IARC *p*53 database (http://www.iarc.fr/p53/index.html) suggests that sporadic breast cancers, compared to germline (Li-Fraumeni) cases and cancers in hormone-independent tissues such as lung, bladder and brain, show mutational hotspots at codons 163 and 179 in the p53 gene [unpublished results]. An increased frequency of A.T to G.C mutations could be seen at these sites. In the in vitro transformation of human breast epithelial cells (HBEC) by E_2 or 4-OHE_2, a 5-bp deletion in TP53 exon 4 of chromosome 17 (marker TP53-Dint located in exon 4 of TP53) was also reported by Russo et al. [48]. In addition, BRCA1/2-related inherited breast cancers also show similarly increased frequencies of A.T to G.C mutations and hotspots at several codons of the p53 gene, including codon 163 [89].

Our studies suggest that the chief contributor to estrogen genotoxicity in breast cancer is $E_1(E_2)$-3,4-Q, the ultimate carcinogenic form of the 4-CE. An important link in the hypothesis that estrogens are genotoxic would be a demonstration that a major E_2 metabolite, 4-OHE_2, is mutagenic under conditions where it can be metabolized to the putative ultimate mutagenic metabolite, E_2-3,4-Q. Further evidence supporting this hypothesis would be a demonstration of the mutagenic activity of E_2-3,4-Q.

In early studies, E_2 and some of its metabolites were reported to be negative in a number of in vitro mutagenesis assays [88], but recently in different systems and under different conditions, we have observed that both compounds are mutagenic.

5.1. In vitro mutagenesis

The Big Blue® (BB®) rat2 embryonic cell line (Stratagene, La Jolla, CA) was used to detect mutagenesis. This is a rat embryonic cell line transfected with the lambda-LIZ vector. It enables the host cell to detect mutations in the *lac*I and/or *c*II genes. The host cells contain approximately 60 copies of the vector per cell. The *c*II assay was employed.

Initial experiments conducted at doses from 10 to 6800 nM 4-OHE_2 failed to detect any significant increase in mutant fraction after a single 16 hr treatment, and therefore multiple treatments were performed. 4-OHE_2 induced a dose-dependent increase in mutant fraction up to 200 nM [35]. This was marginally apparent at three treatments and clearer after six treatments. The mutant fraction (in units of mutants/10^5 pfu) increased from 2.6±1.3 in controls to 4.5±0.7 and 5.8±0.3 at 100 and 200 nM, respectively, after six treatments. After three and six 200-nM treatments, the increase over controls was statistically significant. The mutant fraction declined at 400 nM after both three and six treatments. Using similar protocols, for single and multiple treatments, it was not possible to detect any induction of mutagenesis over background by 2-OHE_2. E_2-3,4-Q was also found to be similarly mutagenic.

The mutational spectra from the 4-OHE_2 treated and control plates were compared, and a major apparent difference between the two groups was the higher percentage of mutations at A:T base pairs in the mutants from the 4-OHE_2 -treated cells than in controls (*ca.* 24% vs. 6%). The mutational spectrum of E_2-3,4-Q is currently being analyzed.

Although the mutagenic activity of 4-OHE_2 has not been previously reported, 4-OHE_2 and its precursor, E_2, induce DNA strand breaks, detectable in the comet assay [90,91]. It is not known, however, whether these strand breaks actually lead to mutations, as they may be repaired. In an oxidative damage modification of the comet assay, 4-OHE_2 and 2-OHE_2 were similarly effective in producing strand breaks [90]. In addition, assays on DNA damage in vitro indicate that oxidative damage under Fenton-like conditions is produced by both 2-OHE_2 and 4-OHE_2 with similar efficiency [92]. Taken together, the results here and in the previous studies suggest that 2-OHE_2 and 4-OHE_2 both induce oxidative damage with similar efficiencies, but these processes do not account for differences in the carcinogenic and mutagenic potencies of these compounds. If much of the initial oxidative damage to DNA is rapidly and accurately repaired, mutagenesis may result from the mis-repair of AP sites resulting from depurinating estrogen-DNA adducts, and residual oxidative damage.

4-OHE_2 was a weak mutagen and the BB® rat2 cells required multiple treatments to detect mutagenesis. This and the observation that mutagenesis is only observed over a narrow dose range may explain previous negative reports on mutagenesis by 4-OHE_2 and other estrogens or metabolites. The observation that 4-OHE_2, but not 2-OHE_2, exhibited mutagenic activity in BB® rat2 cells correlates with studies on the relative carcinogenicity [18,43,44] and cell transforming abilities [46] of these compounds, and thus provides additional evidence that genotoxicity plays a role in estrogen-induced carcinogenesis.

5.2. Mutagenesis induced by 4-OHE_2 and E_2-3,4-Q in experimental animals

Studies in experimental animals have shown that E_2 and 4-OHE_2 are carcinogenic, whereas 2-OHE_2 is only marginally active [18,43,44].

treatment	n	mean MF	SD
E_2 + 4-OHE_2	6	1.93*	0.32
4-OHE_2	8	1.65	0.69
E_2	4	2.06*	0.49
	6	0.95	0.50

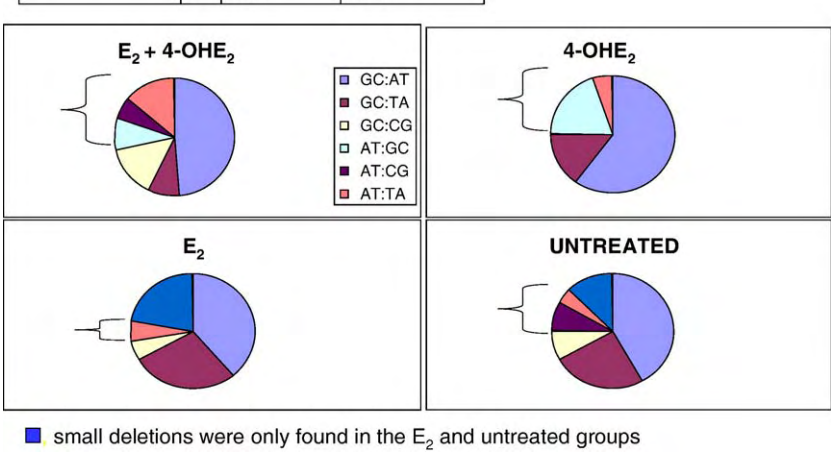


Fig. 4. Mutant fractions (MF) in mammary tissue from BB rats treated with 5 mg of 4-OHE_2 + 5 mg of E_2, 5 mg of 4-OHE_2, or 5 mg of E_2, or left untreated, and the mutational distribution in each group. Brackets refer to mutations at A:T base pairs. Mutagenesis was assayed after 20 weeks. *$P<0.05$ vs. control (2 -tailed t-test). For 4-OHE2, $P=0.06$.

Table 1
Mutations induced by E_2-3,4-Q in mouse skin and rat mammary gland

Animal	Treatment	Mutations after 6–24 h	Frequency	
			Mutation/no. of clones	Mutations/total mutations
SENCAR mouse skin	Control	A.T>G.C	1/36 (3%)	1/1
	E_2-3,4-Q	A.T>G.C	9/59 (15%)	9/13 (69%)
	+TDG	A.T>G.C	0/74	0/4
ACI rat mammary gland	Control	A.T>G.C	18/95 (19%)	18/24 (75%)
	E_2-3,4-Q	A.T>G.C	30/63 (48%)	30/39 (77%)
	+TDG	A.T>G.C	16/79 (20%)	16/20 (80%)

5.2.1. The BB® rat model

The BB® rat is a Fisher 344 rat that contains about 80 copies of the Lambda-LIZ vector in every cell of the animal. The transgene is not expressed and has no effect on the biochemistry or physiology of the animal. Rats were administered 5 mg of 4-OHE_2, 5 mg of E_2 or a combination of 5 mg of each in silastic tubing, placed in the scapular space. In addition, an untreated control group was assayed. The rats were euthanized after 20 weeks, DNA was extracted from inguinal mammary fat pads, and mutagenesis and mutational spectra in *cII* were then assayed.

The mutant fraction in the three treated groups was about twice that in untreated controls and that difference was significant (Fig. 4), indicating that E_2 and 4-OHE_2 were mutagenic. Based on previous carcinogenesis and cell transformation assays [18,43,44,46], it was anticipated that E_2 would be less mutagenic than 4-OHE_2 and perhaps the combination of the agents would be most effective, if E_2 resulted in increased cell proliferation, which enhanced mutagenesis. However, there was no significant difference between the mutant frequencies of the three treated groups. E_2 alone was somewhat toxic, probably as a result of excessive prolactin production in response to this agent, and only 4 rats in the E_2-alone group survived and the small number of rats in this group may have reduced the accuracy of the measurement of the mutant frequency for this group.

The mutational spectrum of the groups receiving 4-OHE_2 was different from the other groups (Fig. 4). The major difference was the higher fraction of mutations at A:T base pairs, and in particular, AT:GC transitions. As described above, these mutations are consistent with those expected for the 4-OHE_2-1-N3Ade, and the fact that they are more frequent in the groups receiving 4-OHE_2 is consistent with the hypothesis that 4-OHE_2 contributes to mutagenesis in BB® rat mammary tissue.

5.2.2. SENCAR mouse and ACI rat models

Our studies in SENCAR mouse skin and ACI rat mammary gland suggest that 4-OHE_2 or E_2-3,4-Q can induce mutations similar to those associated with breast cancer (Table 1). The

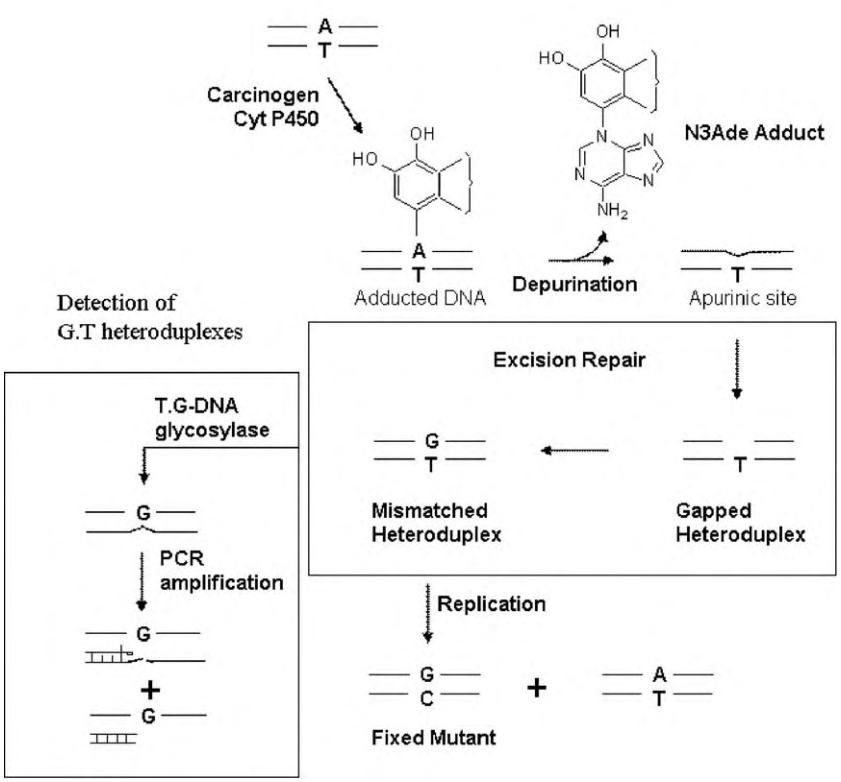


Fig. 5. Induction of A to G mutations from the rapidly-depurinating N3Ade adducts generated by E_2-3,4-Q treatment. The adducted Ade is spontaneously lost from DNA, forming an apurinic site, which undergoes base excision repair. Our studies suggest that this repair frequently commits errors (mutation frequency $\sim 10^{-4}$), generating mutations that are initially formed as G.T heteroduplexes. Left: the G.T pairs are detected by T.G-DNA glycosylase treatment. This enzyme removes the T bases from G.T heteroduplexes, generating abasic sites that are refractory to PCR amplification. As a result, the mutation spectra show a drastic reduction in the frequency of A.T to G.C mutations.

initial study was conducted in the SENCAR mouse model by administering a single dose (200 nmol in acetone) of E_2-3,4-Q and examining the H-*ras* gene as the target of mutagenesis. We studied early induction of mutations (12 h–3 d after the treatment) to make correlations with DNA adducts [32]. The results showed that E_2-3,4-Q induced predominantly A.T to G.C mutations. Next, we examined the ACI rat mammary gland, considered to be a model of breast cancer, for mutagenesis by E_2-3,4-Q. Similar mutations were again observed (Table 1) [33]. These mutations correlated with the rapidly-depurinating N3Ade adducts, while the slowly-depurinating N7Gua adducts did not appear to be major sources of mutagenesis in the early period. These depurinating adducts are spontaneously lost from DNA, forming apurinic sites. Since the N3Ade adducts depurinate rapidly, they will induce a rapid burst of apurinic sites in DNA. Exposure of cells to agents that induce abasic sites results in an early, adaptive induction of base excision repair (BER) genes, along with repression of DNA replication [93]. We found that coinciding with mutagenesis, E_2-3,4-Q treatment of the ACI rat mammary gland induces short-patch BER genes [AP endonuclease 1, DNA polymerase β, poly (ADP-ribose) phosphorylase 1 and ligase III] [93]. The abundant formation of depurinating adducts and induction of BER genes during mutagenesis suggest that erroneous BER could be the mechanism for induction of mutations. During BER, a short section of the AP site-containing strand is excised, and the gap is filled by DNA synthesis. Therefore, errors in BER would generate mutations in the newly-synthesized strand, i.e., mismatched heteroduplexes. A.T to G.C mutations can form either G.T heteroduplexes (if the mutations are A to G) or A.C heteroduplexes (if the mutations are T to C). Using a glycosylase that is specific for the G.T heteroduplexes, we determined that the A.T to G.C mutations are initially (6 h–1 d) induced as G.T mispairs, supporting the idea that the mutations are A to G transitions and were induced from the de-adenylated sites produced by the rapidly-depurinating N3Ade adducts. These ideas are described in a cartoon in Fig. 5.

5.3. Conclusions

4-OHE_2 and E_2-3,4-Q have now been assayed in both in vivo and in vitro systems and found to be mutagenic under appropriate assay conditions. The marginally carcinogenic E_2 metabolite, 2-OHE_2, was non-mutagenic in BB® rat2 cells under conditions in which 4-OHE_2 was mutagenic. This result parallels cell transformation assays (see Section 6 below) [46] and provides further evidence that estrogens can contribute to carcinogenesis via a genotoxic pathway. In addition, the proposed ultimate mutagenic metabolite, E_2-3,4-Q, was mutagenic in rodent breast and skin, and also in BB® rat2 cells in culture, providing further evidence that E_2-3,4-Q is the ultimate mutagenic metabolite of E_2. Finally, the mutational spectra, both in vivo and in vitro, were also consistent with those expected from the DNA adducts produced by 4-OHE_2 and E_2-3,4-Q. Taken together, the results obtained from mutagenesis studies of 4-OHE_2 and E_2-3,4-Q support the hypothesis that estrogens can contribute to carcinogenesis by a genotoxic pathway.

6. An in vitro/in vivo model of estrogen-induced carcinogenesis

To fully demonstrate that estrogens are carcinogenic in the human breast and for testing potential mechanisms of action, an experimental system is required in which the natural estrogen E_2 by itself or its metabolites, 2-OHE_2, 4-OHE_2, and 16α-OHE_2, respectively, would induce neoplastic transformation of HBEC in vitro to a degree at least similar to that induced by the chemical carcinogen benzo[*a*]pyrene (BP) [94,95]. The transforming potential of estrogens on human breast epithelium was evaluated by treating the spontaneously immortalized ER-α negative MCF-10F cells with 0.007 nM, 70 nM and 1 μg/ml of E_2, 2-OHE_2, 4-OHE_2, 16α-OHE_2, or cholesterol [94]. Treatments with estrogens alone or in the presence of the antiestrogens tamoxifen (TAM) or ICI-182,780 were carried out for 24 h twice a week for 2 weeks to mimic the intermittent exposure of the breast to endogenous estrogens. At the end of the second week of treatment, and in successive passages thereafter, the cells were evaluated for assessing the expression of phenotypes indicative of cell transformation [46,94–96], namely, determination of colony formation in agar-methocel, or colony efficiency, ductulogenic capacity in collagen matrix, invasiveness in a reconstituted basement membrane using the Boyden chamber, genomic analysis by capillary electrophoresis, and tumorigenic assay in severe compromised immune-deficient (SCID) mice [97,98].

At all passages tested, MCF-10F cells treated with BP, E_2, 2-OHE_2, 4-OHE_2, or 16α-OHE_2 formed colonies in agar-methocel that were greater than 80 μm in diameter. Cells treated with cholesterol did not form colonies. Colony efficiency was dose dependent and similar in cells treated with BP or E_2; 2-OHE_2-treated cells had lower colony efficiency than the two previous compounds, and in 4-OHE_2-treated cells colony efficiency was greater at the 0.007 nM dose, reaching a plateau at the two higher doses. Ductulogenesis, which was evaluated by estimating the ability of cells plated in collagen to form tubules, revealed that cholesterol-treated control MCF-10F cells formed ductule-like structures, mimicking the normal branching pattern of the mammary parenchyma. The ductulogenic capacity was lost in BP and E_2 treated cells, which formed, instead, solid masses [46]. The metabolites of estrogen also impaired the formation of ductules. Histological analysis showed that the ductules that control cells formed in the collagen matrix were lined by a single layer of cuboidal epithelial cells. The E_2-metabolite-treated cells formed spherical masses filled by large cuboidal cells. The invasive capacity of MCF-10F cells was significantly increased by BP; E_2 or 4-OHE_2 treatments increased even further the invasiveness of the cells; 2-OHE_2 treatment stimulated invasiveness, but to a lower degree than BP. Neither TAM nor ICI-182,780 abrogated the transforming efficiency of estrogen metabolites [47].

Genomic analysis revealed that MCF-10F cells transformed with E_2 or 4-OHE_2, either alone or in combination with ICI-182,780, exhibited loss of heterozygosity (LOH) in the region 13q12.3 (D13S893 marker located at approximately 0.8 cM telomeric to BRCA2) at all the doses tested; 2-OHE_2 induced the same change only at the highest dose used (1 μg/ml) [48].

Another significant genomic change observed was a 5-bp deletion in TP53 exon 4 of chromosome 17 (marker TP53-Dint located in exon 4 of TP53) in cells treated with E_2 or 4-OHE_2 at the doses of 0.007 nM or 70 nM and with 2-OHE_2 only at a highest dose used. These changes were considered to be specific for E_2 and its metabolites, since the observed mutations in HBEC at D13S893 and TP53 exon 4 loci were not induced by BP treatment [48]. Injection of $10–15 \times 10^6$ control or treated cells in the inguinal fat pad of SCID mice failed to induce tumors up to the 9th passage. To determine whether more aggressive phenotypes could be selected, cells in their 9th passage after transformation with E_2 were seeded in a Boyden chamber, and those cells crossing the membrane were collected, expanded, and designated E_2-70-B2,B3,B4,B5,C2,C3,C4 and C5 for those transformed with 70 nM and 1-B2, 1-B3, 1-B4, 1-B5, 1-C2, 1-C3, 1-C4, and 1-C5 for those transformed with 1 μg/ml E_2. These cells were injected in SCID mice for assay of tumorigenicity. Only E_2-70-C3 and E_2-70-C5 were tumorigenic in 2/12 and 9/10 animals injected, respectively [99]. The tumors were poorly differentiated adenocarcinomas, ER-α, ER-β and progesterone receptor negative, expressing immunocytochemically high molecular weight basic keratin (+++), E Cadherin (+) and CAM5.2 (+). RNA was collected from E_2-70-C3 and E_2-70-C5 cells for cDNA microarray analysis. The genomic profile of E_2-70-C5

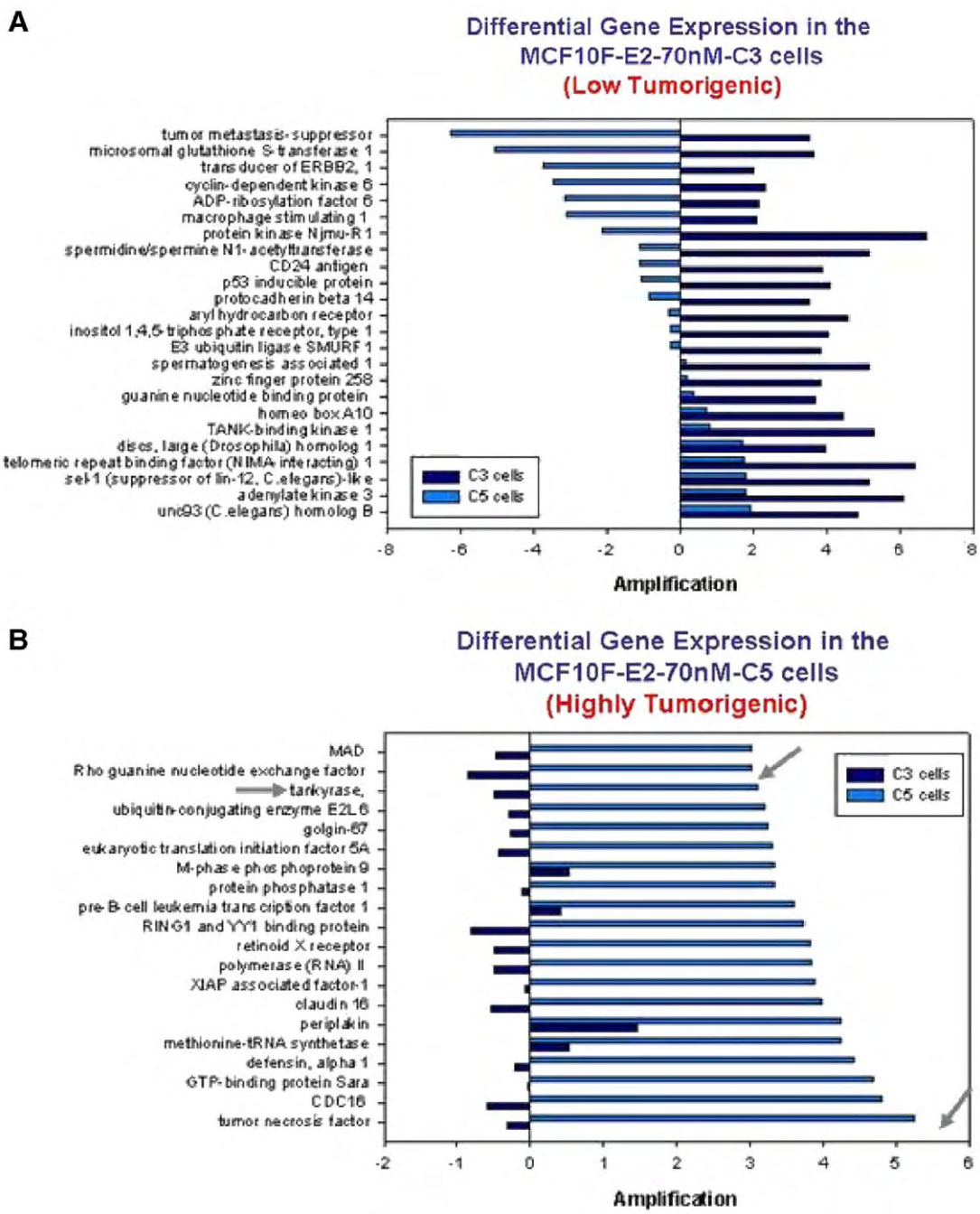


Fig. 6. Schematic representation of the genes up- and down-regulated in (A) C3 cells and (B) C5 cells.

cells, which differed from that of E_2-70-C3 cells, showed that they overexpressed by more than 5-fold genes such as tankyrase (TRF1-interacting ankyrin related ADP ribose polymerase), claudin 1, homeobox C10, and Notch homolog 3; it also exhibited down-regulation of telomeric repeat binding factor and tumor metastasis-suppressor gene, all genes that have been shown to be altered in primary breast cancer (Fig. 6). From the 9 tumors obtained from E_2-70-C5 cells, four tumoral cell lines designated C5-A1-T1,C5-A4-T4, C5-A6-T6 and C5-A8-T8 were derived. Fingerprint analysis confirmed that all these cells originated from MCF-10F cells.

To simultaneously explore copy number abnormalities and loss of heterozygosity occurring throughout the transformation of cells and proceeding from MCF-10F cells to cells derived from tumors in SCID mice, we analyzed genomic DNA from each of the cell types using the Affymetrix 100k SNP GeneChip Mapping Array set. Shown in Fig. 7 are the progressive changes in the structure of chromosomes 1–22 and X. This analysis clearly demonstrates the progression of cancer occurring throughout cell transformation of MCF-10F. Gross copy number abnormalities are rarely observed in the MCF-10F cells transformed with E_2 (Fig. 7, E_2 lanes). The earliest event is a gain in chromosome 1p, 1p36.12–1p36.21. In the Boyden chamber-selected cells and its sub-clones (Fig. 7, C5 and Clone lanes) additional gains are seen in chromosome 1p, 1p36.12-1pter, and chromosome 5q, 5q21.1–5q35.3; losses are observed at chromosome 4 and chromosome 8p, 8p11.1–8p23.1. In cells derived from tumors in SCID mice (Fig. 7, Tumor lanes), additional losses are seen at chromosome 3p, 3p12.1–3p14.1, chromosome 9p, 9p22.1–9p24.3, and chromosome 18q, 18q11.2–18q23.

In summary, we have accumulated evidence indicating that E_2 and its metabolites are mutagenic as an early event in the process of transformation of the human breast epithelium. The fact that an antiestrogen did not prevent these mutations indicates that the carcinogenic effect of this hormone and its metabolites is independent of the receptor pathway.

7. Analysis of possible biomarkers for human prostate cancer

The estrogen metabolites, GSH conjugates and depurinating DNA adducts provide several possible biomarkers for risk of developing estrogen-initiated cancers. Spectroscopic studies of 4-OHE_1-1-N3Ade, 4-OHE_1-1-N7Gua, 4-OHE_2-1-N3Ade, and 4-OHE_2-1-N7Gua adduct standards have been performed at different temperatures [100]. Upon high-energy laser excitation at 257 nm, the 4-OHE_1- and 4-OHE_2-derived N7Gua and N3Ade adducts are strongly phosphorescent at liquid nitrogen temperature (T=77 K). No phosphorescence was observed at room temperature (~300 K). The limit of detection (LOD) for the N3Ade and N7Gua adducts, based on phosphorescence measurements, is in the low femtomole range (about 10^{-9} M) [100]. The LOD in capillary electrophoresis with field amplified sample stacking (FASS) and absorbance detection is about 3×10^{-8} M [100,101]. We have demonstrated that CE-Q-derived DNA adducts can be identified in tissue extracts from

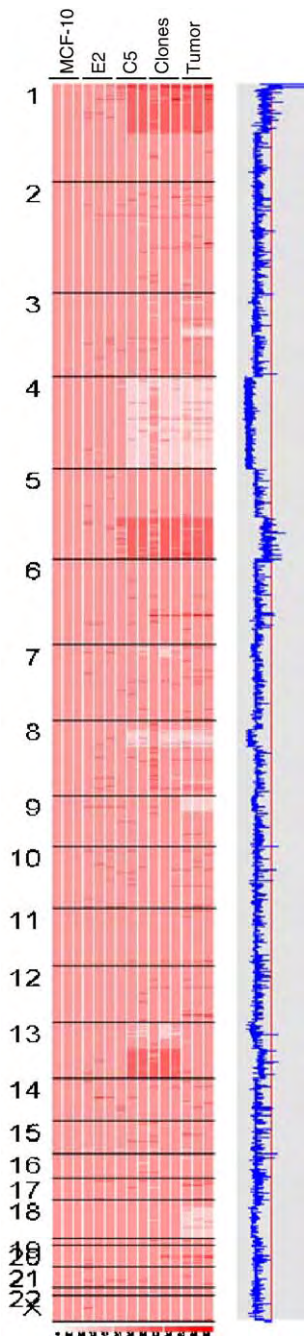


Fig. 7. Analysis of chromosomal copy number throughout cell transformation. The complete genome view of copy number determined by 100 k SNP analysis of genomic DNA is shown. Three individual samples of each group are identified at the top of the panel. The chromosomes are identified at the left from 1 to 22 and X. Darker areas indicate regions of gain; lighter areas indicate regions of loss. The average copy number is shown to the right. The analysis was performed using dCHIP software (Chen Li, Harvard University). DNA was isolated from: MCF-10F, the immortal, non-transformed HBEC; E_2, cells transformed with 70 nM E2; C5, cells selected for invasive growth using a Boyden chamber (E_2-70-C5 in text above); Clones, sub-clones of C5; Tumor, cells established from tumors produced in SCID mice. The changes seen in chromosome 13 were not consistent between replicated samples and did not reach statistical significance.

breast cancer patients [100]. To determine whether this type of DNA damage can be detected in human urine, urine samples from men with prostate cancer, benign tumors, or benign

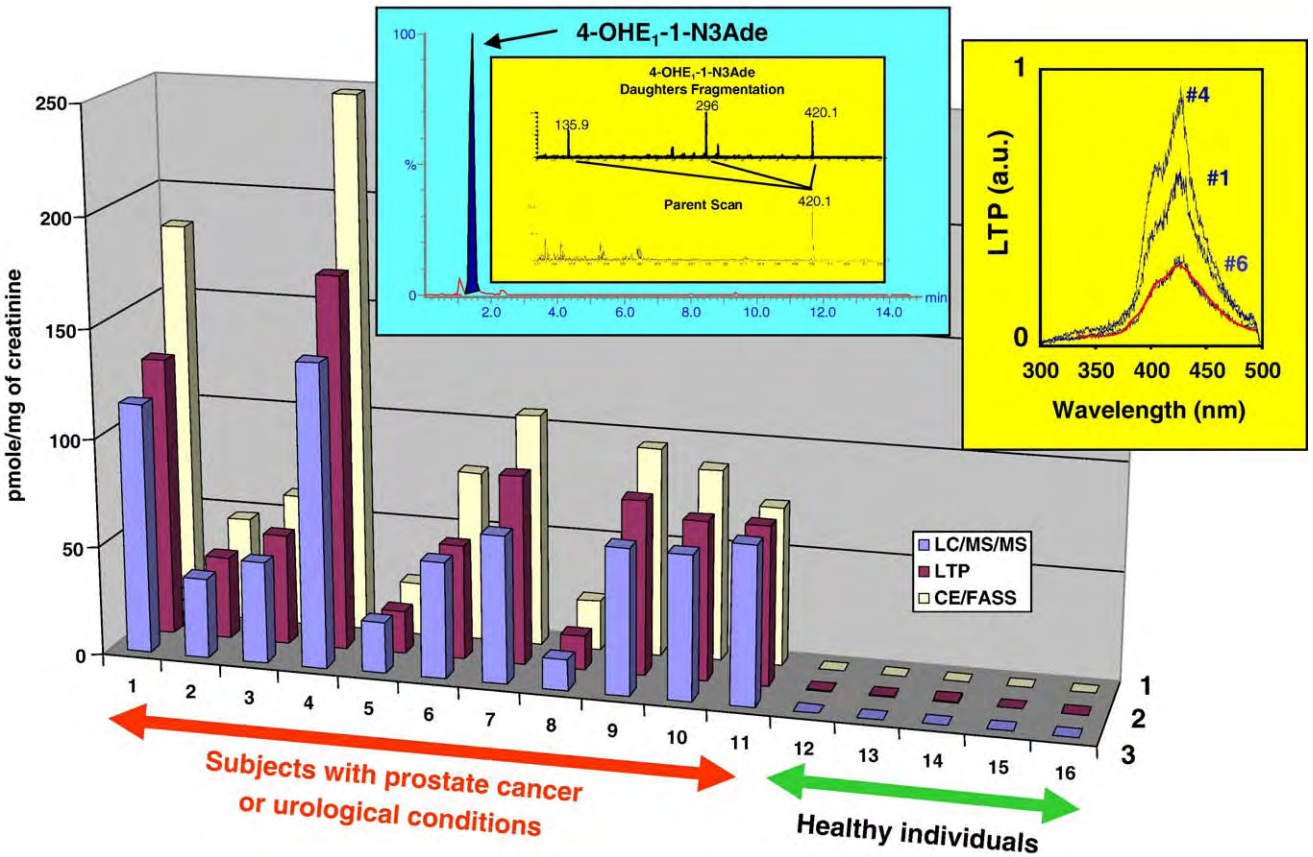


Fig. 8. Identification of the 4-OHE$_1$-1-N3Ade adduct in human urine samples from men with prostate cancer or urological conditions and healthy men as controls. Right inset: the spectra labeled 1, 4 and 6 refer to individual samples 1, 4 and 6, respectively; the red spectrum is that of the standard adduct. Left inset: identification of 4-OHE1-1-N3Ade by LC/MS/MS. The *m/z* 420.1 corresponds to the molecular weight of the parent compound and *m/z* 135.9 and 296 are the fragmentation daughters selected for the unequivocal identification of the adduct.

prostate hyperplasia, as well as healthy males, were analyzed in a blind study to determine the presence of 4-OHE$_1$(E$_2$)-1-N3Ade, one of the major adducts formed by CE-Q.

Urine samples (20 ml each) from sixteen subjects were analyzed by using several detection methods (Fig. 8). All specimens were initially analyzed using affinity column purification, i.e., the adducts of interest were extracted from the urine samples using home-built columns equipped with monoclonal antibody (MAb) specifically developed for the 4-OHE$_1$(E$_2$)-1-N3Ade adducts and purified on a multiple antigenic peptide resin bead column ([101] and unpublished results). This purified MAb was immobilized on an agarose bead column and used to detect 4-OHE$_1$-1-N3Ade. We have demonstrated that this MAb binds with high affinity to 4-OHE$_1$-1-N3Ade adducts ($K_a = 10^8$ M^{-1}), highly discriminating against a large spectrum of closely related CE-Q-derived metabolites ([102] and unpublished results). Eluted extracts from the immunoaffinity columns were analyzed by laser-excited low-temperature phosphorescence spectroscopy and liquid chromatography interfaced with tandem mass spectrometry (LC/MS/MS). In addition, urine samples, after lyophilization and methanol extraction, were pre-concentrated and the analytes therein were separated by capillary electrophoresis with FASS and detected by absorbance-based electropherograms. A spiking procedure with synthesized DNA adduct-standards and absorption spectroscopy was used to identify the biomarkers of interest.

In Fig. 8, the bars in the first row correspond to the integrated (normalized) area of the absorbance-based capillary electrophoresis electropherogram peaks assigned to 4-OHE$_1$(E$_2$)-1-N3Ade. Only the samples from the subjects with prostate cancer or urological conditions contained 4-OHE$_1$(E$_2$)-1-N3Ade adduct, with concentration levels of about 15–240 pmol per mg of creatinine. The identity of the N3Ade adduct in these samples was confirmed by low-temperature (77K) luminescence spectroscopy, as shown by the bars in the second row in Fig. 8. An example of the phosphorescence spectrum obtained for sample #11 is shown in the right inset of Fig. 8. This spectrum is nearly indistinguishable from the spectrum of the N3Ade adduct standard ([100,101], unpublished data)]. The amount of this adduct in the 11 samples detected by using low temperature phosphorescence-based calibration curves was about 10–150 pmol per mg of creatinine. With a detection limit of about 10^{-9} M [100], no 4-OHE$_1$-1-N3Ade adducts were observed in the five control samples, in agreement with the capillary electrophoresis/FASS results. The observed emission intensity was near the background level.

Finally, LC/MS/MS was used for further validation of the above findings using the samples eluted from the immunoaffinity

columns as shown in the third row of Fig. 8. Although similar adduct distribution is observed in all samples using the three different methodologies, the relative adduct concentrations observed in eluents from immunoaffinity columns were somewhat smaller than that observed by capillary electrophoresis/FASS with absorbance detection. This is not surprising, since the recovery from a typical column is 70–80% [100,101]. An example of the LC/MS/MS obtained for sample #11 is shown in the left inset of Fig. 8; the major peak of the LC chromatogram corresponds to the 4-OHE_1-1-N3Ade adduct and indicates that the eluent from the immunoaffinity column was relatively pure. The upper spectrum corresponds to the daughters, *m*/*z* 135.9 and 296.0, which were obtained from fragmentation of the adduct parent ion, *m*/*z* 420.1. Thus, 4-OHE_1-1-N3Ade is excreted into the urine of subjects with prostate cancer, suggesting that this adduct may be a biomarker for risk of developing prostate cancer.

In addition to the three analytical methods used in the initial study of N3Ade adducts in the urine of prostate cancer patients and control men, other promising methods are being explored. To analyze the estrogen-DNA adducts and/or estrogen-GSH conjugates in human serum or urine, novel MAb-based biosensor methodologies and improved microfluidic devices are being developed. These methodologies offer the promise of clinically useful assays. CE metabolites [42], CE-Q-derived DNA adducts [100] and/or CE-Q-derived conjugates [101,102] could serve as biomarkers to investigate the hypothesis that metabolically activated endogenous estrogens might be involved in initiating cancer of both the prostate and the breast. In addition, these CE-Q-derived DNA adducts identified in the urine of breast and prostate cancer patients could serve as biomarkers to assess cancer risk.

8. Overall conclusions

Exposure to estrogens is associated with increased risk of breast, prostate and other types of human cancer. Cancer is expressed as perhaps as many as 200 diseases, but we hypothesize that many of the most prevalent, most lethal types of cancer are initiated by a common, estrogen-initiated mechanism. This mechanism derives from specific estrogen metabolites that react with DNA to form predominantly depurinating adducts. These adducts are released from DNA to generate apurinic sites [28–31]. If these sites are in critical oncogenes and/or tumor suppressor genes, they could initiate breast, prostate and other cancers.

Estrogens may become tumor initiators when estrogen metabolism is unbalanced and the CE-Q are formed. These quinones can react with DNA to form depurinating N3Ade and N7Gua adducts as the predominant adducts (>99%). Analogous depurinating adducts are formed by the catechol quinones of the human carcinogen diethylstilbestrol, the leukemogen benzene [80] and the neurotransmitter dopamine [80].

Evidence for the role of the CE-Q in the initiation of breast and prostate cancer has been acquired from a variety of studies. A key point has been the demonstration of mutagenic activity by the estrogen metabolite 4-OHE_2 in a reporter gene in the mammary gland of BB® rats, as well as BB® rat2 cells [34,35]. The E_2-3,4-Q also has been shown to be mutagenic in the H-*ras* oncogene in mouse skin and rat mammary gland [32,33]. These results demonstrate the genotoxicity of estrogen metabolites. The mutations induced by estrogens could give rise to abnormal cell proliferation that through various processes, including hormone receptor-mediated ones, promote the development of cancer.

Mammary tumors develop in female transgenic, knock-out ERKO/Wnt-1 mice, despite the lack of ER-α [82,83]. The development of these tumors in ovariectomized ERKO/Wnt-1 mice depends on the amount of E_2 administered to them [86], and tumor development is not reduced by simultaneous administration of the anti-estrogen ICI-182,780 [86]. Malignant transformation of MCF-10F cells by E_2 and 4-OHE_2 occurs despite the lack of ER-α in these cells and in the presence of the anti-estrogen ICI-182,780 [45–48]. These transformed MCF-10F cells accumulate genetic changes, and the aggressively transformed MCF-10F cells induce poorly differentiated adenocarcinomas in SCID mice [48].

Further evidence has been obtained from studies of women with and without breast cancer and men with and without prostate cancer. Non-tumor breast tissue from women with breast carcinoma contained significantly higher amounts of 4-OHE_2 and CE-Q conjugates of GSH, compared to breast tissue from women without breast cancer [42]. In addition, enzymes involved in the metabolism of estrogens to CE-Q had higher expression in breast tissue of women with breast cancer, whereas expression of protective enzymes was lower [71]. The depurinating DNA adduct 4-OHE_1-1-N3Ade was detected at a much higher level in breast tissue from a woman with breast cancer, compared to a control woman [100]. Finally, the 4-OHE_1-1-N3Ade adduct is excreted in the urine of men with prostate cancer, whereas the level in urine of healthy men is virtually nil.

These data suggest that some of the estrogen-DNA adducts, estrogen-GSH conjugates and estrogen metabolites may serve as biomarkers for risk of developing breast, prostate and other cancers. We think they would be detected long before tumors appear. All of these results lay the groundwork for strategies to assess risk and prevent disease.

Acknowledgements

We thank Dr. David Longfellow, Coordinator of the Cancer Cube, a focus group on estrogen genotoxicity leading to cancer, for his valuable and extensive advice and support. We also thank the other members of the Cancer Cube for many valuable scientific discussions and Ms. K. Grotzinger for her outstanding facilitation of Cancer Cube activities. We thank Drs. G. Balogh, S. Fernandez, N. Gaikwad, Y. Huang, M. Khmelnitsky, Y. Markushin, M. Saeed, S. Singh, B. Trock, D. Venugopal, W. Yue, M. Zahid, and Z.-L. Zhao, Ms. S. Goodwin, Ms. S. Higginbotham, and Ms. P. Mailander for their contributions to this research. This work was supported by grants DAMD17-00-1-0247, DAMD17-03-1-0229, P01 CA49210, P20 RR15563. Core support at the Eppley Institute was provided by grant P30 CA36727 from the National Cancer Institute.

References

[1] J.F. Dorgan, C. Longcope, H.E. Stephenson Jr., R.T. Falk, R. Miller, C. Franz, L. Kahle, W.S. Campbell, J.A. Tangrea, A. Schatzkin, Relation of prediagnostic serum estrogen and androgen levels to breast cancer risk, Cancer Epidemiol. Biomark. Prev. 5 (1996) 533–539.

[2] J.F. Dorgan, F.Z. Stanczyk, C. Longcope, H.E. Stephenson Jr., L. Chang, R. Miller, C. Franz, R.T. Falk, L. Kahle, Relationship of serum dehydroepiandrosterone (DHEA), DHEA sulfate, and 5-androstate-3β, 17β-diol to risk of breast cancer in postmenopausal women, Cancer Epidemiol. Biomark. Prev. 6 (1997) 177–181.

[3] H.V. Thomas, T.J. Key, D.S. Allen, J.W. Moore, M. Dowsett, I.S. Fentiman, D.Y. Wang, A prospective study of endogenous serum hormone concentrations and breast cancer risk in postmenopausal women on the island of Guernsey, Br. J. Cancer 76 (1997) 401–405.

[4] S.E. Hankinson, W.C. Willett, J.E. Manson, G.A. Colditz, D.J. Hunter, D. Spiegelman, R.L. Barbieri, F.E. Speizer, Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women, J. Natl. Cancer Inst. 90 (1998) 1292–1299.

[5] P.G. Toniolo, M. Levitz, A. Zeleniuch-Jacquotte, S. Banerjee, K.L. Koenig, R.E. Shore, P. Strax, B.S. Pasternack, A prospective study of endogenous estrogens and breast cancer in postmenopausal women, J. Natl. Cancer Inst. 87 (1995) 190–197.

[6] A. Zeleniuch-Jacquotte, P.F. Bruning, J.M. Bonfrer, K.L. Koenig, R.E. Shore, M.Y. Kim, B.S. Pasternack, P. Toniolo, Relation of serum levels of testosterone and dehydroepiandrosterone sulfate to risk of breast cancer in postmenopausal women, Am. J. Epidemiol. 145 (1997) 1030–1038.

[7] F. Berrino, P. Muti, A. Micheli, G. Bolelli, V. Krogh, R. Sciajno, P. Pisani, S. Panico, G. Secreto, Serum sex hormone levels after menopause and subsequent breast cancer, J. Natl. Cancer Inst. 88 (1996) 291–296.

[8] E. Barrett-Connor, N.J. Friedlander, K.T. Khaw, Dehydroepiandrosterone sulfate and breast cancer risk, Cancer Res. 50 (1990) 6571–6574.

[9] C.F. Garland, N.J. Friedlander, E. Barrett-Connor, K.T. Khaw, Sex hormones and postmenopausal breast cancer: a prospective study in an adult community, Am. J. Epidemiol. 135 (1992) 1220–1230.

[10] M. Kabuto, S. Akiba, R.G. Stevens, K. Neriishi, C.E. Land, A prospective study of estradiol and breast cancer in Japanese women, Cancer Epidemiol. Biomark. Prev. 9 (2000) 575–579.

[11] J.A. Cauley, F.L. Lucas, L.H. Kuller, K. Stone, W. Browner, S.R. Cummings, Elevated serum estradiol and testosterone concentrations are associated with a high risk for breast cancer. Study of Osteoporotic Fractures Research Group, Ann. Intern. Med. 130 (1999) 270–277.

[12] G.B. Gordon, T.L. Bush, K.J. Helzlsouer, S.R. Miller, G.W. Comstock, Relationship of serum levels of dehydroepiandrosterone and dehydroepiandrosterone sulfate to the risk of developing postmenopausal breast cancer, Cancer Res. 50 (1990) 3859–3862.

[13] K.J. Helzlsouer, A.J. Alberg, T.L. Bush, C. Longcope, G.B. Gordon, G.W. Comstock, A prospective study of endogenous hormones and breast cancer, Cancer Detec. Prev. 18 (1994) 79–85.

[14] Endogenous Hormones and Breast Cancer Collaborative Group, Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies, J. Natl. Cancer Inst. 94 (2002) 606–616.

[15] R. Kaaks, F. Berrino, T. Key, S. Rinaldi, L. Dossus, C. Biessy, G. Secreto, P. Amiano, S. Bingham, H. Boeing, H.B. Bueno de Mesquita, J. Chang-Claude, F. Clavel-Chapelon, A. Fournier, C.H. van Gils, C.A. Gonzalez, A.B. Gurrea, E. Critselis, K.T. Khaw, V. Krogh, P.H. Lahmann, G. Nagel, A. Olsen, N.C. Onland-Moret, K. Overvad, D. Palli, S. Panico, P. Peeters, J.R. Quiros, A. Roddam, A. Thiebaut, A. Tjonneland, M.D. Chirlaque, A. Trichopoulou, D. Trichopoulos, R. Tumino, P. Vineis, T. Norat, P. Ferrari, N. Slimani, E. Riboli, Serum sex steroids in premenopausal women and breast cancer risk within the European Prospective Investigation into Cancer and Nutrition (EPIC), J. Natl. Cancer Inst. 97 (2005) 755–765.

[16] H.S. Feigelson, B.E. Henderson, Estrogens and breast cancer, Carcinogenesis 17 (1996) 2279–2284.

[17] R.B. Dickson, G.M. Stancel, Estrogen receptor-mediated processes in normal and cancer cells, in: E. Cavalieri, E. Rogan (Eds.), JNCI Monograph 27, Estrogens as Endogenous Carcinogens in the Breast and Prostate, Oxford Press, 2000, pp. 135–145.

[18] J.G. Liehr, W.F. Fang, D.A. Sirbasku, A. Ari-Ulubelen, Carcinogenicity of catechol estrogens in Syrian hamsters, J. Steroid Biochem. 24 (1986) 353–356.

[19] S. Nandi, Role of hormones in mammary neoplasia, Cancer Res. 38 (1978) 4046–4049.

[20] J.J. Li, Estrogen carcinogenesis in hamster tissues: update, Endocr. Rev. 14 (1993) 94–95.

[21] R. Lang, U. Redmann, Non-mutagenicity of some sex hormones in the Salmonella/microsome test, Mutat. Res. 67 (1979) 361–365.

[22] R. Lang, R. Reiman, Studies for a genotoxic potential of some endogenous and exogenous sex steroids. I. Communication: examination for the induction of gene mutations using the Ames Salmonella/microsome test and the HGPRT test in V79 cells, Environ. Mol. Mutagen. 21 (1993) 272–304.

[23] C. Drevon, C. Piccoli, R. Montesano, Mutagenicity assays of estrogenic hormones in mammalian cells, Mut. Res. 89 (1981) 83–90.

[24] J. Furth, Hormones as etiological agents in neoplasia, in: F.F. Becker (Ed.), Cancer. A Comprehensive Treatise. 1. Etiology: Chemical and Physical Carcinogenesis, Chapt. 4, Cancer Plenum Press, New York, NY, 1982, pp. 89–134.

[25] J.J. Li, S.A. Li, Estrogen carcinogenesis in hamster tissues: a critical review, Endocr. Rev. 11 (1990) 524–531.

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

[27] W.C. Hahn, R.A. Weinberg, Rules for making tumor cells, N. Engl. J. Med. 347 (2002) 1593–1603.

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

[29] E. Cavalieri, K. Frenkel, J.G. Liehr, E. Rogan, D. Roy, Estrogens as endogenous genotoxic agents: DNA adducts and mutations, in: E. Cavalieri, E. Rogan (Eds.), JNCI Monograph 27: Estrogens as Endogenous Carcinogens in the Breast and Prostate, Oxford Press, 2000, pp. 75–93.

[30] E. Cavalieri, E. Rogan, D. Chakravarti, The role of endogenous catechol quinones in the initiation of cancer and neurodegenerative diseases, in: H. Sies, L. Packer (Eds.), Methods in Enzymology, Quinones and Quinone Enzymes, Part B, vol. 382, Elsevier, Duesseldorf, Germany, 2004, pp. 293–319.

[31] K.-M. Li, R. Todorovic, P. Devanesan, S. Higginbotham, H. Köfeler, R. Ramanathan, M.L. Gross, E.G. Rogan, E.L. Cavalieri, Metabolism and DNA binding studies of 4-hydroxyestradiol and estradiol-3,4-quinone in vitro and in female ACI rat mammary gland in vivo, Carcinogenesis 25 (2004) 289–297.

[32] D. Chakravarti, P.C. Mailander, K.-M. Li, S. Higginbotham, H.L. Zhang, M.L. Gross, J.L. Meza, E.L. Cavalieri, E.G. Rogan, Evidence that a burst of DNA depurination in SENCAR mouse skin induces error-prone repair and forms mutations in the H-ras gene, Oncogene 20 (2001) 7945–7953.

[33] D. Chakravarti, P.C. Mailander, S. Higginbotham, E.L. Cavalieri, E.G. Rogan, The catechol estrogen-3,4-quinone metabolite induces mutations in the mammary gland of ACI rats, Proc. Am. Assoc. Cancer Res. 44 (2003) 180.

[34] J.B. Guttenplan, Effects of estradiol, 4-hydroxyestradiol and estradiol 2,3- and 3,4-quinones on mutagenesis in vivo and in vitro, U.S. Army Breast Cancer Research Program Era of Hope meeting, June 8–11, 2005.

[35] Z. Zhao, W. Kosinska, M. Khmelnitsky, E.L. Cavalieri, E.G. Rogan, D. Chakravarti, P. Sacks, J.B. Guttenplan, Mutagenic activity of 4-hydroxyestradiol, but not 2-hydroxyestradiol, in BB2 rat embryonic cells, and the mutational spectrum of 4-hydroxyestradiol, Chem. Res. Toxicol. 19 (2006) 475–479.

[36] E.L. Cavalieri, E.G. Rogan, Mechanisms of tumor initiation by polycyclic aromatic hydrocarbons in mammals, in: A.H. Neilson (Ed.), The

Handbook of Environmental Chemistry: PAHs and Related Compounds, vol. 3J, Springer, Heidelberg, Germany, 1998, pp. 81–117.
[37] E.L. Cavalieri, E.G. Rogan, K.-M. Li, R. Todorovic, F. Ariese, R. Jankowiak, N. Grubor, G.J. Small, Identification and quantification of the depurinating DNA adducts formed in mouse skin treated with dibenzo[*a,l*] pyrene (DB[*a,l*]P) or its metabolites and in rat mammary gland treated with DB[*a,l*]P, Chem. Res. Toxicol. 18 (2005) 976–983.
[38] D. Chakravarti, J.C. Pelling, E.L. Cavalieri, E.G. Rogan, Relating aromatic hydrocarbon-induced DNA adducts and c-Harvey-ras mutations in mouse skin papillomas: the role of apurinic sites, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 10422–10426.
[39] D. Chakravarti, P.C. Mailander, E.L. Cavalieri, E.G. Rogan, Evidence that error-prone DNA repair converts dibenzo[*a,l*]pyrene-induced depurinating lesions into mutations: formation, clonal proliferation and regression of initiated cells carrying H-ras oncogene mutations in early preneoplasia, Mutat. Res. 456 (2000) 17–32.
[40] E.L. Cavalieri, S. Kumar, R. Todorovic, S. Higginbotham, A.F. Badawi, E.G. Rogan, Imbalance of estrogen homeostasis in kidney and liver of hamsters treated with estradiol: implications for estrogen-induced initiation of renal tumors, Chem. Res. Toxicol. 14 (2001) 1041–1050.
[41] E.L. Cavalieri, P. Devanesan, M.C. Bosland, A.F. Badawi, E.G. Rogan, Catechol estrogen metabolites and conjugates in different regions of the prostate of Noble rats treated with 4-hydroxyestradiol: implications for estrogen-induced initiation of prostate cancer, Carcinogenesis 23 (2002) 329–333.
[42] E.G. Rogan, A.F. Badawi, P.D. Devanesan, J.L. Meza, J.A. Edney, W.W. West, S.M. Higginbotham, E.L. Cavalieri, Relative imbalances in estrogen metabolism and conjugation in breast tissue of women with carcinoma: potential biomarkers of susceptibility to cancer, Carcinogenesis 24 (2003) 697–702.
[43] J.J. Li, S.A. Li, Estrogen carcinogenesis in Syrian hamster tissue: role of metabolism, Fed. Proc. 46 (1987) 1858–1863.
[44] R.R. Newbold, J.G. Liehr, Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens, Cancer Res. 60 (2000) 235–237.
[45] J. Russo, M.H. Lareef, Q. Tahin, Y.F. Hu, C. Slater, X. Ao, I.H. Russo, 17Beta-estradiol is carcinogenic in human breast epithelial cells, J. Steroid Biochem. Mol. Biol. 80 (2002) 149–162.
[46] J. Russo, M.H. Lareef, G. Balogh, S. Guo, I.H. Russo, Estrogen and its metabolites are carcinogenic agents in human breast epithelial cells, J. Steroid Biochem. Mol. Biol. 87 (2003) 1–25.
[47] M.H. Lareef, J. Garber, P.A. Russo, I.H. Russo, R. Heulings, J. Russo, The estrogen antagonist ICI-182-780 does not inhibit the transformation phenotypes induced by 17-beta-estradiol and 4-OH estradiol in human breast epithelial cells, Int. J. Oncol. 26 (2005) 423–429.
[48] S.V. Fernandez, I.H. Russo, J. Russo, Estradiol and its metabolites 4-hydroxyestradiol and 2-hydroxyestradiol induce mutations in human breast epithelial cells, Int. J. Cancer 118 (2006) 1862–1868.
[49] F.P. Guengerich, Characterization of human microsomal cytochrome P-450 enzymes, Annu. Rev. Pharmacol. Toxicol. 29 (1989) 241–264.
[50] C.P. Martucci, J. Fishman, P450 Enzymes of estrogen metabolism, Pharmacol. Ther. 57 (1993) 237–257.
[51] B.T. Zhu, A.H. Conney, Functional role of estrogen metabolism in target cells: review and perspectives, Carcinogenesis 9 (1998) 1–27.
[52] M. Zahid, E. Kohli, M. Saeed, E. Rogan, E. Cavalieri, The greater reactivity of estradiol-3,4-quinone versus estradiol-2,3-quinone with DNA in the formation of depurinating DNA adducts. Implications for tumor-initiating activity, Chem. Res. Toxicol. 19 (2005) 164–172.
[53] M. Saeed, M. Zahid, S.J. Gunselman, E. Rogan, E. Cavalieri, Slow loss of deoxyribose from the N7deoxyguanosine adducts of estradiol-3,4-quinone and hexestrol-3′,4′-quinone. Implications for mutagenic activity, Steroids 70 (2005) 29–35.
[54] P.T. Männistö, S. Kaakola, Catechol-*O*-methyltransferase (COMT): biochemistry, molecular biology, pharmacology and clinical efficacy of the new selective COMT inhibitors, Pharmacol. Rev. 51 (1999) 593–628.
[55] J.A. Mobley, A.S. Bhat, R.W. Brueggemeier, Measurement of oxidative DNA damage by catechol estrogens and analogues in vitro, Chem. Res. Toxicol. 12 (1999) 270–277.
[56] E. Cavalieri, Minisymposium on endogenous carcinogens: the catechol estrogen pathway. An introduction, Polycycl. Aromat. Compd. 6 (1994) 223–228.
[57] L. Ernester, R.W. Estabrook, P. Hochstein, S. Orrenius (Eds.), DT Diaphorase-A Quinone Reductase with Special Functions in Cell Metabolism and Detoxication, Chemica Scripta, vol. 27A, 1987.
[58] D. Roy, J.G. Liehr, Temporary decrease in renal quinone reductase activity induced by chronic administration of estradiol to male Syrian hamsters, J. Biol. Chem. 263 (1988) 3646–3651.
[59] W.R. Miller, J. O'Neill, The importance of local synthesis of estrogen within the breast, Steroids 50 (1987) 537–548.
[60] E.R. Simpson, M.S. Mahendroo, G.D. Means, M.W. Kilgore, M.M. Hinshelwood, S. Graham-Lorence, B. Amarneh, Y. Ito, C.R. Fisher, M.D. Michael, C.R. Mendelson, S.E. Bulun, Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis, Endocr. Rev. 15 (1994) 342–355.
[61] C.R. Jefcoate, J.G. Liehr, R.J. Santen, T.R. Sutter, J.D. Yager, W. Yue, S.J. Santner, R. Tekmal, L. Demers, R. Pauley, F. Naftolin, G. Mor, L. Berstein, in: E. Cavalieri, E. Rogan (Eds.), Tissue-Specific Synthesis and Oxidative Metabolism of Estrogens, JNCI Monograph 27: Estrogens as Endogenous Carcinogens in the Breast and Prostate, Oxford Press, 2000, pp. 95–112.
[62] S.J. Santner, P.D. Feil, R.J. Santen, In situ estrogen production via the estrone sulfatase pathway in breast tumors: relative importance versus the aromatase pathway, J. Clin. Endocrinol. Metab. 59 (1984) 29–33.
[63] J.R. Pasqualini, G. Chetrite, C. Blacker, M.C. Feinstein, L. Delalonde, M. Talbi, C. Maloche, Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients, J. Clin. Endocrinol. Metab. 81 (1996) 1460–1464.
[64] G.S. Chetrite, J. Cortes-Prieto, J.C. Philippe, F. Wright, J.R. Pasqualini, Comparison of estrogen concentrations, estrone sulfatase and aromatase activities in normal, and in cancerous, human breast tissues, J. Steroid Biochem. Mol. Biol. 72 (2000) 23–27.
[65] C.L. Hayes, D.C. Spink, B.C. Spink, J.Q. Cao, N.J. Walker, T.R. Sutter, 17β-Estradiol hydroxylation catalyzed by human cytochrome P450 1B1, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 9776–9781.
[66] D.C. Spink, B.C. Spink, J.Q. Cao, J.A. DePasquale, B.T. Pentecost, M.J. Fasco, Y. Li, T.R. Sutter, Differential expression of CYP1A1 and CYP1B1 in human breast epithelial cells and breast tumor cells, Carcinogenesis 19 (1998) 291–298.
[67] K. Mitrunen, A. Hirvonen, Molecular epidemiology of sporadic breast cancer. The role of polymorphic genes involved in oestrogen biosynthesis and metabolism, Mutat. Res. 544 (2003) 9–41.
[68] P. Devanesan, R.J. Santen, W.P. Bocchinfuso, K.S. Korach, E.G. Rogan, E. Cavalieri, Catechol estrogen metabolites and conjugates in mammary tumors and hyperplastic tissue from estrogen receptor alpha knock out (ERKO)/Wnt 1 mice: implications for initiation of mammary tumors, Carcinogenesis 22 (2001) 1573–1576.
[69] J.G. Liehr, M.J. Ricci, 4-Hydroxylation of estrogens as marker of human mammary tumors, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 3294–3296.
[70] G.I. Murray, M.C. Taylor, M.C. McFayden, J.A. McKay, W.F. Greenlee, M.D. Burke, W.T. Melvin, Tumor-specific expression of cytochrome P450 CYP1B1, Cancer Res. 57 (1997) 3026–3031.
[71] S. Singh, D. Chakravarti, J.A. Edney, R.R. Hollins, P.J. Johnson, W.W. West, S.M. Higginbotham, E.L. Cavalieri, E.G. Rogan, Relative imbalances in the expression of estrogen-metabolizing enzymes in the breast tissue of women with breast carcinoma, Oncol. Rep. 14 (2005) 1091–1096.
[72] A.L. Herbst, H. Ulfelder, D.C. Poskanzer, Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women, N. Engl. J. Med. 284 (1971) 878–881.
[73] J.J. Li, S.A. Li, J.K. Klicka, J.A. Parsons, L.K. Lam, Relative carcinogenic activity of various synthetic and natural estrogens in the Syrian hamster kidney, Cancer Res. 43 (1983) 5200–5204.
[74] J.G. Liehr, A.M. Ballatore, B.B. Dague, A.A. Ulubelen, Carcinogenicity and metabolic activation of hexestrol, Chem.-Biol. Interact. 55 (1985) 157–176.

[75] H. Haaf, M. Metzler, In vitro metabolism of diethylstilbestrol by hepatic, renal and uterine microsomes of rats and hamsters, Biochem. Pharmacol. 34 (1985) 3107–3115.
[76] G. Blaich, M. Gåttlicher, P. Cikryt, M. Metzler, Effects of various inducers on diethylstilbestrol metabolism, drug-metabolizing enzyme activities and the aromatic hydrocarbon (Ah) receptor in male Syrian golden hamster livers, J. Steroid Biochem. 35 (1996) 201–204.
[77] M. Metzler, A. McLachlan, Oxidative metabolism of the synthetic estrogens hexestrol and dienestrol indicates reactive intermediates, Adv. Exp. Med. Biol. 136A (1981) 829–837.
[78] S.T. Jan, P.D. Devanesan, D.E. Stack, R. Ramanathan, J. Byun, M.L. Gross, E.G. Rogan, E.L. Cavalieri, Metabolic activation and formation of DNA adducts of hexestrol, a synthetic nonsteroidal carcinogenic estrogen, Chem. Res. Toxicol. 11 (1998) 412–419.
[79] M. Saeed, S.J. Gunselman, S. Higginbotham, E. Rogan, E. Cavalieri, Formation of the depurinating N3adenine and N7guanine adducts by reaction of DNA with hexestrol-3′,4′-quinone or enzyme-activated 3′-hydroxyhexestrol. Implications for a unifying mechanism of tumor initiation by natural and synthetic estrogens, Steroids 70 (2005) 37–45.
[80] M. Saeed, E. Rogan, E. Cavalieri, Mechanism of tumor initiation by the human carcinogen diethylstilbestrol. The defining link to natural estrogens, Proc. Am. Assoc. Cancer Res. 46 (2005) 2129.
[81] E.L. Cavalieri, K.-M. Li, N. Balu, M. Saeed, P. Devanesan, S. Higginbotham, J. Zhao, M.L. Gross, E.G. Rogan, Catechol ortho-quinones: the electrophilic compounds that form depurinating DNA adducts and could initiate cancer and other diseases, Carcinogenesis 23 (2002) 1071–1077.
[82] W.P. Bocchinfuso, K.S. Korach, Mammary gland development and tumorigenesis in estrogen receptor knockout mice, J. Mammary Gland Biol. Neoplasia 2 (1997) 323–334.
[83] W.P. Bocchinfuso, W.P. Hively, J.F. Couse, H.E. Varmus, K.S. Korach, A mouse mammary tumor virus-Wnt1 transgene induces mammary gland hyperplasia and tumorigenesis in mice lacking estrogen receptor-alpha, Cancer Res. 59 (1999) 1869–1876.
[84] W. Yue, R.J. Santen, J.P. Wang, Y. Li, M.F. Verderame, W.P. Bocchinfuso, K.S. Korach, P. Devanesan, R. Todorovic, E.G. Rogan, E.L. Cavalieri, Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis, J. Steroid Biochem. Mol. Biol. 86 (2003) 477–486.
[85] R.J. Santen, W. Yue, W. Bocchinfuso, K. Korach, J.-P. Wang, E. Rogan, Y. Li, E. Cavalieri, J. Russo, P. Devanesan, M. Verderame, Estradiol-induced carcinogenesis via formation of genotoxic metabolites, in: J.N. Ingle, M. Dowsett (Eds.), Advances in Endocrine Therapy of Breast Cancer: Proceedings of the 2003 Gleneagles Conference, Marcel Dekker, New York, NY, 2004, pp. 163–177.
[86] W. Yue, J.-P. Wang, P. Fan, E. Rogan, E. Cavalieri, R.J. Santen, Estradiol can cause breast cancer through a non-receptor mediated mechanism in ERα knock-out/Wnt-1 transgenic mice, Proc. Am. Assoc. Cancer Res. 46 (2005) 2096.
[87] W. Yue, R. Todorovic, J.-P. Wang, R.J. Santen, E.L. Cavalieri, Aromatization of androgens and production of genotoxic estrogen metabolites in aromatase-transfected MCF-7 human breast cancer cells, Proc. Am. Assoc. Cancer Res. 40 (1999) 46.
[88] J.G. Liehr, Is estradiol a genotoxic mutagenic carcinogen? Endocr. Rev. 21 (2000) 40–54.
[89] M.S. Greenblatt, P.O. Chappuis, J.P. Bond, N. Hamel, W.D. Foulkes, TP53 mutations in breast cancer associated with BRCA1 or BRCA2 germ-line mutations: distinctive spectrum and structural distribution, Cancer Res. 61 (2001) 4092–4097.
[90] N. Rajapakse, M. Butterworth, A. Kortenkamp, Detection of DNA strand breaks and oxidized DNA bases at the single-cell level resulting from exposure to estradiol and hydroxylated metabolites, Environ. Mol. Mutagen. 45 (2005) 397–404.
[91] E. Yared, T.J. McMillan, F.L. Martin, Genotoxic effects of oestrogens in breast cells detected by the micronucleus assay and the Comet assay, Mutagenesis 17 (2002) 345–352.
[92] P.H. Lin, J. Nakamura, S. Yamaguchi, S. Asakura, J.A. Swenberg, Aldehydic DNA lesions induced by catechol estrogens in calf thymus DNA, Carcinogenesis 24 (2003) 1133–1141.
[93] G.A. Khan, D. Chakravarti, Imbalanced induction of short-patch base excision repair genes in dibenzo[*a,l*]pyrene-treated mouse skin and estradiol-3,4-quinone-treated rat mammary gland is associated with XRCC1 insufficiency, Proc. Am. Assoc. Cancer Res. 46 (2005) 719.
[94] J. Russo, G. Calaf, I.H. Russo, A critical approach to the malignant transformation of human breast epithelial cells, CRC Crit. Rev. Oncog. 4 (1993) 403–417.
[95] G. Calaf, J. Russo, Transformation of human breast epithelial cells by chemical carcinogens, Carcinogenesis 14 (1993) 483–492.
[96] J. Russo, I.H. Russo, Genotoxicity of steroidal estrogens, Trends Endocrinol. Metab. 15 (2004) 211–214.
[97] P.A. Russo, M.H. Lareef, J. Garber, I.H. Russo, S. Fernandez, R. Fernbaugh, B. Balsara, F. Sheriff, G.A. Balogh, D. Mailo, R. Heulings, J. Russo, An in vitro–in vivo model of estrogen induced human breast carcinogenesis, Proc. Am. Assoc. Cancer Res. 46 (2005) 2097.
[98] J. Russo, Estrogens as carcinogens in the human breast, Proc. Am. Assoc. Cancer Res. 46 (2005) 1464.
[99] J. Russo, G.A. Balogh, J. Chen, S.V. Fernandez, R. Fernbaugh, R. Heulings, D.A. Mailo, R. Moral, P.A. Russo, F. Sheriff, J.E. Vanegas, R. Wang, I.H. Russo, The concept of stem cell in the mammary gland and its implication in morphogenesis, cancer and prevention, Front. Biosci. 11 (2006) 151–172.
[100] Y. Markushin, W. Zhong, E.L. Cavalieri, E.G. Rogan, G.J. Small, E.S. Yeung, R. Jankowiak, Spectral characterization of catechol estrogen quinone (CEQ)-derived DNA adducts and their identification in human breast tissue extracts, Chem. Res. Toxicol. 16 (2003) 1107–1117.
[101] R. Jankowiak, Y. Markushin, E.L. Cavalieri, G.J. Small, Spectroscopic characterization of the 4-hydroxy catechol estrogen quinones-derived GSH and N-acetylated Cys conjugates, Chem. Res. Toxicol. 16 (2003) 304–311.
[102] Y. Markushin, P. Kapke, M. Saeed, H. Zhang, A. Dawoud, E.G. Rogan, E.L. Cavalieri, R. Jankowiak, Development of monoclonal antibodies to 4-hydroxyestrogen-2-N-acetylcysteine conjugates: immunoaffinity and spectroscopic studies, Chem. Res. Toxicol. 18 (2005) 1520–1527.

SPECIFIC AIM 1 – CAVALIERI

A. Introduction

The basic hypothesis guiding this research is that endogenous estrogens can be oxidatively metabolized to catechol estrogen quinones that react with DNA to form specific DNA adducts to generate tumor-initiating mutations. These DNA adducts are potential biomarkers for breast cancer and risk of developing breast cancer. In fact, the level of the 4-hydroxyestrone(estradiol)-1-N3Adenine adduct [4-OHE$_1$(E_2)-1-N3Ade] has been observed to be significantly higher in urine from men with prostate cancer or other urological conditions than in urine from healthy control men, in which the adduct is at background levels [1]. The results obtained in animal models, cell culture and human breast tissue led us to select several compounds to prevent the genotoxicity of estrogens that we think is at the origin of breast cancer. The selected compounds target different steps involved in the mechanism of tumor initiation. Prevention studies will demonstrate that estrogen genotoxicity plays a critical role in the initiation of breast cancer. In addition, the results will lay the groundwork for designing a clinical research study of breast cancer prevention and developing bioassays for susceptibility to this disease. With these goals in mind, we have obtained data demonstrating the utility of the depurinating estrogen-DNA adducts as biomarkers for risk of developing breast cancer and for use in prevention studies..

B. Body

B-i. Methods and Procedures

Research was previously completed on Specific Aim 1a.

In Specific Aim 1b, we continued analyzing urine from women with and without breast cancer by using ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS).

B-ii. Results

Specific aim #1a: Investigate the prevention of estradiol (E_2)-induced tumors in the mammary gland of female ACI rats by analyzing the profiles of estrogen metabolites, conjugates and depurinating DNA adducts in treated animals at various time-points and the development of tumors in the animals.

No further research was conducted on this specific aim because (1) the experiments were completed, (2) we realized that the planned experiments would not yield useful results, and (3) we had found that we could conduct more informative studies in humans.

Specific Aim #1b: Analyze the profiles of estrogen metabolites, conjugates and depurinating DNA adducts in ductal lavage samples from women with and without breast cancer.

In our initial study (not in this grant) we found that men with prostate cancer or other urological conditions had high levels of the estrogen-DNA adducts in their urine, while the healthy control men had background levels of the adducts [1]. This is the first demonstration of a quantifiable relationship between formation of depurinating estrogen-DNA adducts and human cancer. The success of this study led us to undertake in the COE a broader study of estrogen metabolism and formation of adducts in women, based on the knowledge that adducts formed in tissues are excreted in urine. The estrogen metabolites, conjugates and depurinating DNA

adducts in spot urine samples (~50 ml) from 46 healthy control women, 12 women at high risk of breast cancer (Gail model score > 1.66%) and 17 women with breast cancer were preconcentrated from 2-ml aliquots by solid-phase extraction (SPE) and analyzed by ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) [2]. The urine samples from healthy control women were provided by Dr. Paola Muti (under a different IRB protocol) or obtained at UNMC. Those from high-risk women were provided by Dr. James Ingle at the Mayo Clinic, and the ones from women with breast cancer were collected at the Mayo Clinic and at UNMC. The ratio of the adducts to their respective estrogen metabolites and conjugates was significantly higher in urine from the women with breast cancer ($p<0.001$) and high-risk women ($p<0.001$) than in urine from the healthy control women (Fig. 1) [2]. The levels in high-risk women and those with breast cancer did not differ ($p=0.62$). This study provides the first data showing that elevation of these DNA adducts is associated with high risk of developing breast cancer.

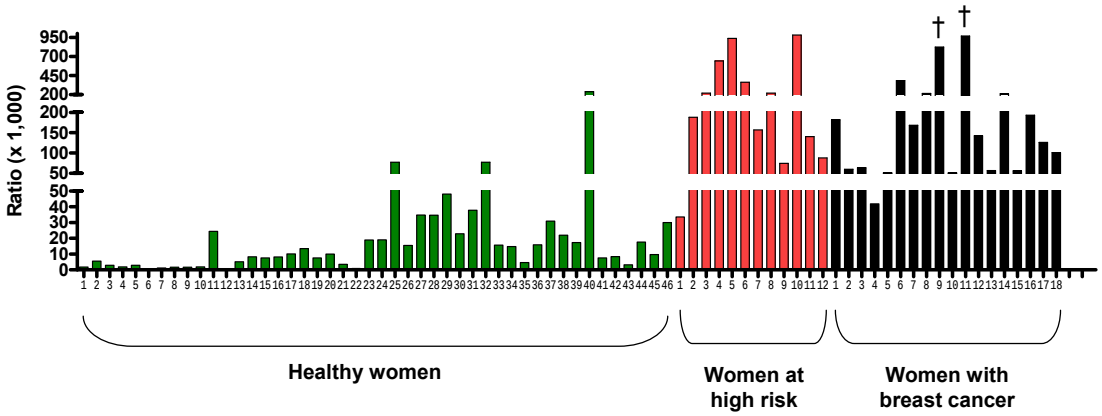


Figure 1. Depurinating estrogen-DNA adducts in the urine of healthy women, high-risk women and women with breast cancer [2]. The ordinate of this bar graph corresponds to the ratio of depurinating DNA adducts divided by their respective estrogen metabolites and conjugates:

$$\left(\frac{\text{4-OHE}_1(\text{E}_2)\text{-1-N3Ade} + \text{4-OHE}_1(\text{E}_2)\text{-1-N7Gua}}{\text{4-catechol estrogens + 4-catechol estrogen conjugates}} + \frac{\text{2-OHE}_1(\text{E}_2)\text{-6-N3Ade}}{\text{2-catechol estrogens + 2-catechol estrogen conjugates}} \right) \text{x } 1000$$

†These are two urine samples from the same subject, collected 11 weeks apart.

These results suggest that the estrogen-DNA adducts can be used as biomarkers for breast cancer. If the formation of estrogen-DNA adducts is the first critical event in the initiation of breast cancer, as we hypothesize, then these adducts could be used as biomarkers for early diagnosis of breast cancer risk. These adducts also will serve as surrogate endpoint biomarkers in studies of potential agents to prevent breast cancer. In fact, in an initial study with five healthy subjects (funded elsewhere), we have found that daily ingestion of 600 mg of the dietary supplement *N*-acetylcysteine for 30 days results in a significant decrease in the levels of estrogen-DNA adducts in urine and a concomitant increase in methoxy catechol estrogens, which represent the most important protective pathway in estrogen metabolism. These results provide preliminary evidence for one of the areas under study in the COE, the use of selected dietary supplements to reduce estrogen-DNA adduct formation and, presumably, reduce the risk of developing breast cancer.

C. Key Research Accomplishments

1. We have concluded that the Sprague-Dawley rat is not a model for estrogen-initiated tumors.
2. We have found in a small set of samples that nipple aspirate fluid from women with breast cancer contains estrogen metabolites, conjugates and depurinating DNA adducts, but nipple aspirate fluid from healthy control women does not contain the DNA adducts at levels detectable by UPLC/MS/MS. The adducts 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua in nipple aspirate fluid are potential biomarkers for breast cancer.
3. Most importantly, we have demonstrated that urine from women with breast cancer and women at high risk of breast cancer (Gail Model score >1.66%) contains significantly higher levels of 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua than does urine from healthy control women. The adducts 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua in urine are potential biomarkers for risk of developing breast cancer and for demonstrating the efficacy of possible agents to prevent breast cancer.
4. We have demonstrated that serum from women without breast cancer (healthy control women) contains baseline levels of estrogen metabolites, estrogen conjugates and the estrogen-DNA adducts 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua. The adducts 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua in serum are potential biomarkers for breast cancer.

D. Reportable Outcomes

a. Publications

1. Rogan, E.G., Badawi, A.F., Devanesan, P.D., Meza, J.L., Edney, J.A., West, W.W., Higginbotham, S.M. and Cavalieri, E.L. Relative imbalances in estrogen metabolism and conjugation in breast tissue of women with carcinoma: Potential biomarkers of susceptibility to cancer. Carcinogenesis, 24:697-702, 2003.

2. Yue, W., Santen, R.J., Wang, J-P., Li, Y., Verderame, M.F., Bocchinfuso, W.P., Korach, K.S., Devanesan, P., Todorovic, R., Rogan, E.G. and Cavalieri, E.L. Genotoxic metabolites of estradiol in breast: Potential mechanism of estradiol induced carcinogenesis. J. Steroid Biochem. Mol. Biol., 86:477-486, 2003.

3. Markushin, Y., Zhong, W., Cavalieri, E.L., Rogan, E.G., Small, G.J., Yeung, E.S. and Jankowiak, R. Spectral characterization of catechol estrogen quinone (CEQ)-derived DNA adducts and their identification in human breast tissue extract. Chem. Res. Toxicol., 16:1107-1117, 2003.

4. Li, K.-M., Todorovic, R., Devanesan, P., Higginbotham, S., Köfeler, H., Ramanathan, R., Gross, M.L., Rogan, E.G. and Cavalieri, E.L. Metabolism and DNA binding studies of 4-hydroxyestradiol and estradiol-3,4-quinone *in vitro* and in female ACI rat mammary gland *in vivo*. Carcinogenesis, 25:289-297, 2004.

5. Rogan, E.G. and Cavalieri, E.L. The role of specific estrogen metabolites in the initiation of breast and other human cancers. Int. J. Cancer Prev., 1:243-249, 2004.

6. Saeed, M., Zahid, M., Gunselman, S.J., Rogan, E. and Cavalieri, E. Slow loss of deoxyribose from the N7deoxyguanosine adducts of estradiol-3,4-quinone and hexestrol-3′,4′-quinone. Implications for mutagenic activity. Steroids, 70:29-35, 2005.

7. Saeed, M., Gunselman, S.J., Higginbotham, S., Rogan, E. and Cavalieri, E. Formation of the depurinating N3adenine and N7guanine adducts by reaction of DNA with hexestrol-3′-4′-quinone or enzyme-activated 3′-hydroxyhexestrol. Implications for a unifying mechanism of tumor initiation by natural and synthetic estrogens. Steroids, 70:37-45, 2005.

8. Yue, W., Wang, J.-P., Li, Y., Bocchinfuso, W., Korach, K., Devanesan, P., Rogan, E., Cavalieri, E. and Santen, R.J. Tamoxifen versus aromatase inhibitors for breast cancer prevention. Clin. Cancer Res., 11:925s-930s, 2005.

9. Saeed, M., Zahid, M., Rogan, E. and Cavalieri, E. Synthesis of the catechols of natural and synthetic estrogens by using 2-iodoxybenzoic acid (IBX) as the oxidizing agent. Steroids, 70:173-178, 2005.

10. Singh, S., Chakravarti, D., Edney, J.A., Hollins, R.R., Johnson, P.J., West, W.W., Higginbotham, S.M., Cavalieri, E.L. and Rogan, E.G. Relative imbalances in the expression of estrogen-metabolizing enzymes in the breast tissue of women with breast carcinoma. Oncology Reports, 14:1091-1096, 2005.

11. Markushin, Y., Kapke, P., Saeed, M., Zhang, H., Dawoud, A., Rogan, E.G., Cavalieri, E.L. and Jankowiak, R. Development of monoclonal antibodies to 4-hydroxyestrogen-2-*N*-acetylcysteine conjugates: Immunoaffinity and spectroscopic studies. Chem. Res. Toxicol., 18:1520-1527, 2005.

12. Zahid, M., Kohli, E., Saeed, M., Rogan, E. and Cavalieri, E. The greater reactivity of estradiol-3,4-quinone versus estradiol-2,3-quinone with DNA in the formation of depurinating adducts. Implications for tumor-initiating activity. Chem. Res. Toxicol., 19:164-172, 2006.

13. Zhao, Z., Kosinska, W., Khmelnitsky, M., Cavalieri, E.L., Rogan, E.G., Chakravarti, D., Sacks, P. and Guttenplan, J.B. Mutagenic activity of 4-hydroxyestradiol, but not 2-hydroxyestradiol, in BB rat2 embryonic cells, and the mutational spectrum of 4-hydoxyestradiol. Chem. Res. Toxicol., 19:475-479, 2006.

14. Markushin, Y., Gaikwad, N., Zhang, H., Kapke, P., Rogan, E.G., Cavalieri, E.L., Trock, B.J., Pavlovich, C. and Jankowiak, R. Potential biomarker for early risk assessment of prostate cancer. Prostate, 66:1565-1571, 2006.

15. Chakravarti, D., Zahid, M., Backora, M., Myers, E.M., Gaikwad, N., Weisenburger, D.D., Cavalieri, E.L., Rogan, E.G. and Joshi, S.S. *Ortho*-quinones of benzene and estrogens induce hyperproliferation of human peripheral blood mononuclear cells. Leuk. Lymphoma, 47:2635-2644, 2006.

16. Saeed, M., Rogan, E., Fernandez, S.V., Sheriff, F., Russo, J. and Cavalieri, E. Formation of depurinating N3Adenine and N7Guanine adducts by MCF-10F cells cultured in the presence of 4-hydroxyestradiol. Int. J. Cancer, 120:1821-1824, 2007.

17. Zhang, Y., Gaikwad, N.W., Olson, K., Zahid, M., Cavalieri, E.L. and Rogan, E.G. Cytochrome P450 isoforms catalyze formation of catechol estrogen quinones that react with DNA. Metabolism, in press, 2007.

18. Lu, F., Zahid, M., Saeed, M., Cavalieri, E.L. and Rogan, E.G. Estrogen metabolism and formation of estrogen-DNA adducts in estradiol-treated MCF-10F cells. The effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induction and catechol-*O*-methyltransferase inhibition. J. Steroid Biochem. Mol. Biol., doi 10.1016/jsbmb.2006.12.102, in press, 2007.

19. Gaikwad, N.W., Rogan, E.G. and Cavalieri, E.L. Evidence for NQO1-catalyzed reduction of estrogen *ortho*-quinones. Mass spectrometric determination of enzyme-substrate complex. Free. Radic. Biol. Med., in press, 2007.

20. Gaikwad, N.W., Muti, P., Meza, J., Yang, L., Pruthi, S., Ingle, J., Rogan, E.G. and Cavalieri, E.L. The molecular etiology of breast cancer and biomarkers of risk. Submitted, 2007.

b. Reviews and Book Chapters

1. Cavalieri, E., Rogan, E. and Chakravarti, D. The role of endogenous catechol quinones in the initiation of cancer and neurodegenerative diseases. In: Methods in Enzymology, Quinones and Quinone Enzymes, Part B (H. Sies & L. Packer, Eds.), Vol. 382, Elsevier, Duesseldorf, Germany, 293-319, 2004.

2. Santen, R.J., Yue, W., Bocchinfuso, W., Korach, K., Wang, J.-P., Rogan, E.G., Li, Y., Cavalieri, E., Russo, J., Devanesan, P. and Verderame, M. Estradiol-induced carcinogenesis via formation of genotoxic metabolites. In: Advances in Endocrine Therapy of Breast Cancer, (J.N. Ingle & M. Dowsett, Eds.), Marcel Dekker, New York, 163-177, 2004.

3. Rogan, E.G. and Cavalieri, E.L. Estrogen metabolites, conjugates and DNA adducts: Possible biomarkers for risk of breast, prostate and other human cancers, In: Adv. Clin. Chem., (G.S. Makowski, G. Nowacki & K.-J. Hsiao, Eds.) 38:135-149, 2004.

4. Cavalieri, E.L. and Rogan, E.G. A unifying mechanism in the initiation of cancer and other diseases by catechol quinones, Ann. NY Acad. Sci., 1028:247-257, 2004.

5. Jankowiak, R., Rogan, E.G. and Cavalieri, E.L. The role of fluorescence line-narrowing spectroscopy and related luminescence-based techniques in the elucidation of mechanisms of tumor initiation by polycyclic aromatic hydrocarbons and estrogens. J. Phys. Chem. B, 108:10266-10283, 2004.

6. Cavalieri, E., Chakravarti, D., Guttenplan, J., Hart, E., Ingle, J., Jankowiak, R., Muti, P., Rogan, E., Russo, J., Santen, R. and Sutter, T. Catechol estrogen quinones as initiators of

breast and other human cancers. Implications for biomarkers of susceptibility and cancer prevention. BBA-Reviews on Cancer, 1766:63-78, 2006.

7. Cavalieri, E. and Rogan, E. Catechol quinones of estrogen in the initiation of breast, prostate, and other human cancers. Ann. NY Acad. Sci., 1089:286-301, 2006.

c. Invited Presentations and Abstracts

1. Cavalieri, E. Meccanismo unificatore nell'etiologia del cancro e di altre malattie. La prevenzione è una realtà a portata di mano. First International Convention of the Italian Scientists in the World, Roma, Italy, March 10-12, 2003.

2. Rogan, E.G., Singh, S., Chakravarti, D., Higginbotham, S. and Cavalieri, E.L. Differential expression of estrogen-metabolizing enzymes in four quadrants of human breast. Proc. Amer. Assoc. Cancer Res., 44:307, 2003.

3. Cavalieri, E., Kohli, E., Zahid, M. and Rogan, E. Greater reactivity of estradiol-3,4-quinone *vs* estradiol-2,3-quinone with DNA in the formation of depurinating DNA adducts. Proc. Amer. Assoc. Cancer Res., 44:180, 2003.

4. Chakravarti, D., Mailander, P.C., Higginbotham, S., Cavalieri, E.L. and Rogan, E.G. The catechol estrogen-3,4-quinone metabolites induces mutations in the mammary gland of ACI rats. Proc. Amer. Assoc. Cancer Res., 44:180, 2003.

5. Cavalieri, E., Saeed, M., Gunselman, S. and Rogan, E. Depurination of adenine and guanine adducts occurs in DNA, but only very slowly in RNA. Proc. Amer. Assoc. Cancer Res., 44:1324, 2003.

6. Cavalieri, E. Initiation of cancer and neurodegenerative diseases by catechol quinones: A unifying mechanism. Erice, Sicily, Italy, October 24-31, 2003.

7. Cavalieri, E. La prevenzione è la cura del cancro. Lyons Club, Correggio Emilia, Italy, November 3, 2003.

8. Cavalieri, E. Unifying mechanism in the etiology of cancer and neurodegenerative diseases. Prevention is a reality at hand. Eppley Cancer Center Grand Rounds, UNMC, Omaha, NE, November 10, 2003.

9. Gunselman, S.J., Saeed, M., Higginbotham, S., Rogan, E.G. and Cavalieri, E.L. Formation of the depurinating N3adenine and N7guanine adducts by reaction of DNA with hexestrol-3′,4′-+quinone or enzyme-activated 3′-hydroxyhexestrol. Implications for a unifying mechanism of tumor initiation by natural and synthetic estrogens. Proc. Amer. Assoc. Cancer Res., 45:1564, 2004.

10. Saeed, M., Zahid, M., Gunselman, S.J., Rogan, E. and Cavalieri, E. Slow loss of deoxyribose from the N7deoxyguanosine adducts of estradiol-3,4-quinone and hexestrol-3′-4′-quinone. Implications for mutagenic activity. Proc. Amer. Assoc. Cancer Res., 45:1555, 2004.

11. Singh, S., Bosland, M.C., Cavalieri, E.L. and Rogan, E.L. Effect of treatment with estradiol or testosterone on the expression of CYP19, CYP1B1, COMT and NQO1 in the prostate of male Noble rats. Proc. Amer. Assoc. Cancer Res., 45:14, 2004.

12. Zhang, Y., Schoomaker, S., Cavalieri, E. and Rogan, E. Metabolism of estradiol by cytochrome P450 in rat liver microsomes to form quinones that bind to DNA. Proc. Amer. Assoc. Cancer Res., 45:1886, 2004.

13. Krisnamachari, V., Olson, K., Rogan, E. and Cavalieri, E.L. Reaction of dopamine *ortho*-quinone in the formation of depurinating adducts. Implications for neurodegenerative disorders. Proc. Amer. Assoc. Cancer Res., 45:1556, 2004.

14. Raghavanpillai, A., Zahid, M., Olson, K., Rogan, E. and Cavalieri, E. Slow loss of deoxyribose from N7deoxyguanosine adducts of catechol quinone: Possible relevance for mutagenic activity. Proc. Amer. Assoc. Cancer Res., 45:1553, 2004.

15. Cavalieri, E. Meccanismo unificatore nell'eziologia del cancro e di alter malattie. La prevenzione è una realtà a portata di mano. Center for High Technology and Education in Biomedical Sciences, Catholic Uhiversity, Campobasso, Italy, September 23, 2004.

16. Cavalieri, E., What is the scientific evidence that estrogen compounds induce genotoxic events initiating cancer? Molecular Biology Etiology Program, Eppley Research Institute, UNMC, October 26, 2004.

17. Cavalieri, E. and Rogan, E. Catechol quinones in the initiation of cancer and other diseases: A target for prevention. Keystone Symposia Meeting, Hormonal Regulation of Tumorigenesis, Monterey, California, February 20-25, 2005.

18. Saeed, M., Rogan, E. and Cavalieri, E. Mechanism of tumor initiation by the human carcinogen diethylstilbestrol: The defining link to natural estrogens. Proc. Amer. Assoc. Cancer Res., 46:2129, 2005.

19. Yue, W., Wang, J.-P., Fan, P., Rogan, E., and Cavalieri, E. Estradiol can cause breast cancer through a non-receptor mediated mechanism in ERα knockout/Wnt-1 transgenic mice. Proc. Amer. Assoc. Cancer Res., 46:2096, 2005.

20. Cavalieri, E.L. and Rogan, E.G. The etiology of breast cancer. Prevention is now the solution. Era of Hope, Department of Defense Breast Cancer Research Meeting, Philadelphia, PA, June 8-11, 2005

21. Cavalieri, E.L., Saeed, M. and Rogan, E. The mechanism of tumor initiation by the human carcinogen diethylstilbestrol is similar to that of natural estrogens. Era of Hope, Department of Defense Breast Cancer Research Meeting, Philadelphia, PA, June 8-11, 2005

22. Rogan, E.G. and Cavalieri, E.L. The role of estrogen metabolism in the initiation of human breast cancer. Era of Hope, Department of Defense Breast Cancer Research Meeting, Philadelphia, PA, June 8-11, 2005

23. Yue, W., Wang, J.-P., Fan, P., Rogan, E., Cavalieri, E., and Santen, R. Estradiol can cause breast cancer through a non-receptor mediated mechanism in ERα knockout/Wnt-1 transgenic mice. Era of Hope, Department of Defense Breast Cancer Research Meeting, Philadelphia, PA, June 8-11, 2005

24. Cavalieri, E., Russo, J., Guttenplan, J., Santen, R., Sutter, T., Rogan, E., Ingle, J., and Hart, E. Estrogen-induced depurination of DNA: A novel target for breast cancer prevention. Center for Excellence Meeting, Department of Defense Breast Cancer Research Program, Baltimore, MD, October 6-7, 2005.

25. Cavalieri, E. L'origine del cancro e dialtre malattie. Biblioteca Mandotti, Guastalla, Italy, September 12, 2005.

26. Cavalieri, E. L'origine commune du cancer et d'antres maladies. Rotary Club Lyon East, Lyon, France, October 17, 2005.

27. Cavalieri, E. The common mechanism in the initiation of cancer and other diseases. Department of Chemistry, Kansas State University, Manhattan, KS, November 17, 2005.

28. Gaikwad, N.W., Markushin, Y., Saeed, M., Jankowiak, R., Trock, B.J., Rogan, E.G. and Cavalieri, E.L. The depurinating 4-hydroxyestrone(estradiol)-1-N3Ade DNA adducts as biomarkers of prostate cancer risk. Proc. Amer. Assoc. Cancer Res., 47:78, 2006.

29. Gaikwad, N.W., Cavalieri, E.L. and Rogan, E.G. NQO1-catalyzed reduction of estradiol-3,4-quinone. Implications for tumor initiation by estrogens. Proc. Amer. Assoc. Cancer Res., 47:445, 2006.

30. Lu, F., Cavalieri, E.L. and Rogan, E.G. TCDD-induced expression of cytochrome P450 enzymes and formation of estrogen metabolites in human breast epithelial MCF-10F cells. Proc. Amer. Assoc. Cancer Res., 47:445, 2006.

31. Rogan, E.G., Zhang, Y., Gaikwad, N.W., Olson, K., Zahid, M. and Cavalieri, E.L. Cytochrome P450 isoforms catalyze oxidation of catechol estrogens to their quinones, which react with DNA. Proc. Amer. Assoc. Cancer Res., 47:446, 2006.

32. Zahid, M., Saeed, M., Olson, K., Gaikwad, N., Rogan, E., Cavalieri, E. Inhibition of the formation of the N3Ade and N7Gua depurinating adducts after reaction of estradiol-3,4-quinone or lactoperoxidase-oxidized 4-hydroxyestradiol with DNA. Proc. Amer. Assoc. Cancer Res., 47:446, 2006.

33. Saeed, M., Rogan, Ed., Cavalieri, E., Sheriff, F., Fernandez, S. and Russo, J. Formation of the DNA depurinating N3Ade and N7Gua adducts of 4-hydroxyestradiol by MCF-10F cells cultured with the carcinogenic estrogen metabolite 4-hydroxyestradiol. Proc. Amer. Assoc. Cancer Res., 47:447, 2006.

34. Singh, S., Zahid, M., Gaikwad, N., Chakravarti, D., Cavalieri, E.L. and Rogan, E.G. The effect of NAD(P)H:quinone oxidoreductase 1 polymorphisms on estrogen metabolism in relation to initiation of breast cancer. Proc. Amer. Assoc. Cancer Res., 47:835, 2006.

35. Cavalieri, E. Symposium Co-Chairman, Special Topic Symposium, Catechol Estrogen Quinones as Initiators of Breast and Other Human Cancers, 81st Annual Meeting "Science and the Next Generation" AAAS-SWARM Meeting, Tulsa, OK, April 5-8, 2006

36. Cavalieri, E. Introductory Remarks and A unifying mechanism in the initiation of cancer and other diseases. 81st Annual Meeting "Science and the Next Generation" AAAS-SWARM Meeting, Tulsa, OK, April 5-8, 2006.

37. Rogan, E. Estrogen Metabolism in Human Breast Cancer, AAAS-SWARM Division Annual Meeting, Science and the Next Generation, Tulsa, OK, April 5-8, 2006.

38. Rogan, E. Xenoestrogens, Biotransformation, and Differential Risks for Breast Cancer, The 13th International Symposium on Functional Medicine, Managing Biotransformation: The Metabolic, Genomic, and Detoxification Balance Points, Tampa, FL, April 19-22, 2006.

39. Cavalieri, E. Catechol quinones of estrogens and dopamine in the initiation of cancer and neurodegenerative diseases. 132nd Advanced Course on Estrogens and Human Diseases, Erice, Italy, May 15-21, 2006.

40. Cavalieri, E. From aromatic hydrocarbons to estrogens and then back to benzene and naphthalene. Naphthalene State-of-the-Science Symposium, Monterey, CA, October 9-12, 2006.

41. Rogan, E. The role of estrogen metabolism in the initiation of breast, prostate and other human cancers, The Michigan Society of Toxicology Fall Meeting, East Lansing, MI, November 3, 2006.

42. Rogan, E., The Role of Estrogen Metabolism in the Initiation of Breast, Prostate and Other Human Cancers, CAMExpo West, Los Angeles, CA, November 10-12, 2006

43. Rogan, E., Estrogen-DNA Adducts in the Etiology and Prevention of Breast and Prostate Cancer, Functional Medicine of Colorado, Boulder, CO, February 7, 2007

E. Conclusions

1. We have not been successful in finding an animal model for estrogen-initiated mammary tumors.

2. We have detected estrogen metabolites, GSH conjugates and depurinating DNA adducts in our initial studies of nipple aspirate fluid. The adducts, 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua, were detected in nipple aspirate fluid from women with breast cancer, but not from women without breast cancer.

3. We have detected estrogen metabolites, GSH conjugates and depurinating DNA adducts in spot urine samples. The adducts, 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua, were detected at significantly higher levels in urine from women with breast cancer and women at high risk of breast cancer (Gail Model score >1.66%), compared to urine from women without breast cancer (Fig. 1) [2]. These adducts are potential biomarkers for risk of developing breast cancer and for demonstrating the efficacy of possible agents to prevent breast cancer.

4. We have detected estrogen metabolites, GSH conjugates and depurinating DNA adducts in our initial studies of serum. The adducts, 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua, were detected at baseline levels in serum from women without breast cancer. These adducts are potential biomarkers for breast cancer.

5. In our hypothesis, estrogen-3,4-quinones are initiators not only of breast cancer, but also of other types of human cancer, for example, prostate, non-Hodgkin's lymphoma, ovary, etc. Therefore, we hypothesize that higher levels of estrogen-DNA adducts in urine would be biomarkers for all of these cancers. Detection of depurinating estrogen-DNA adducts in nipple aspirate fluid would be a specific test for breast cancer.

F. References

1. Markushin, Y., Gaikwad, N., Zhang, H., Rogan, E., Cavalieri, E., Trock, B., Pavlovich, C. and Jankowiak, R. Potential biomarker for early risk assessment of prostate cancer. Prostate, 66, 1565-1571, 2006.

2. Gaikwad NW, Muti P, Meza J, Yang L, Pruthi S, Ingle J, Rogan EG, Cavalieri E. The molecular etiology of breast cancer and biomarkers of risk. Submitted, 2007.

A-INTRODUCTION

Breast cancer is a malignancy whose dependence on ovarian function was first recognized through the regression of both advanced cancer and metastatic disease induced by oophorectomy in premenopausal women. The correlation of ovarian function with estrogen production, and the isolation of the estrogen receptor protein, combined with the observed greater incidence of estrogen receptor positive tumors in postmenopausal women, led to the identification of a strong association of estrogen dose and length of exposure with increased breast cancer risk. Despite the epidemiological and clinical evidence linking cumulative and sustained exposure to estrogens with increased risk of developing breast cancer (1-4), ***there are, however, three interrelated biological process that needs to be elucidated. First is the understanding of the mechanisms by which estrogen induces cancer, second the cell of origin that is the target of the estrogenic effect and the last one the role of estrogen if any in the epithelial mesenchymal transition in breast cancer.***

Among the mechanisms postulated by which estrogen induce breast cancer, the most widely acknowledged is its binding to its specific nuclear receptor alpha (ER-α) for exerting a potent stimulus on breast cell proliferation through its direct and/or indirect actions on the enhanced production of growth factors. However, the fact that ER-α knockout mice expressing the Wnt-1 oncogene (ERKO/Wnt-1) develop mammary tumors provides direct evidence that estrogens may cause breast cancer through a genotoxic, non-ER-α-mediated mechanism. This postulate is further supported by the observations that when ovariectomized mice are supplemented with E_2 they develop a higher tumor incidence with shorter latency time than controls, even in the presence of the pure antiestrogen ICI-182,780. Experimental studies on estrogen metabolism, formation of DNA adducts, carcinogenicity, mutagenicity, and cell transformation have supported the hypothesis that reaction of specific estrogen metabolites, namely, catechol estrogen-3,4-quinones (CE-3,4-Q) and to a much lesser extent, CE-2,3-Q, can generate critical DNA mutations that initiate breast, prostate and other cancers (5-8).

The second important biological concept is which is the progenitor or target cells or the stem cells in which the estrogen exerts these carcinogenic properties. The content of ER and PgR in the normal breast tissue, as detected immunocytochemically, varies with the degree of lobular development, in a linear relationship with the rate of cell proliferation of the same structures (9). The utilization of a double labeling immunocytochemical technique for staining in the same tissue section of steroid hormone receptors and proliferating cells, i.e. Ki67 positive, has allowed to determine that the expression of the receptors occurs in cells other than the proliferating cells, confirming results reported by other authors. The findings that proliferating cells are different from those that are ER and PgR positive support data that indicate that estrogen controls cell proliferation by an indirect mechanism. ER positive cells treated with antiestrogens secrete TGF-β to inhibit the proliferation of ER negative cells. The proliferative activity and the percentage of ER and PgR positive cells are highest in Lob l in comparison with the various lobular structures composing the normal breast. These findings provide a mechanistic explanation for the higher susceptibility of these structures to be transformed by chemical carcinogens *in vitro*, supporting as well the observations that Lobl are the site of origin of ductal carcinomas. However, the relationship between ER positive and ER negative breast cancers is not clear. We have reported that Lob l contains at least three cell types, ER positive cells that do not proliferate, ER negative cells that are capable of proliferating, and a small proportion of ER positive cells that can proliferate as well. Furthermore, we have observed that when Lob l of normal breast tissue are placed in culture they lose the ER positive cells, indicating that only proliferating cells, that are also ER negative, can survive, and constitute the stem cells. These observations are supported by the fact that MCF-10F, a spontaneously immortalized human breast epithelial cell line derived from breast tissues containing Lob. l and Lob. 2, is ER negative. Recent data on the genomic classification of breast tumors is helping to understand the cell type involved in the emergence of ER positive and ER negative tumors. Breast cancer can be subdivided into five major subtypes based on gene expression profiling: basal-like, Her2(HRBB2)-overexpressing, normal breast tissue-like, and two luminal-like (luminal A and luminal B) subtypes (10). The luminal-like subtypes display moderate to high expression of ERα and luminal cytokeratins, while the basal-like subtype is characterized by ERα(-), ERBB2(-) and high expression of basal cytokeratins 5 and 17. The ERBB2-overexpressing subtype is ERα(-) and is characterized by high

expression of several genes in the ERBB2 amplicon at 17q22.24 (11). ERα(-) tumors are more aggressive than ERα(+) tumors, and the loss of ERα is associated with poor prognosis and poor response to hormonal therapy (12). The outcomes, measured as time to development of distal metastasis, were worst for basal-like and ERBB2-overexpressing, best for luminal A, and intermediate for luminal B subtypes (10, 13, 14). Altogether these data support the concept that estrogen receptors positive and negative may be originated from two different cell populations as postulated earlier (9).

The third biological problem inherent to breast cancer is that their progression toward malignancy is accompanied by loss of epithelial dedifferentiation and a shift towards a mesenchymal phenotype. This process has been referred to as epithelial to mesenchymal transition or EMT, that exacerbates motility and invasiveness of many cell types and is often considered a prerequisite for tumor infiltration and metastasis.

In order to definitively outline the pathways through which estrogens act as carcinogens in the human breast either through the receptor pathway or through a genotoxic effect in a specific cell type of the breast and how these early events are associated with specific EMT phenotype it is needed an experimental system in which E_2 by itself or its metabolites induce transformation of human breast epithelial cells (HBEC) in a well controlled environment, preferentially *in vitro.* Towards this purpose we have developed an *in vitro*/*in vivo* system in which the carcinogenic action of estrogen on human breast epithelial cells (HBECs) is demonstrated, *in vitro*, by estrogen-mediated transformation of the spontaneously immortalized HBEC line, MCF-10F. Treatment of these cells with either 17β-estradiol (E_2) or its DNA reactive catechol metabolites results in acquisition of transformed phenotypes including colony formation in agar methocel, decreased ductulogenesis, and increased invasiveness (6). Of great interest, MCF-10F cells do not have detectable levels of ERα, and the *in vitro* cell transformation is not abrogated by co-treatment of these cells with antiestrogen ICI-182-780, supporting a non ERα-mediated mechanism (16). Recently, complete neoplastic transformation of MCF-10F cells has been demonstrated by the formation of tumors in an appropriate heterologous host. MCF-10F cells transformed with 70 nM E2 and then selected by Matrigel invasion chambers form tumors when injected into the mammary fat pad of the abdominal region of severe combined immune depressed (SCID) mice. Cell lines established from these tumors also form tumors in SCID mice. The tumors were poorly differentiated adenocarcinomas characteristic of primary breast tumor (15) that are ER (-), PgR(-) and ERB2 (-), mimicking one the basal cell type described earlier(11).

Furthermore, as a model of estrogen-mediated malignant transformation of HBECs, this is a unique system for identifying the temporal acquisition of changes in genome structure and gene expression that correspond to the transformed phenotype culminating in tumorigenesis. For this purpose, we performed Affymtrix 100k single nucleotide polymorphism (SNP) arrays to measure chromosomal copy number (CN) and loss of heretozygosity (LOH), and HG-U133_Plus_2 array to measure mRNA expression. By integrating these data we were able to identify associations between CN changes, LOH and tumorigenic phenotype, as well as the related changes in transcript expression. Furthermore, the functional influences of these changes on the biological processes and canonical pathways were elaborated by combining the gene expression profiles with their functional annotations in various knowledge bases using sophisticated algorithms and software. Progressive changes in both genome structure and gene expression profiles were observed, and these changes may be responsible for the malignant cell transformation. The up- and down-regulated genes were enriched in the regions of CN amplification and deletion, respectively. The breast cancer stem cell markers CD44 and CD24 (17) displayed $CD44H^{high}/CD44E^{-}/CD24^{-}$ pattern in the tumorigenic cells in current study. These changes will be described by Dr Thomas Sutter in section IV. Moreover, these cells demonstrated a phenotype of epithelial-mesenchymal transition (EMT). Functional analysis identified several deregulated pathways, including integring signaling, glutathione metabolism and apoptosis. Together, these analyses revealed new aspects of the cell biology of E_2-mediated malignant cell transformation in ERα(-) HBECs.

B-BODY

B-i- Methods and procedures.

B-i-a- *Ductulogenic assay.*

MCF-10F, E2 70 and C5-T8 (9) were suspended at a final concentration of 7.5 X 10^3 cells/ml in 89.3% (Vitrogen100) collagen matrix (Collagen Co., Palo Alto. CA, USA) and plated into 24-well chambers pre-coated with 89.3% of collagen base. They were fed with fresh high calcium media. The cells were examined under an inverted microscope for seven days. At the end of observation period the structures were photographed, fixed in 70% alcohol solution and processed for histological examination.

B-i-b-Histological and immunohistochemical analyses

Tissues fixed in alcohol 70%, dehydrated ad embedded in paraffin were cut at 5 μm thickness and stained with hematoxylin and eosin for histological analysis. For immunohistochemical analysis, tissue sections were mounted on aminoalkylsilane-coated or positively charged slides, deparaffinazed, rehydrated and incubated in 2% hydrogen peroxide at room temperature for 15 minutes for quenching endogenous peroxidase activity. The sections were sequentially incubated in two changes of Target Retrieval Solution at 98°C for 5 minutes each. All tissue sections were incubated in diluted normal blocking serum for 20 minutes. Excess serum was blotted from the slides and sections were incubated with the following antibodies: HHF35 a mouse anti-human muscle actin primary antibody, epithelial membrane antigen (EMA) clone E29, AE1, anti-human low molecular weight cytokeratin (Biogenex, San Ramon,CA), E-Cadherin, (Becton Dickinson Biosciences), Vimentin monoclonal mouse anti-human antibody (Dako Cytomation Colorado Inc.), and Fibronectin P1H11 mouse monoclonal raised against a cell binding domain of fibronectin of human origin (Santa Cruz Biotechnology, Inc., CA). After incubation in a humidity chamber at 4°C overnight, sections were washed in buffer and incubated with horse biotinylated secondary antibody (Vector Laboratories. Inc., Burlingame, CA, USA) at room temperature for 30 minutes followed by a 30 minutes incubation with Vectastain Elite avidin-biotin complex kit (Vector Laboratories), washed in PBS buffer, and incubated in peroxidase substrate solution containing hydrogen peroxide and 3, 3' – diaminobenzidine-HCL for 2 minutes. Sections incubated with no immune serum were used as negative controls. All sections were lightly counterstained with hematoxylin. . Immunostaining was evaluated by examination of slides under a bright field microscope, and graded according to the intensity of the brown staining.

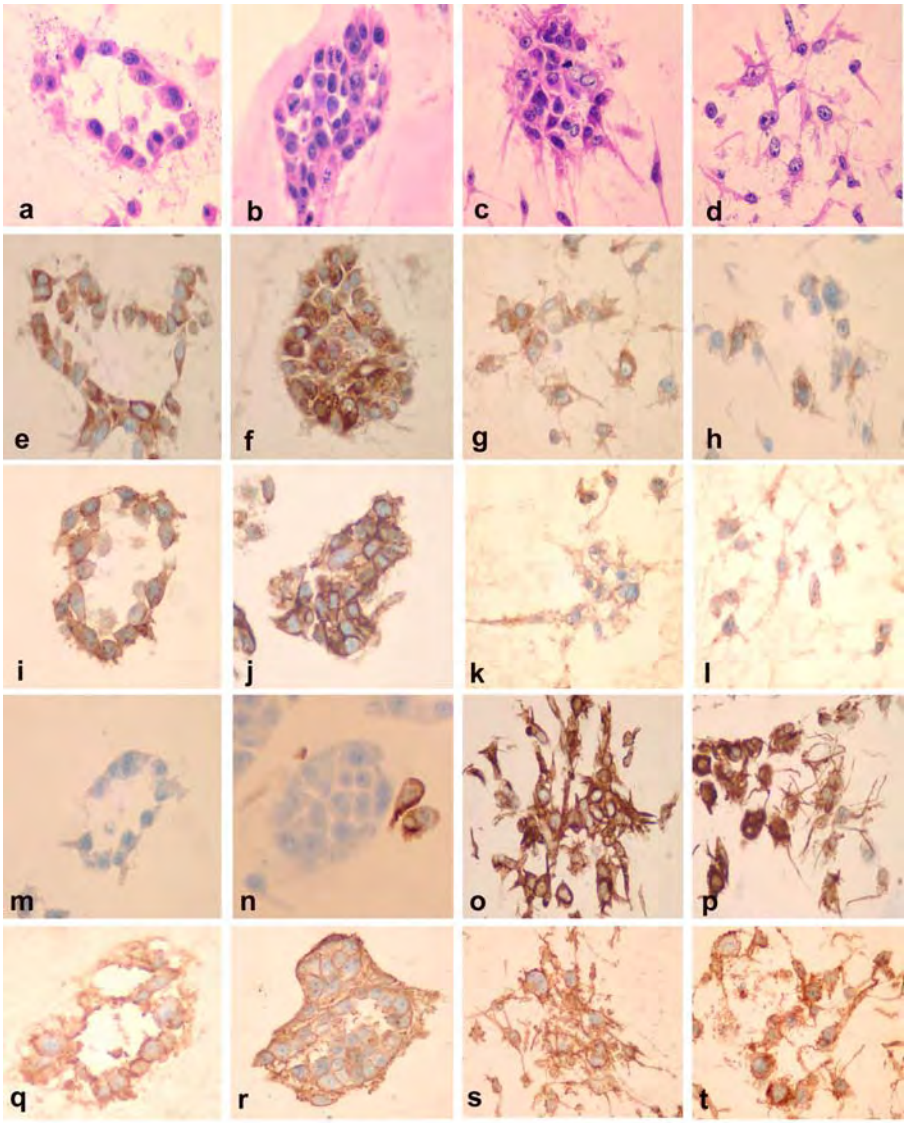
B-i-c-Real time RT-PCR.

Total RNA was isolated from growing cells at 70% - 80% confluence using Trizol (Life Technologies, Inc.) according to manufacture's instructions. The RNA was treated with DNase I (Invitrogen) and cleaned using RNeasy kit (Quiagen). The concentration and quality of RNA were determined spectrophotometrically and by capillary gel eletrophoresis (Agilent 2100 Bioanalyser, Palo Alto, CA). Real time reverse transcriptase PCR (Real time RT-PCR) was used to quantify the expression of E-cadherin, TGFß1, TGFß2, h-RAS, TWIST1, SNAIL2, SMAD5, FN1, CEACAM1 and JAG1. The TaqMan One Step RT-PCR kit (Applied Biosystems) was used and the assays ran using Applied Biosystems 7900 HT instrument. The Ct (threshold cycle) was calculated using three 25ng of RNA sample for each cell line. The TATA box-binding protein (TBP) was used as endogenous RNA control and each sample was normalized on the basis of its TBP content. The Ct was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe pass a fixed threshold above baseline. The SDS 2.1 software based on the comparative Ct method was used for data analysis. The comparative method calculates the relative gene expression using the following equation: relative quantity= $2^{-\Delta\Delta Ct}$ (User Bulletin 2, Applied Biosystems). For each gene, the expression level was compared to expression in the parental cell line MCF-10F.

B-ii- Results.

B-ii- a- ***Ductulogenic assay.***

MCF-10F, E2 70 and C5-T8 cell lines presented with different phenotypes when growing in collagen matrix. MCF-10F exhibits a duct-like growth pattern (Figure 1a) whereas E2 70 cell line forms spherical masses that in the histological sections resemble a ductal hyperplasia or carcinoma in situ (Figure 1b). The cell line C5-T8 grows in an invasive spread pattern with no structure formation (Figure 1c) or as single spindle cells resembling an invasive ductal carcinoma (Figure 1d).



In order to further characterize the cell phenotype we studied the expression of muscle actin protein using HHF35 primary antibody and the expression of epithelial membrane antigen using EMA Mc-5 primary antibody (Table 1). There was no HHF35 staining in MCF-10F, E2 70 and C5-T8 cells (Table 1) indicating that they are not myoepithelial cells, otherwise, all cell lines were positive for EMA (Figure 1 e-h) revealing their breast. epithelial nature. Keratin was significantly reduced from the MCF10F to the C5-T8 cells (Table 1). E cadherin was strongly positive in MCF10F cells and start decreasing the reactivity in the E2 70 cells (Figures 1i and 1j), for being almost negligible in C5-T8 cells (Figures 1k and 1l). Analyzing vimentin expression, MCF-10F and E2 70 cell lines are negative (Figures 1m and 1n), whereas the C5-T8 cells have a strong dark brown staining (Figures 1o and 1p). The staining for fibronectin also shows an increase in the intensity for
the C5-T8 (Table 1).

Figure 1: a: Histological section of MCF10F cells growing in collagen matrix, H&E x40; b:E2 70 cells growing in collagen matrix, H&E x40;c and d: C5-T8 cells growing in collagen matrix, H&E x40;; e, i, m and q:MCF10F cells reacted with EMA, E-Cadherin, Vimentin, and Fibronectin respectively (40X); f, j, n and r; E2 70 transformed cells reacted EMA, E-cadherin, Vimentin and fibronectin respectively (40X); g, h, k, l, o, p and s t: C5-T8 cells reacted with EMA, E-cadherin, Vimentin and fibronectin respectively (40X);

Table 1. Immunohistochemical expression profile of human breast epithelial cells transformed with estradiol

Antibody	MCF-10F cells	E2 70 cells	C5-T8 cells
EMA	++++	++++	++
HHF35	-	-	-
AE1 cytokeratin	++++	++	-
E-cadherin	++++	++	-
Vimentin	+*	-	++++
Fibronectin	+++	++	++++

Negative (-), weak (+), moderate (++), marked (+++) and strong (++++).

B-ii-b-Gene expression study.

We have determined by RT-PCR the expression of E-cadherin, TGFß1, TGFß2, h-RAS, TWIST1, SNAIL2, SMAD5, FN1, CEACAM1 and JAG1 genes (Figure 2). We observed a reduction in E-cadherin expression in E2 70 cells and a completely lost in C5-A8-T8 cells. TGFß1, TGFß2, CEACAM1 and JAG1 were down regulated in E2 70 and C5-A8-T8 cells. SMAD5 and h-RAS were up regulated in the tumorigenic C5-A8-T8 cells whereas FN1, TWIST1 and SNAIL2 were up regulated in C5-A8-T8 and down regulated in E2 70. Figure 2 shows the relative expression of EMT related genes during MCF-10F cell transformation.

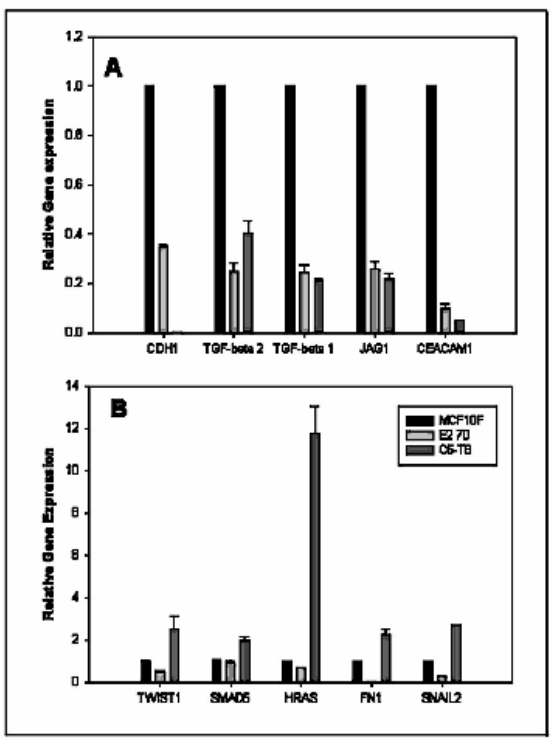


Figure 2: Comparative expression of genes related to EMT process during the neoplastic transformation of breast epithelial cells by estrogen.

B-iii next proposed Plan of research

To publish all the information gathered during this grant award period.

C-KEY RESEARCH ACCOMPLISHMENTS

1- Treatment of MCF-10F cells with 17β-estradiol results in malignant cell transformation. In this model, cells from the initially 17 beta estradiol transformed foci are nontumorigenic, whereas cells selected from this population by migration through Matrigel invasion chambers form solid tumors in SCID mice. Integrated analysis of genotyping and gene expression arrays revealed progressive genomic changes. Chromosomal amplifications were found in 1p36.12-pter, 5q21.1-qter and 13q21.31-qter; losses were detected in 8p11.21-23.1 and all of chromosome 4. In cell line established from tumors, additional losses were found in 3p12.1-14.1, 9p22.1-pter and 18q11.21-qter. Functional profiling of dysregulated genes revealed inhibition of apoptosis and glutathione metabolism pathway, epithelial-mesenchymal transition and $CD44H^{high}/CD44E^{-}/CD24^{-}$ phenotype in tumorigenic cells, and deregulation of integrin signaling pathway in both nontumorigenic and tumorigenic cells.

2. We have demonstrated that Epithelial-mesenchymal transition (EMT) in epithelial cells is an important component of 17 beta estradiol induced neoplastic transformation. We have evaluated the expression of different genes related to EMT such as E-cadherin, TGFß1, TGFß2, h-

RAS, TWIST1, SNAIL2, SMAD5, FN1, CEACAM1 and JAG1 using the *in vitro- in vivo* model of the estrogen induced cell transformation developed in our laboratory. The E2-transformed MCF-10F (E2 70) cells and the tumorigenic cell line C5-A8-T8 (C5-T8) exhibit progressive loss of ductulogenesis as demonstrated by growth in collagen matrix. MCF-10F cells form ductal structures while E2 70 cells form solid spherical masses that in histological sections exhibit a pattern of growth resembling ductal hyperplasia or carcinoma *in situ.* The tumorigenic cells C5-T8 did not form structures on collagen acquiring an invasive pattern with spindle like features. We have observed a reduction in E-cadherin expression in E2 70 cells and a completely lost in C5-T8 cells. TGFß1, TGFß2, CEACAM1 and JAG1 were down regulated in E2 70 and C5-T8 cells. SMAD5 and h-RAS were up regulated in the tumorigenic C5-T8 cells whereas FN1, Twist1 and Snail2 were up regulated in C5-T8 and down regulated in E2 70. We conclude that the lost of expression of TGFß1, TGFß2, CEACAM1 and JAG1 are related to ductulogenesis and branching and the overexpression of h-RAS with loss of E-cadherin expression and up modulation of TWIST1, SNAIL2 and SMAD5 expressions are involved in the EMT modulation.

D-REPORTABLE OUTCOMES

1. Russo, J. and Russo, I.H. Mechanism Involved in the carcinogenesis of the Breast. In: Breast Cancer: Prognosis, Prevention and Treatment (J.R. Pasqualini, Ed.) Marcel Dekker, Inc. New York. Pp1-18, 2002.
2. Russo, J, Lareef M.H., Balogh, G. Guo, S., and Russo I.H. Estrogen and its metabolites are carcinogenic in human breast epithelial cells. J. of Steroid Biochemistry & Molecular Biology 87:1-25, 2003.
3. Soares, R., Guo, S., Gartner, F., Schmitt, F.C. and Russo, J. 17 ~ estradiol-mediated vessel assembly and stabilization in tumor angiogenesis requires TGF and EGFR crosstalk Angiogenesis 6:271-281, 2003.
4. Soares, R., Guo, S., Russo, J. and Schmitt, F.C. Role of estrogen antagonist ICI 182, 780 vessel assembly and apoptosis of endothelial cells. Ultrastructural Pathology 27:33-39, 2003.
5. Russo, J., Russo, I.H. Genotoxicity of Steroidal Estrogens. TRENDS in Endocrinology and Metabolism, 15(5):211-214, 2004.
6. Soares R., Balogh, G., Guo S., Gartner, G.F., Russo J., and Schmitt, F. Evidence for the notch signaling pathway on the role of estrogen and angiogenesis. Molecular Endocrinology 18:1-10, 2004.
7. Santen, R.J., Yue, W., Wang, J-P., Li, Y., Russo, J., Verderame, M., Bocchinfuso, W., Korach, K., Devanesan, P., Rogan, E., and Cavalieri, E., Estradiol induced carcinogenesis via formation of genotoxic metabolites. In: Advances in Endocrine Therapy of Breast Cancer. Proceeding of the 2003 Gleneagles Conference (Ingle, J.N. and Dowsett, M. Eds), Marcel Dekker, New York, NY, pp.173-177, (2004).
8. Russo, J., Santen, R., and Russo, I.H. Hormonal control of the breast development. In: Endocrinology (Fifth Edition) Edited by L. J. DeGroot and J.L. Jameson. W.B. Saunders Company. Philadelphia, 2005, Vol.3, pp 3045-3055.
9. Lareef, M.H. Garber, J. Russo, P.A., Russo, I.H., Heulings, R. and Russo, J. The estrogen antagonist ICI-182-780 does not inhibit the transformation phenotypes induced by 17- -estradiol and 4-OH estradiol in human breast epithelial cells. Int. J. of Oncology 26:423-429, 2005.
10. Fernandez, SV., Russo, IH., Lareef, MH., Balsara, B., and Russo, J. Comparative Genomic Hybridization of Human Breast Epithelial Cells Transformed by Estrogen and its Metabolites. Int J Oncol. 2005 Mar; 26(3):691-5.
11. Come SE, Buzdar AU, Ingle JN, Arteaga CL, Brodie AM, Colditz GA, Johnston SR, Kristensen VN, Lonning PE, McDonnell DP, Osborne CK, Russo J, Santen RJ, Yee D, Hart Proceedings of the Fourth International Conference on Recent Advances and Future Directions in Endocrine Manipulation of Breast Cancer: conference summary statement. Clin Cancer Res. 2005 Jan 15; 11(2 Pt 2):861s-4s.

12. Chen, J-Q, Yager, J.D. and Russo, J. Regulation of mitochondrial respiratory chain structure and function by estrogens/estrogen receptors and potential physiological/pathophysiological implications: Review. Biochimica et Biophysica Acta XX: 1-17, 2005.
13. Fernandez, S.V., Lareef, M.H., Russo, I.H., Balsara, B.R., Testa, J.R. and Russo, J. Estrogen and its metabolites 4-Hydroxy-estradiol induce mutations in TP53 and LOH in chromosome 13q12.3 near BRCA2 in human breast epithelial cells. International Journal of Cancer 118(8):1862-8, 2006.
14. Cavalieri, E., Chakravarti, D., Guttenplan, J.,Hart, E., Ingle, J., Jankowiak, R., Muti, P., Rogan, E., Russo, J., Santen, R., Sutter, T. Catechol Estrogen Quinones as Initiators of Breast and Other Human Cancers. Implications for Biomarkers of Susceptibility and Cancer Prevention. Review. Biochimica et Biophysica Acta 1766:63-78, 2006
15. Russo, J., Fernandez, S.V., Russo, P.A., Fernbaugh, R., Sheriff, F.S., Lareef, H.M.,Garber, J., and Russo,I.H. 17 beta estradiol induces transformation and tumorigenesis in human breast epithelial cells. FASEB J 20:1622-1634, 2006.
16. Russo, J. and Russo, I.H. The role of estrogen in the initiation of breast cancer. The Journal of Steroid Biochemistry & Molecular Biology, 102:89-96, 2006.
17. Saeed M, Rogan E, Fernandez SV, Sheriff F, Russo J, Cavalieri E. Related Articles, Links Formation of depurinating N3Adenine and N7Guanine adducts by MCF-10F cells cultured in the presence of 4-hydroxyestradiol. Int J Cancer. 2007 Jan 17; [Epub ahead of print] PMID: 17230531 [PubMed - as supplied by publisher]
18. Mello, M.L., Vidal, B.C., Lareef, M.H.Russo, I.H. and Russo, J. DNA content and chromatin Texture of Human breast epithelial cells treated with 17- estradiol and the estrogen antagonist ICI 182,780 as assessed by Image Analysis. Mutation Research 617:1-7, 2007.
19. Russo, J., Lareef, H., Tahin, Q. , and Russo, I.H. Pathways of carcinogenesis in the human breast. Climacteric: S1: S-27-02, 2002.
20. Lareef, H.M. Russo I.H. Sheriff, F., Tahin, Q., and Russo, J. Genomic Changes induced by Estrogens in human breast epithelial cells (HBEC). Proc. Am. Assoc. Cancer Research. 44:904a, 2003.
21. Russo, J.; Lareef, H.M.; Balogh, G.; Russo, I.H. Estrogen is a carcinogenic agent in the human breast. In. J. of Mol. Medicine 12: (Supp. 1) 147a, 2003.
22. Fernandez, S.V., Lareef, M.H., Russo, I.H., Balsara, B.B., Testa, J. and Russo, J. Estrogen and its metabolite 4-OH-E2 induce LOH at 13q12.3, at a locus 0.8cM from the BRCA2 gene in human breast epithelial cells. Proc. Am. Assoc. Cancer Res.45:7a, 2004.
23. Russo, P.A., Balogh, G.V., Russo, I.H., and Russo, J. Estrogen-induced transformation of human breast epithelial cells involves mutations in mismatch repair genes. Proc. Am. Assoc. Cancer Res.45:2629a, 2004.
24. Lareef, M.H., Heulings, R., Russo, P.A., Garber, J., Russo, I.H., and Russo, J. The estrogen antagonist ICI-182-270 does not inhibit the proliferative activity and invasiveness induced in human breast epithelial cells by estradiol and its metabolite 4-OH-Estradiol. Proc. Am. Assoc. Cancer Res.45:11a, 2004.
25. Vanegas, J., Russo, P.A., Russo, I.H., and Russo, J. Estrogen-Transformed Human Breast Epithelial Cells Exhibit an Altered Migratory Behavior *in Vitro*. Proc. Am. Assoc. Cancer Res.46: 2005.
26. Fernandez, S.V., Russo, I.H. and Russo, J. Estrogen and its metabolites 4-hydroxy-estradiol and 2-hydroxy-estradiol induce mutation of p53 and LOH in a gene located 0.8cM of BCR2 in human breast epithelial cells. Proc. Am. Assoc. Cancer Res.46:2098a, 2005.
27. Russo, P.A., Lareef, M.H., Garber, J., Russo, I.H., Fernandez, S., Fernbaugh, R., Balsara,B.R., Sheriff, F., Balogh, G.A., Mailo, D., Heulings, R. Russo, J. An in Vitro-in Vivo Model of Estrogen Induced Human Breast Carcinogenesis. Proc. Am. Assoc. Cancer Res.46: 2097a, 2005.
28. Russo, J. Estrogens as carcinogens in the human breast. Proc. Am. Assoc. Cancer Res.46:SY14-1, 2005.
29. Vanegas, J.E., Russo, P.A., Russo, I.H., Russo, J. Estrogen and its metabolites alter the migratory behavior of human breast epithelial cells. Proc. Era of Hope, June 2005
30. Russo, P.A., Lareef, M.H., Garber, J., Russo, I.H., Fernandez, S.V., Fernbaugh, R., Balsara, B., Sheriff, F., Balogh, G., Mailo, D., Heulings, R. Russo, J. A model of estrogen induced human breast carcinogenesis. Proc. Era of Hope, June 2005.

31. Fernandez, S.V., Russo, I.H., Russo, J. Estrogen and its metabolites 4-Hydroxy estradiol and 2-Hydrox-yestradiol induce mutations in human breast epithelial cells. Proc. Era of Hope, June 2005.
32. Chen, J.Q, Russo, P, Russo, IH, and Russo, J ER shifts from mitochondria to nucleus during 17 -estradiol induced neoplastic transformation of human breast epithelial cells. Proceeding of the Am. Assoc. Cancer Res. 47:2932a, 2006.
33. Huang,Y., Fernandez,S., Goodwin, S., Russo, P.A., Russo, P.A., Sutter, T.R., Russo, J. Genomic profile of the estrogen induced neoplastic transformation of the human breast epithelial cell MCF10F. Proceeding of the Am. Assoc. Cancer Res. 47:149a, 2006.
34. Fernandez, S.V., Wu,Y-Z., Russs,I.H., Plass C., Russo, J. The role of DNA methylation in estrogen-induced transformation of human breast epithelial cells. Proc Am. Assoc. Cancer Res. 47: 1590a, 2006
35. Saeed, M., Rogan, E., Cavalieri, E., Sheriff, F., Fernandez, S.V., Russo, J. Formation of the DNA depurinating N3Ade and N7Gua adducts of 4-Hydroxyestradiol by MCF10F cells cultured with the carcinogenic estrogen metabolite 4-Hydroxyestradiol. Proceeding of the Am. Assoc. Cancer Res. 47:1895a, 2006.
36. Sandra V. Fernandez, Yue-Zhong Wu, Irma H. Russo, Christoph Plass, and Jose Russo.DNA Methylation in Human Breast Epithelial Cells transformed by estradiol. Proceeding Endocrine Society, 2006.
37. Chen, JQ, Russo, PA, Russo, IH, and Russo, J. Mitochondria-to-nucleus shift of the estrogen receptor beta during estrogen-induced transformation of human breast epithelial cells. Proceeding Endocrine Society, 2006.

E-CONCLUSIONS

During the fourth year of our award we have clearly demonstrated for the first time, that 17 -estradiol induces complete *in vitro* transformation of human breast epithelial cells, as evidenced by the expression of anchorage independent growth, loss of ductulogenesis in collagen, invasiveness in Matrigel, and tumorigenesis in SCID mice. Functional profiling of dysregulated genes revealed inhibition of apoptosis and glutathione metabolism pathway, epithelial-mesenchymal transition and $CD44H^{high}/CD44E^{-}/CD24^{-}$ phenotype in tumorigenic cells, and deregulation of integrin signaling pathway in both nontumorigenic and tumorigenic cells. We also have concluded that the lost of expression of TGFß1, TGFß2, CEACAM1 and JAG1 are related to ductulogenesis and branching and the overexpression of h-RAS with loss of E-cadherin expression and up modulation of TWIST1, SNAIL2 and SMAD5 expressions are involved in the EMT modulation.

F. REFERENCES

1-Henderson, B.E., Ross, R., Bernstein, L. (1988). Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation award lecture.Cancer Res. *48*, 246-53.

2-Hilakivi-Clarke, L. (1997). Estrogen-regulated non-reproductive behaviors and breast cancer risk: animal models and human studies. Breast Cancer Res Treat. *46*,143-59.

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

4-Landis, S.H., Murray, T., Bolden, S., Wingo, P.A. (1999). Cancer statistics, Cancer J Clin. *49*, 8-31, 1.

5-Henderson, B. E. and Feigelson, H. S. (2000). Hormonal carcinogenesis. Carcinogenesis, *21:* 427-433

6-Russo, J., Lareef, H.M., Tahin, Q., Hu, Y.F., Slater, C., Ao, X., Russo, I.H. (2002). 17Beta-estradiol is carcinogenic in human breast epithelial cells. J Steroid Biochem Mol Biol. *80*, 149-62.

7-Russo, J., Hasan Lareef, M., Balogh, G., Guo, S., and Russo, I. H. (2003). Estrogen and its metabolites are carcinogenic agents in human breast epithelial cells. J Steroid Biochem Mol Biol, *87:* 1-25

8-Russo, J. and Russo, I. H. (2004). Genotoxicity of steroidal estrogens. Trends Endocrinol Metab, *15:* 211-214.

9- Russo, J., Ao, X., Grill, C., and Russo, I.H. Estrogen and progesterone receptor positive cells are not the proliferating cells in the mammary gland. Proc. Am. Assoc. Cancer Res. 39:3756a, 1998.

10-Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., et al. (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A, *100:* 8418-8423

11-Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., et al. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A, *98:* 10869-10874

12-Clark Prognostic and predictive factors. In: Harris J, editor. Diseases of the breast. Vol.2. Philadelphia: Lippincott, Williams & Wilkins; p 489- 514, 2000.

13-Signoretti, S., Di Marcotullio, L., Richardson, A., Ramaswamy, S., Isaac, B., Rue, M., Monti, F., Loda, M., and Pagano, M. (2002). Oncogenic role of the ubiquitin ligase subunit Skp2 in human breast cancer. J Clin Invest, *110:* 633-641

14-van de Rijn, M., Perou, C. M., Tibshirani, R., Haas, P., Kallioniemi, O., Kononen, J., Torhorst, J., Sauter, G., Zuber, M., Kochli, O. R., et al. (2002). Expression of cytokeratins 17 and 5 identifies a group of breast carcinomas with poor clinical outcome. Am J Pathol, *161:* 1991-1996

15-Russo, J., Fernandez, S.V., Russo, P.A., Fernbaugh, R., Sheriff, F.S., Lareef, H.M., Garber, J., Russo I.H. (2006). 17-Beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells. FASEB J. *10*, 1622-34.

16-Lareef, M. H., Garber, J., Russo, P. A., Russo, I. H., Heulings, R., and Russo, J. (2005). The estrogen antagonist ICI-182-780 does not inhibit the transformation phenotypes induced by 17-beta-estradiol and 4-OH estradiol in human breast epithelial cells. Int J Oncol, *26:* 423-429

17-Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. (2003). Prospective identification of tumorigenic breast cancer cells. PNAS, *100*, 3983-8.

SPECIFIC AIM 3 - GUTTENPLAN

A. Introduction

Epidemiological studies have implicated steroidal estrogens in the etiology of breast cancer [1, 2]. Stimulation of proliferation in ER positive cells has been hypothesized to increase the probability that some endogenous damage to DNA will be converted to mutations before it is repaired [1, 3]. However, it has also been proposed that estrogen metabolites themselves may be genotoxic and give rise to DNA damage and mutations [3-5]. Metabolism of estradiol to the catechol estrogens, 2-OHE_2 and 4-OHE_2, followed by oxidation to E_2-2,3 or E_2-3,4-semiquinone and then E_2-2,3 or E_2-3,4-quinone (Fig. 1) has been proposed to lead to mutagenesis by redox cycling between the semiquinone and quinone forms, with the concomitant production of ROS [3-5]. It has also been shown that the E_2-3,4-quinone reacts with DNA in vitro leading predominantly to the depurinating adducts, 4-OHE_2-1-N3adenine and 4-OHE_2-N7guanine [5]. E_2-2,3-quinone also gives rise to a depurinating adduct, 2-OHE_2-6-N3adenine and stable guanine and adenine adducts[1] [5]. Under equivalent conditions, E_2-3,4-quinone forms much higher levels of depurinating adducts than E_2-2,3 quinone [5]. 4-OHE_2 and 2-OHE_2 have been detected in breast tissue from women with breast carcinoma, but the former compound was present in much higher levels and was also present in higher levels than in breast tissue from women without breast cancer [6, 7]. To further support the genotoxic hypothesis, it is important to demonstrate that 4-OHE_2 is mutagenic and, as a corollary, the mutagenic activity should be greater than that of 2-OHE_2.

Early studies on genotoxicity of estrogens and their metabolites failed to detect any mutagenic activity (reviewed in [3]), perhaps because of inappropriate concentrations of estrogens or inadequate test systems or conditions [3]. More recent studies in cultured cells have reported that the 4-OHE_2 precursor, E_2, is weakly mutagenic in V-79 cells in the HGPRT assay [8]. The dose-response curve was unusual in that mutagenesis decreased at higher doses [8]. A small number of mutants were sequenced, and mutations at A:T base pairs were most common, but a number of mutations were identical, suggesting they resulted from clonal expansion rather than independent mutations. Small deletions were also observed with similar frequencies. Other studies have reported that E_2, 4-OHE_2, and 2-OHE_2 all led to strand breaks as detected in the comet assay in MCF-7 cells, but differences in potencies between these compounds were not apparent [8]. However, cells were assayed immediately after treatments, so that accurate repair of at least some of the DNA damage would likely not have had time to occur. It has also been reported that E_2, and 4-OHE_2, transforms cells [9, 10].

Certain in vivo studies also demonstrate that estradiol or its metabolites are carcinogenic or mutagenic in rodents. E_2-3,4-quinone induces mutations in the H-ras gene of SENCAR mouse skin [11]. 4-OHE_2 is also carcinogenic in Syrian Golden hamsters and CD-1 mice [3] whereas the 2-OHE_2 is only marginally carcinogenic at best [3]. An important link in the evidence for a genotoxic mechanism contributing to carcinogenicity of estrogens would be a demonstration that catechol estrogens are mutagenic. We have measured the mutagenic activities of 4-OHE_2 and 2-OHE_2 in lacI rats and a lacI rat embryonic cell line, BB® rat2, and determined the mutational spectrum of 4-OHE_2.

[1] Zahid, et al., Chem. Res. Tox., submitted

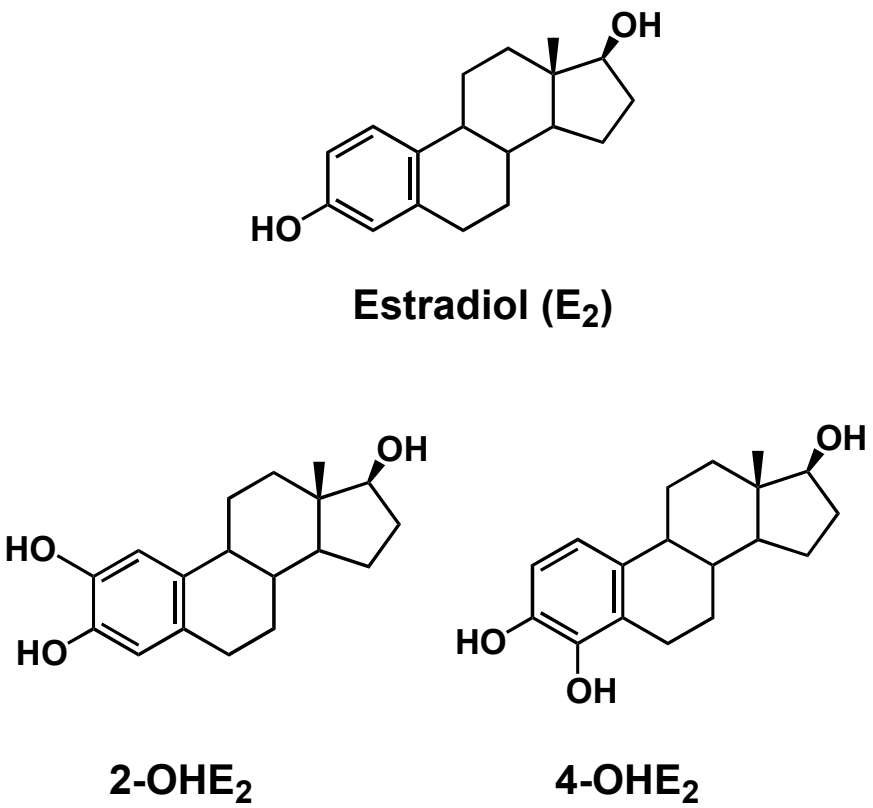


Figure 1 Structures of estradiol and hydroxylated metabolites.

B. Body

Methods

Treatment conditions
Studies in cells.

Cells were grown in 10 cm^2 petri dishes in phenol red-free Dulbecco's Modified Eagle Medium (Invitrogen Corp., Grand Island, NY) containing 10% charcoal/dextran-treated fetal calf serum (Hyclone, Logan UT) to a density of about 10^6 cells/dish. Glutamine, G418-sulfate and penicillin-streptomycin (Mediatech, Inc., Hemdom, VA) were added to concentrations of 0.29 mg/ml, 50 i.u., 50 ug/ml and 0.22 mg/ml, resp. When treated with catechol estrogens or solvent alone, the medium was changed to phenol red-free DMEM without serum, and after each treatment, the medium was removed, cells were rinsed with PBS, and the phenol red-free DMEM containing charcoal-treated fetal calf serum was added. All treatments were carried out on two or three replicate plates and at the doses shown in Figs. 2 and 3, the entire experiment was repeated. Values in figures represent the average MF from each separate dose in the two separate experiments. When treated multiple times, treatments were for 18 hr, every other day, except weekends. After three treatments, cells were trypsinized one portion was used for a mutagenesis assay and the rest was replated at a density of 2 x 10^6 cells/dish for subsequent treatment. The lineage of each plate was retained after plating. After 6 treatments the cells were

grown to about 80% confluence and harvested. The doubling time was about 24 hr in DMEM containing serum.

An initial dose-response experiment for single treatments was carried out to determine a dose range with minimal toxicity. Cells from each treatment group were counted after three treatments and surviving cell numbers/plate were, within experimental error, the same for all doses except the highest dose tested (400 nM for 4-OHE_2 and 800 nM for 2-OHE_2) where there were slightly fewer cells (ca. 80% cell survival). For single treatments, doses above 400nM for 4-OHE_2 and 1360 nM for 2-OHE_2 resulted in toxicity of > 50%.

Studies in LacI rats

Groups of 6-8 Fisher 344 *lacI* rats were implanted with E_2, 4-OHE_2, 2-OHE_2, and 4-OHE_2 + E_2 in silastic tubing and euthanized 20 weeks later. DNA was isolated from 4 upper inguinal mammary fat pads from each rat. Mutagenesis in each DNA was analyzed separately, and the 4 individual mutant fractions (MF's) averaged to obtain the MF for each rat. The treatments were carried out twice for E_2, 4-OHE_2 and the control, with similar results; and the combined results along with those for other treatment groups is given in the table below.

Mutagenesis

After treatment of the cells or rats, DNA was extracted using a Recoverase kit (Stratagene, LaJolla, CA) as per manufacturer's instructions, which involves isolation of nuclei, cell lysis, digestion with protease K and RNAse and dialysis on a membrane. Phage packaging was carried out using a phage packaging mix prepared from bacterial strains E coli NM759 and BHB2688 generously supplied by Dr. Peter Glazer (Yale, Univ. School of Medicine, New Haven, CT) according to published methods [12]. The cII mutagenesis assay was then employed.

The BB® rat2 cell line contains a lambda shuttle vector that includes the bacterial lacI locus and also the cII gene, which is the target for the mutagenesis studies. It also obviates the potential for ex vivo mutations that could complicate results. This assay detects mutations at the cII locus and possibly the regulator cI locus [13] [14-17]. The cII protein is a positive regulator of gene transcription that controls the decision between lytic or lysogenic development pathways in phage-infected cells. In appropriate E. coli (E. coli 1250) host cells, under specified conditions (25° C) only mutants give rise to phage plaques, whereas at 37° C all infected cells give rise to plaques, providing a phage titer [13] [14-17]. The ratio of mutant to non-mutant plaques is the measure of mutagenesis, the mutant fraction (MF). Each DNA sample was assayed at least twice and at least 15 mutants were accumulated for each DNA sample.

Amplification and sequencing

Mutants were cored from petri dishes and the agar plug was mixed with 100 ul phage buffer. 10 ul of the buffer was then spread on a selective plate to confirm mutant phenotype and purify mutant phages. Both control and 4-OHE_2- induced mutant plaques were randomly selected from selective plates containing packaged DNA samples isolated from several plates/dose. The purified mutant plaques were the subjected to amplification and sequencing of the cII gene by PCR. Sequencing was performed by Roderick Haesevoets, University of Victoria, B.C., Canada.

Amplification: Primer sequences, forward: 5'-AAAAAGGGCATCAAATTAAACC-3', reverse: 5'-CCGAAGTTGAGTATTTTTGCTGT-3'
Reaction Mixture (100.0 μL reaction) H_2O 59.1 μL, 100mM dNTP mix,1.0 μL10X buffer,10.0 μL cII forward primer (10.0 μM), 2.0 μL cII reverse primer (10.0 μM) 2.0 μL, 50mM $MgCl_2$3.5 μL, Taq, 2.4 μL, sample, 20.0 μL.10X buffer:100 mM Tris HCl pH 9.0, 500 mM KCl, 1.0% TritonX100.
PCR conditions: 94.0^o, 4.0 min; 30 cycles: 95.0^o, 30 sec; 55.0^o; 30 sec; 72.0^o, 2.5 min; 4.0^o, hold.
Purification: PCR Product Pre-Sequencing Kit (USB); as per kit instructions.
Sequencing: Primer sequences,cII forward: 5'-ACCACACCTATGGTGTATG-3',
cII reverse, 5'-GTCATAATGACTCCTGTTGA-3' (only used to confirm a mutation if the sequence from cII forward primer is not clear)
Reaction Mixture: CEQ Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman-Coulter).
PCR conditions: 96.0^o, 5.0 min, 30 cycles: 96.0^o, 20 sec; 52.0^o, 25 sec; 60.0^o, 4.0 min;
4.0^o, hold
Electrophoresis/Base Calling/Trace Generation: Sequencing was done on a CEQ8000 Capillary Electrophoresis DNA Sequencer (Beckman-Coulter).
Analysis software: SeqMan II v6.1 (DNASTAR).

Statistics

Differences in MF's between the 4-OHE_2 -treated and control groups were analyzed for significance using a two-tailed Student's *t*-test. At least four measurements (2 experiments and duplicate measurements for each dose) were used for each point.

Results

Studies in cells

Mutant fractions:

Initial experiments conducted at doses from 10 - 6800 nM 4-OHE_2 failed to detect any significant increase in mutant fraction. Since previous studies had indicated that 4-OHE_2 was not mutagenic, and its precursor, E_2, was more mutagenic at certain lower doses than higher doses, we tested multiple low dose exposures to 4-OHE_2. 4-OHE_2 induced a dose-dependent increase in mutant fraction from 50 - 200 nM (Fig. 2). This was marginally apparent at three treatments and clearer after six treatments. In both cases the 200 nM treatments resulted in statistically significant increases over controls. Also, the mutant fraction declined at 400 nM after both three and six treatments.

Using similar protocols, for single and multiple treatments, it was not possible to detect any induction of mutagenesis over background by 2-OHE_2 (Fig.3). 2-OHE_2 was about 3X less toxic than 4-OHE_2 and was less effective in cell transformation assays [10] therefore the doses used were about 3X greater than those used for 4-OHE_2.

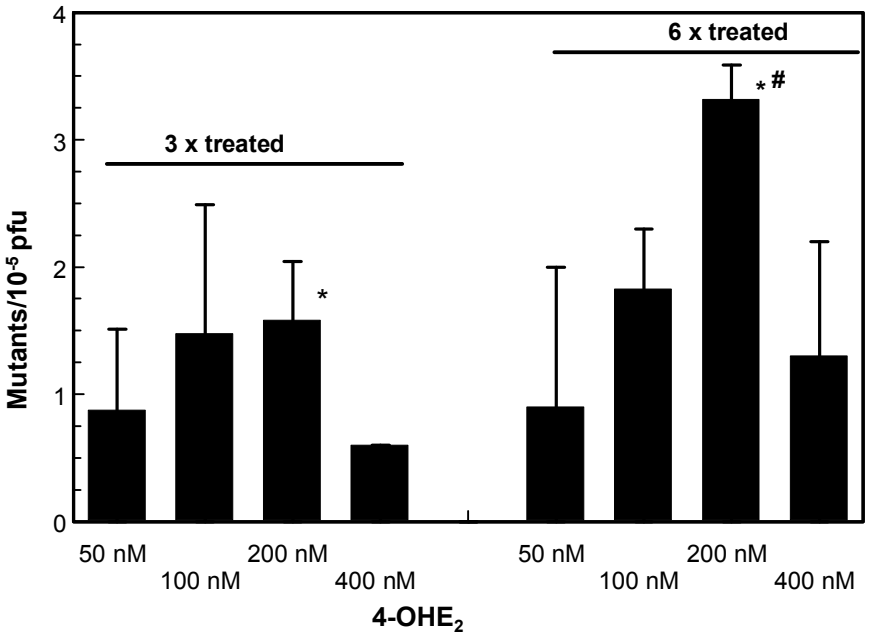


Figure 2. Effects of dose on the mutant fraction in the cII gene from 4-OHE_2 -treated BB® rat2 cells. In order to normalize results from different experiments, background mutant fractions (from solvent controls) have been subtracted from all values. The background obtained in each individual experiment was subtracted from the corresponding mutant fraction of the 4-OHE_2-treated group. Backgrounds ranged from 2.2 - 3.5 mutants/10^5 pfu. Asterisk indicates $P <$ 0.05vs. solvent controls; number sign indicates $P < 0.05$ in corresponding 3x-treated vs. 6x-treated.

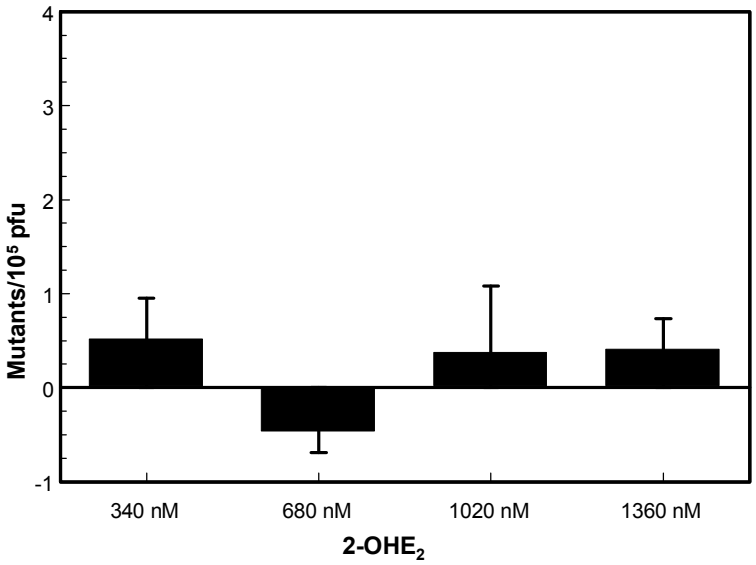


Figure 3. Effects of dose on the mutant fraction in the cII gene from 2-OHE_2 -treated BB® rat2 cells. Cells were treated six times with the above doses.
The mutant fraction from background (solvent alone) plates was 4.3 +/- 0.5 pfu and has been subtracted from the above values.
Error bars, standard errors.

Mutational spectra

The mutational spectra from the 4-OHE_2 treated and control plates were compared (Tables 1-3). A major apparent difference between the two groups was the higher percentage of mutations at A:T base pairs in the mutants from the 4-OHE_2 -treated cells than in controls (*ca.* 24% vs. 6%). Also, there was a higher fraction of GC:AT transitions in controls and a higher fraction of these were at CpG sites. In addition, the fraction of GC>TA mutations was higher in the 4-OHE_2 -treated group than the controls. A number of the mutations in both 4-OHE_2 and control cells (25% and 34% resp.) were identical. For the control cells, it was assumed these all resulted from clonal expansion and redundant mutants were only counted once for each experiment. Two experiments with 2-3 plates/dose were conducted and each experiment utilized a freshly thawed culture of BB® cells. For the 4-OHE_2-treated cells it was assumed that identical mutants from different plates were independent, since they might have been induced by the 4-OHE_2 separately on each plate, but those identical mutants on the same plate presumably resulted from clonal expansion and were only counted once. The clonal mutants have not been included in tables 1 and 2.

Table 1: DNA sequence context of mutations among mutants in the 6 times 4-OHE$_2$-treated BB® rat2 cells[1,2,3,4]

position	change	context
-14	g>a	cta agg aaa
-13	g>t	cta agg aaa
1	A>G	cat ATG GTT
2	T>C	cat ATG GTT
3	G>A	cat ATG GTT
24	C>G	CGC AAC GAG
24	C>G	CGC AAC GAG
24	C>G	CGC AAC GAG
29	C>T	GAG GCT CTA
32	T>C	GCT CTA CGA
42	G>C	ATC GAG AGT
57	C>A	CTT AAC AAA
58	A>G	AAC AAA ATC
65	C>T	ATC GCA ATG
73	G>C	CTT GGA ACT
86	C>A	AAG ACA GCG
118	A>G	CAG ATC AGC
119	T>G	CAG ATC AGC
129	G>T	AGG TGG AAG
148	A>C	CCA AAG TTC
163	C>A	CTG CTT GCT
169	G>C	GCT GTT CTT
179	G>C	GAA TGG GGG
191	A>G	GTT GAC GAC
196	G>A	GAC GAC ATG
205	C>G	GCT CGA TTG
241	A del. (6>5)	ATT AAA AAA
241	A del. (6>5)	AAT AAA AAA
241	A del. (6>5)	AAT AAA AAA
241	A del. (6>5)	AAT AAA AAA
190-192	GAC del. (3>2)	GTT GAC GAC
29-30	CT del. (2>1)	GAG GCT CTA
btwn. 178-179	G insert. (6>7)	GAA TGG GGG
btwn. 178-179	G insert. (6>7)	GAA TGG GGG

1. Altered nucleotides are underlined.
2. Mutants taken from plates treated 6x with 100 and 200 nM 4-OHE$_2$
3. Eleven redundant mutations not included in the table
4. Position no. 1 corresponds to the start of the coding sequence, and upper case letters refer to sequences in the coding region of the cII gene.

Table 2. DNA sequence context of mutations among mutants in 6 times solvent-treated BB® rat2 cells[1,2,3]

position	change	context
24	GAC del. (3>2)	GTT GAC GAC
34	C>T	CTA CGA ATC
52	C>T	TTG CTT AAC
64	G>A	ATC GCA ATG
73	C>G	CGC AAC GAG
88	G del. (6>5)	GAA TGG GGG
113	C>T	AAG TCG CAG
113	C>A	AAG TCG CAG
118	A>G	CAG ATC AGC
179	G del. (6>5)	GAA TGG GGG
179	G>C	CTT GGA ACT
196	G>A	GAC GAC ATG
241	A del. (6>5)	ATT AAA AAA
248	G>C	AAA CGC CCG
190-192	G insert. (6>7)	GAA TGG GGG
btwn. 178-179	G insert. (6>7)	GAA TGG GGG
btwn. 178-179	C>G	CGC AAC GAG

1. Altered nucleotides are underlined.
2. Nine redundant mutations not included in the table.

A summary table is included below.

Table 3. Mutational spectrum of 4-OHE_2 induced and spontaneous mutations: numbers and percentages of mutants

Mutation class		**4-OHE_2**		**control**	
		number	**%**	**number**	**%**
Total		34	100	17	10
Base substitutions		26	76.5	12	70.6
Transitions		10	29.4	7	41.2
	GC:AT	5	14.7	6	35.3
	at CpG sites	1	2.9	4	23.5
	AT:GC	5	14.7	1	5.9
Transversions		16	47.1	5	29.4
	GC:TA	5	14.7	1	5.9
	GC:CG	8	23.5	4	23.5
	AT:CG	3	8.8	0	0
	AT:TA	0	0	0	0
Deletions		5	14.7	4	23.5
	1 bp	3	8.8	3	17.7
	2 or 3 bp	2	2.8	1	5.9
Insertions (-1bp)		3	8.8	1	5.9

Studies in rats

Mutant fractions:

Two experiments were carried out because of the realtively small changes in mutant fractions. In each the rats were treated for 20 weeks. Statistically significant differences in the MF's were observed between the control and the E_2, 4-OHE_2, 4-OHE_2 + E_2 groups (Table 4). The highest mutant fractions was observed for the combination of 4-OHE_2 and E_2. 2-OHE_2 was not mutagenic. We did not observe a dose response for mutagenesis induced by 4-OHE_2. Additionally we investigated whether the estrogenic compounds, diethylstibesterol (DES) or 4-OH-diethylstilbesterol were mutagenic in rats. At 5 mg/rat the MF's for DES and 4-OH-DES were 1.55 +/- 0.39 and 1.43 +/- 0.48 resp.

Table 4. Effects of E_2, 4-OHE_2 and 2-OHE_2 on the mutant fraction in the cII gene from 4-OHE_2 -treated BB® lacI Fisher rats

treatment	Dose (mg)	MF (mutants/ 10^5 pfu)	SD
2-OHE_2	5	0.96	0.25
4-OHE_2	2.5	1.65	0.69 *
4-OHE_2	5	1.48	0.38 *
E_2	5	1.43	0.49
4-OHE_2 +E_2	2.5 + 2.5	1.93	0.50 *
control		1.05	0.41

*, $p < 0.05$ vs. control in 2-tailed t-test

The data for the individual rats is included below

Table 5. Mutant fractions in individual rats treated with E_2, 4-OHE_2 and 2-OHE_2

	Experiment 1				
rat #	**control**	**4-OHE2**	**E2**	**4-OHE2**	**2-OHE2**
1	0.63	1.90	1.18	0.80	
2	0.71	2.15	1.62	2.30	
3	0.90	2.30	2.07	2.15	
4	1.17	2.13	2.43	1.30	
5	1.85	1.78	2.88	0.71	
6	0.55	1.43	2.66	1.90	
				2.43	
	Experiment 2				
7	1.12	1.07	1.42		0.94
8	0.74	1.67	0.98		1.00
9	0.85	1.29	1.09		0.55
10	0.77	1.09	1.37		0.97
11	1.47	1.91	0.74		1.34
12	1.34	1.18	2.33		0.78

13	0.00	1.97	2.19		1.13
14	1.17	1.44			
15	1.64				
16	1.33				

Mutational spectra in rats

The mutational profiles for E_2, 4-OHE_2, 4-OHE_2 + E_2 and control mutations were analyzed. A major difference was that the fraction of mutations at AT base pairs was 19% in the 4-OHE_2 groups, 21% in the 4-OHE_2 + E_2 group, but only 10 and 11% in the E_2 and control groups resp. (Figure 4). This is consistent with ability of 4-OHE_2 to form depurinating adducts with adenine. A table presenting the raw data follows:

Table 6 . Distribution of mutations induced by E_2, 4-OHE_2, E_2 + 4-OHE_2 and in control rat mammary tissue in vivo

	numbers of mutants							
	GC AT	GC TA	GC CG	AT GC	AT CG	AT TA	DEL/INS	total
4-OHE2	63	20	4	9	3	5	12	116
E2 + 4-OHE2	17	3	5	3	2	5	3	38
E2	16	10	3	1	0	2	8	40
control	37	17	6	7	3	1	16	87

The results are also depicted graphically in Fig. 4 in terms of mutant fractions at GC and AT base pairs.

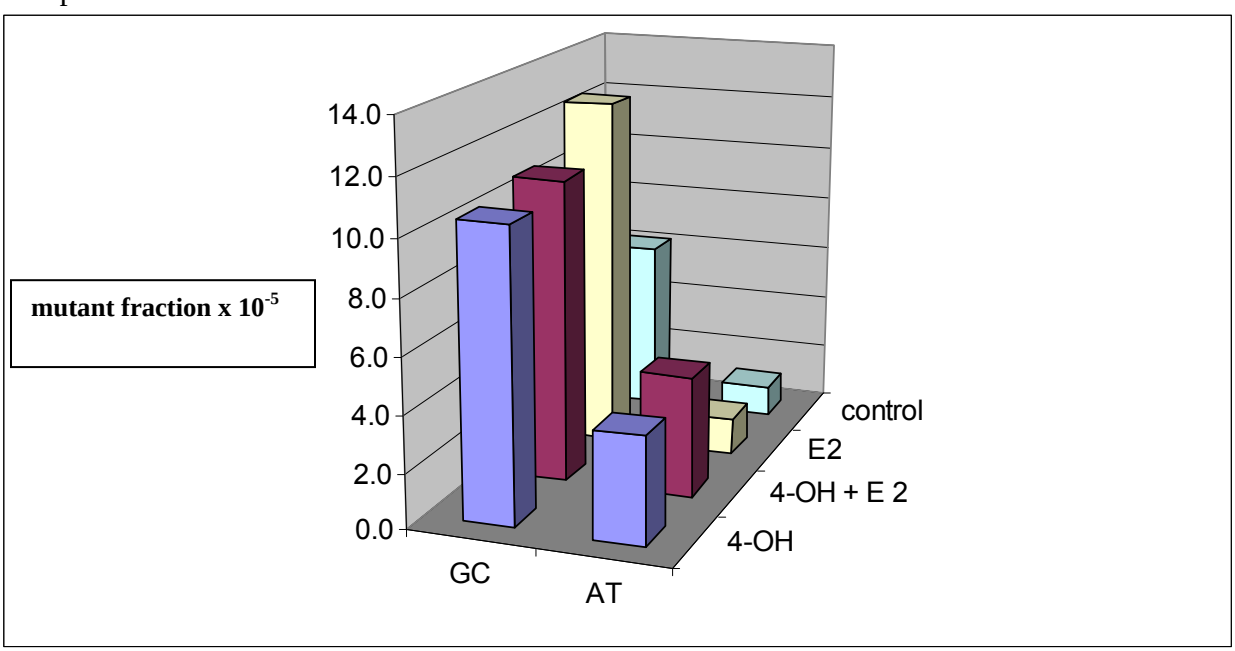


Figure 4. Mutant factions induced at AT and GC base pairs by E_2, 4-OHE_2, E_2 + 4-OHE_2 and in control rat mammary tissue in vivo.

Next proposed plan of research

To finish analyzing data and publish results

C. Key research accomplishments

A major goal of this project was to demonstrate that estradiol and its 4-hydroxymetabolite were mutagenic and that mutagenesis results from a genotoxic mechanism. We have demonstrated that 4-OHE_2 is mutagenic *in vitro* and *in vivo*. Although it could be argued that mutagenicity results from increased cell turnover, we have also observed that there is an increase in mutations at AT base pairs relative to controls, and this provides evidence that DNA modifications occur.

D. Reportable results

1. Zhao Z. Kosinska W. Khmelnitsky M. Cavalieri EL. Rogan EG. Chakravarti D. Sacks PG. Guttenplan JB. Mutagenic activity of 4-hydroxyestradiol, but not 2-hydroxyestradiol, in BB rat2 embryonic cells, and the mutational spectrum of 4-hydroxyestradiol. Chemical Research in Toxicology. 19(3):475-9, 2006.
2. Cavalieri E. Chakravarti D. Guttenplan J. Hart E. Ingle J. Jankowiak R. Muti P. Rogan E. Russo J. Santen R. Sutter T. Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. Biochimica et Biophysica Acta. 1766(1):63-78, 2006.
3. Kosinska, W., Zhao, Z., Khmelnitsky, M., Cavalieri, E., Rogan, E., Chakravarti, D., Sacks, P., and Guttenplan, J.B. Effects of estradiol, 4-hydroxyestradiol and estradiol 3,4-quinone on mutagenesis in vivo and in vitro. Proc Era of Hope 2005
4. Khmelnitsky, M., Kosinska, W., Cavalieri, E.C., Rogan, E.G., Chakravarti, D., Guttenplan, J.B. Mutagenic activity and specificity of estradiol and metabolites in lacI rat mammary gland and liver. Proc Am Assoc Cancer Res 2007
5. Khmelnitsky, M., Kosinska, W., Cavalieri, E.C., Rogan, E.G., Chakravarti, D., Guttenplan, J.B. Mutagenic activity and specificity of estradiol and metabolites in lacI rat mammary gland and liver (manuscript in preparation).

E. Conclusions: In both *in vitro* and *in vivo* systems the 4-OHE_2 led to a modest increase in mutagenesis (ca. 50%). However, as estrogens are present in many tissues and particularly high in premenopausal women, it is not unexpected that they would be weak mutagens. If this were not so, they would lead to high incidences of cancer. As the pathways leading to metabolism of estrogens to genotoxic compounds has been elucidated, this knowledge provides leads for intervention and prevention of estrogen-induced breast cancer.

F. References

1. Henderson, B. E., and Feigelson, H. S. (2000). Hormonal carcinogenesis. *Carcinogenesis 21***,** 427-33.
2. Wu, A. H., Pike, M. C., and Stram, D. O. (1999). Meta-analysis: dietary fat intake, serum estrogen levels, and the risk of breast cancer. *J Natl Cancer Inst 91***,** 529-34.
3. Liehr, J. G. (2000). Is estradiol a genotoxic mutagenic carcinogen? *Endocr Rev*, *21***,** 40-54.
4. Yager, J. D. (2000). Endogenous estrogens as carcinogens through metabolic activation. *Journal of the National Cancer Institute*. *27***,** 67-73.
5. 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***.** *27*, 75-93.

6. 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***,** 3294-6.
7. Rogan, E. G., Badawi, A. F., Devanesan, P. D., Meza, J. L., Edney, J. A., West, W. W., Higginbotham, S. M., and Cavalieri, E. L. (2003). Relative imbalances in estrogen metabolism and conjugation in breast tissue of women with carcinoma: potential biomarkers of susceptibility to cancer. *Carcinogenesis. 24***,** 697-702.
8. Kong, L. Y., Szaniszlo, P., Albrecht, T., and Liehr, J. G. (2000). Frequency and molecular analysis of hprt mutations induced by estradiol in Chinese hamster V79 cells. *Int J Oncol. 17***,** 1141-9.
9. Fernandez, S. V., Russo, I. H., Lareef, M., Balsara, B., and Russo, J. (2005). Comparative genomic hybridization of human breast epithelial cells transformed by estrogen and its metabolites. *Int J Oncol. 26***,** 691-5.
10. Russo, J., Hasan Lareef, M., Balogh, G., Guo, S., and Russo, I. H. (2003). Estrogen and its metabolites are carcinogenic agents in human breast epithelial cells. *J Steroid Biochem Mol Biol. 87***,** 1-25.
11. Chakravarti, D., Mailander, P. C., Li, K. M., Higginbotham, S., Zhang, H. L., Gross, M. L., Meza, J. L., Cavalieri, E. L., and Rogan, E. G. (2001). Evidence that a burst of DNA depurination in SENCAR mouse skin induces error-prone repair and forms mutations in the H-ras gene. *Oncogene. 20***,** 7945-53.
12. Hohn, B. (1979). In vitro Packaging of Lambda and Cosmid DNA. *Methods in Enzymology. 68***,** 299-324.
13. Jakubczak, J. L., Merlino, G., French, J. E., Muller, W. J., Paul, B., Adhya, S., and Garges, S. (1996). Analysis of Genetic Instability During Mammary Tumor Progression Using a Novel Selection-Based Assay for In Vivo Mutations in a Bacteriophage Transgene Target. *Proc Natl Acad Sci, USA. 93***,** 9073-9078.
14. Gollapudi, B. B., Jackson, K. M., and Stott, W. T. (1998). Hepatic lacI and cII mutation in transgenic (lambdaLIZ) rats treated with dimethylnitrosamine. *Mutat Res. 419***,** 131-5.
15. Zimmer, D. M., Harbach, P. R., Mattes, W. B., and Aaron, C. S. (1999). Comparison of mutant frequencies at the transgenic lambda LacI and cII/cI loci in control and ENU-treated Big Blue mice. *Environ Mol Mutagen. 33***,** 249-56.
16. Watson, D. E., Cunningham, M. L., and Tindall, K. R. (1998). Spontaneous and ENU-induced mutation spectra at the cII locus in Big Blue Rat2 embryonic fibroblasts. *Mutagenesis. 13***,** 487-97.
17. Swiger, R. R. (2001). Just how does the cII selection system work in Muta Mouse? *Environmental & Molecular Mutagenesis. 37***,** 290-6.

Progress Report Specific Aim #4

Richard J. Santen MD.

A. Introduction:

Breast cancer occurs in approximately 240,000 women in the United States annually with 50,000 expected deaths (1)). Improved diagnostic and treatment strategies have decreased mortality by 25 % over the past two decades (2,3), but the physical and psychological burdens of surgery, radiotherapy, hormonal therapy and chemotherapy are substantial. Accordingly, breast cancer prevention represents a major focus of current research. (4). Rational strategies for breast cancer prevention require understanding of the molecular mechanisms of carcinogenesis. Genetic factors predominate in a substantial fraction of women with breast cancer and include *known mutations* of *BRCA 1* and *2, CHEK2, TP53, LKB-1*, and *PTEN* in 5-10% (5-7) and *unknown mutations*, as determined by identical twin studies, in another 10-15% (6). Hormonal factors can modulate the development of breast cancer in known mutation carriers as exemplified by the 50% reduction in lifetime incidence by early oophorectomy in *BRCA 1* and *2* carriers (8,9). Hormonal factors also exert major modulating effects on breast cancer incidence in women without a demonstrable genetic component (10). Epidemiologic and experimental data implicate estradiol (E_2) as a key hormone in breast cancer development.

The precise molecular mechanisms whereby E_2 influences breast cancer development are not well understood. Current theories suggest that two separate pathways potentially responsible: one ER dependent and the other, ER independent and mediated by genotoxic metabolites of estradiol (11). While mechanistically distinct, these pathways could act in a synergistic, additive, or independent fashion. Current concepts suggest that ER dependent mechanisms involve increased stimulation of cell proliferation with mutational errors during replication (12,13). ER independent genotoxic metabolites could produce mutations through damage to DNA (14-17). Yager and Davidson recently outlined the four oxidative steps potentially involved (11): **(1)** 16-hydroxylation with covalent binding of 16-hydroxy-E_2 to proteins and consequent DNA damage; **(2)** redox cycling with formation of oxygen free radicals resulting from reversible inter-conversions between the catechol-estrogens and estrogen-quinones; **(3)** 2-hydroxylation with formation of stable DNA adducts resulting from further oxidation to their respective quinones; and **(4)** 4-hydroxylation with formation of unstable, depurinating estrogen-quinone adducts. Data published within the past year by collaborators in Specific Aims #1-3 provide strong support that the 4-hydroxylation pathway can exert mutagenic as well as carcinogenic effects.

The rationale for current preventative strategies with anti-estrogens rests primarily upon the receptor dependent pathway of carcinogenesis (4,18). However, the possibility that E_2 can contribute to the development of breast cancer via ER independent mechanisms is of key clinical importance (11). Newer agents being considered for breast cancer prevention, the aromatase inhibitors, block E_2 synthesis and abrogate both ER dependent and independent pathways whereas the antiestrogens only block the ER (19-21). Provided that genotoxic pathways do contribute to breast cancer development, the

aromatase inhibitors should be superior to the anti-estrogens for the prevention of breast cancer.

Specific Aim #4 was designed to provide proof of the principle that E_2 can influence breast carcinogenesis through an ER independent mechanism in an animal model. Because of the strong genetic components of breast cancer in women, we chose an experimental model carrying a breast cancer oncogene in which to examine the modulatory effects of E_2. This model involved transgenic mice engineered to over-express the Wnt-1 oncogene in the mammary gland which experience a 100% incidence of breast cancer (22). To dissect out the separate roles of ER independent from ER dependent pathways, we cross bred these animals into an ERα knock-out (ERKO) background. E_2, administered to castrate ERKO/Wnt-1 animals, caused a dose response related induction of breast tumors, providing strong evidence of an ER independent effect. To ensure complete blockade of any residual ER function, we administered the selective ER down-regulator fulvestrant to ERKO/Wnt-1 mice and demonstrated that E_2 still resulted in enhanced tumor formation. Our data provide evidence that ER independent as well as ER dependent mechanisms of E_2 carcinogenesis are operative in this animal model and provide a rationale for use of aromatase inhibitors rather than anti-estrogens for prevention of breast cancer in women.

B. Methods and Procedures:

Animals. Wnt-1 transgenic animals were originally obtained from Dr. Harold Varmus and bred at the National Institute of Environmental Health Sciences, Research Triangle Park (22-24). These animals were then cross bred with ERα +/- heterozygous mice to generate Wnt-1 transgenic mice in the presence of ERα. Breeding pairs were then sent to the University of Virginia to establish a colony on site. The mice were housed and treated in accordance with the NIH guide to Humane Use of Animals in Research. All surgical procedures were approved by the Animal Care and Use Committee at the University of Virginia. Genotyping was performed using tail DNA and PCR as previously described (22). Studies providing full characterization of the phenotypic, biologic, and biochemical properties of these animals have been recently reviewed (22,25,26).

Preparation of whole mounts. The whole mounts from excised mammary glands were fixed and stained as previously described (27).

"Estradiol Clamp" method. Silastic tubes of 0.19 cm internal diameter were filled with estradiol/cholesterol mixtures at various ratios. The lengths of the filled part of Silastic tubes were 2.5, 5 or 7.5 mm, respectively. Our prior studies validated the ability to clamp plasma E_2 levels ranging from 20 to 800 pg/ml over a two-month period (28). As further evidence of the validity of this technique, we also demonstrated linear dose responses in uterine weight in castrate mice (29). In the present study, the levels of plasma E_2 were clamped at 5, 10, 80, and 240 pg/ml. The implants used contained the following estradiol/cholesterol ratios and lengths: 1:39/2.5 mm (5 pg/ml); 1:19/2.5 mm (10 pg/ml); 1:3/2.5 mm (80 pg/ml) and 1:3/7.5mm (240 pg/ml). These implants were inserted under the skin in the back of mice and changed every two months. Fulvestrant was administered by subcutaneous injection once per week at a dosage of 5 mg/mouse.

Endogenous and exogenous gene expression assays. The ERE-TATA-luciferase reporter system was previously described in detail (30). Progesterone receptors A and B

were detected on western blots using the monoclonal antibody against the progesterone receptor (Cell Signaling Technology, Beverly, MA) (30).

Measurements of Depurinated Estrogen-adenine conjugate

CE/FASS. The analysis of extracts obtained from tissue samples was performed using the GPA100 capillary electrophoresis system (Groton Biosystems). The UV/VIS detector model Lambda 1010 (Bischoff Chromatography) was used for detection of absorption based electropherograms measured at 214 nm. Capillary electrophoresis (CE) with field amplified sample stacking (CE/FASS) using a reversed polarity mode was used for detection. UV transparent capillaries (80 cm x 74 μm ID and 366 μm OD) were obtained from Polymicro Technologies (48 cm effective length to the detection window were used). Prior to use the capillary was conditioned for 30 minutes with 0.1M sodium hydroxide, water, and running buffer. The pH of the running buffer (Tris 25 mM plus 0.5% SDS) was adjusted to pH = 3.2 using phosphoric acid. The final extract obtained from the 0.5 g of tissue using a solid phase extraction (SPE) and incubated with helix palmitase (sulfatase/beta-glucuronidase enzyme) to release free steroids was dissolved in 100 μL of methanol/water (1:1) mixture and used for subsequent analysis. Typically, about 2 μL of this extract was diluted by a factor of 10 and used in CE analysis. The water plug was injected first into the capillary (p = 35 mba, t = 0.3 min) followed by the sample (p = 35 mba, t = 0.6 min). The applied potential for separation was –5 kV (and/or -3 kV) and typical separation was performed at T = 28^{o}C.

Biochip. IgG 15G8-4H12 monoclonal antibodies (MAb) and 4-OHE$_2$-1- N3Ade-SAMSA conjugates were developed specifically for the detection of estrogen-derived DNA adducts. MAbs were purified through protein G column. PS-10 ProteinChip from Ciphergen Biosystems were used for analysis. The pre-activated chip surface had carbonyl diimidazole (CDI) activated amine surface, which can covalently bind MAb through an amine group. First, each spot of the biochip (with a diameter of ~3 mm) was wetted with 2 μL of 50% acetonitrile/water solution for about 1 min. After removing acetonitrile/water solution, about 2 μL PBS was added to the active surface and incubated in a humidity box for 1 min at room temperature. After removing PBS buffer (following procedures established by Ciphergen Biosystems), about 2 μL of PBS buffer was added again to the active area of the chip along with 3 μL of 15G8-4H12 MAb (c = 2.4 mg/mL). Then the biochip was placed in a humidity box overnight at 4^{o}C. Afterwards, the chip was placed inside of a conical tube (containing 7 mL of 1 M ethanolamine) and incubated on a shaking platform for about 1 hour. Subsequently ethanolamine solution was removed without disturbing the active chip area and the chip was washed two times with PBS and 0.5% triton X-100 at room temperature for 5 min while shaking. Finally, the chip was exposed to 1 μL of sample and 0.5 μL $5 \cdot 10^{-6}$ M 4-OHE$_2$-1- N3Ade-SAMSA conjugate, i.e. a fluorescent reporter molecule. The required concentration of 4-OHE$_2$-1-N3Ade-SAMSA conjugate was established in part based on the calibration curve and the results obtained from CE/FASS experiment. After incubation and careful removal of non-bound analytes, 5 μL PBS was added and pipetted up and down at least 15 times. This washing procedure was repeated several times. To keep the chips wet, 2 μL of fresh PBS buffer was added to each spot and the signal was measured by fluorescence imaging using Leica microscope (Germany) equipped with a UV lamp and CCD camera. The concentration of estrogen-derived DNA adducts was estimated form the calibration curve (data not shown).

Measurement of Estradiol Metabolites: LCMS analyses were carried out with a Waters Acquity ultra performance liquid chromatography (UPLC) system connected with a high performance Quattro Micro triple quadrupole mass spectrometer designed for LC/MS-MS operation. Analytical separations on the UPLC system were conducted using an Acquity UPLC BEH C18 1.7 μ column (1 X 100 mm) at a flow rate of 0.15 ml/min. The gradient started with 80% A (0.1% formic acid in H_2O) and 20% B (0.1% formic acid in CH_3CN), changed to 79% A over 4 min, followed by a 6-min linear gradient to 45% A, resulting in a total separation time of 10 min. The elutions from the UPLC column were introduced to the Quatro Micro mass spectrometer.

The ionization method used for the MS analysis was ESI in both the positive (PI) and negative (NI) ion mode with an ESI-MS capillary voltage of 3.0 kV, an extractor cone voltage of 3 V, and a detector voltage of 650 V. Desolvation gas flow was maintained at 600 L/h. Cone gas flow was set at 60 L/h. Desolvation temperature and source temperature were set to 200 and 100 °C, respectively. MS-MS was performed in a multiple reaction monitoring (MRM) mode to produce structural information about the analytes by fragmenting the parent ions inside the mass spectrometer and identifying the resulting daughter/fragment ions. The resulting data were processed by using QuanLynx software (Waters) to quantify the estrogen metabolites. Pure standards were used to optimize the LC/MS conditions prior to analysis.

Statistical methods. The data describing mouse age at the time of tumor appearance (i.e. first clearly palpable tumors) utilized Kaplan-Meier analyses. The various plots were compared to each other using a life table test to determine whether the rate of tumor development was statistically different among the various treatment groups. This analysis takes into account the time a mouse remains on study tumor free and also accounts for animals removed from study due to sickness.

C. Key research accomplishments:

Estrogen receptor independent effects on tumor development

Removal of the ovaries before day 16 in the ERKO animals allowed examination of the role of estradiol acting in the absence of ERα. This ablative procedure appeared to delay the 50% incidence time point to 24 months and reduced the number of animals with tumors by half relative to intact ERKO mice but this trend was not statistically significant (Fig. 1). In addition to E_2, the ovary produces progesterone and inhibins as well as other factors that could confound our results. Accordingly, we examined the dose response effects of E_2 on tumor formation in castrate animals (22,31). Castrate ERKO/Wnt-1 animals received E_2 over a 24 month period and utilized the "estradiol clamp" method to maintain plasma E_2 levels at 5, 10, 80 and 240 pg/ml. The 240 pg/ml dose in the castrate animals caused tumors to develop earlier with a 50% incidence time point at 10 months versus 24 months in vehicle treated animals **(p=0.0002)** (Fig. 2). As in the intact animals, nearly 100% of E_2 treated animals developed tumors. The 80 pg/ml estradiol dose produced effects intermediate between those of vehicle and 240 pg/ml E_2. The 5 and 10 pg/ml E_2 doses appeared to be sub-threshold with no differences compared to vehicle (data not shown).

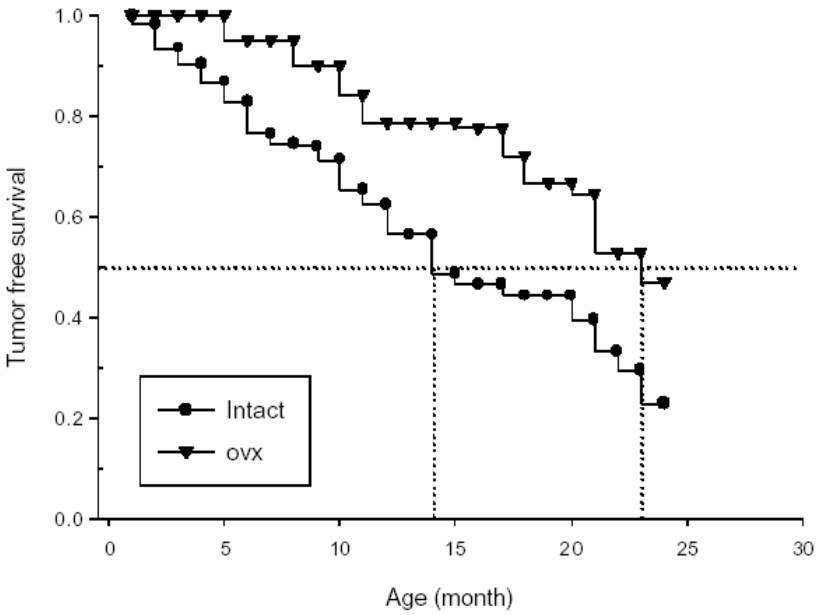


Figure 1. Kaplan –Meyer plots of tumor formation in intact and oophorectecomized (OVX) estrogen receptor knock out (ERKO) transgenic mice.

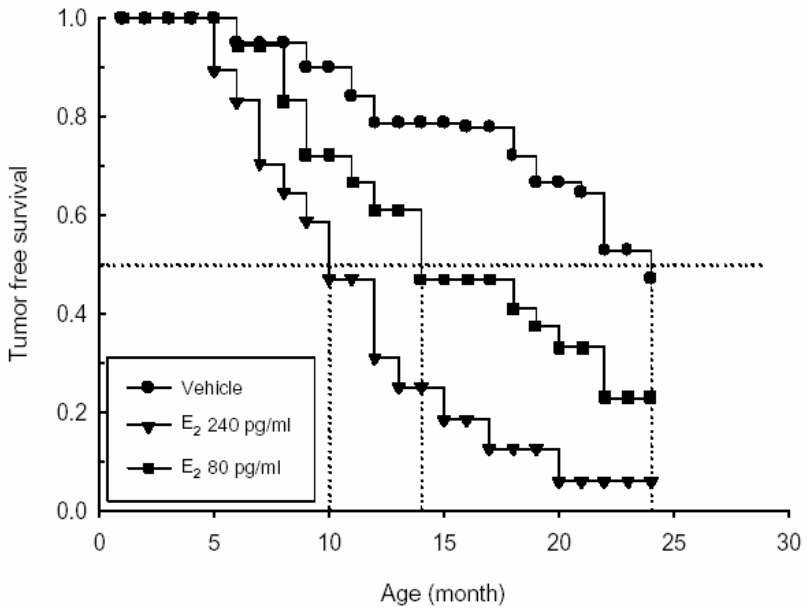


Figure 2. Tumor curves plotted by Kaplan–Meyer analysis in ERKO castrate animals treated with vehicle and with silastic implants delivering estradiol to "clamp" estradiol levels at 80 and 240 pg/ml.

Presence of genotoxic metabolites in mammary tissue: Our working hypothesis suggested that the ER independent effects of E_2 resulted from production of genotoxic metabolites. In order to demonstrate these metabolites in breast tissue, we utilized LC/tandem mass spectrometry methodology to assay the levels of multiple metabolites. In order to enhance the sensitivity of detecting these compounds, we utilized aromatase transfected mice, whose breast tissues contain increased levels of estrogens compared to wild type. The metabolites measured demonstrated the presence of 2- and 4-hydroxylated estrogens, the estradiol quinones, and the depurinated products, 1-N-3-estradiol-adenine and 1-N-7-estradiol-guanine. The 4-OH –estrogen metabolites are shown in Figure 3 and the depurinated compounds in Figure 4. The full range of genotoxic metabolites in ERKO/Wnt-1 tumors are shown in Figure 5. Figure 6 illustrates the levels of the 1-N3-adenine-estrogen depurinated compound as measured by two separate methods to verify its presence in breast tumors.

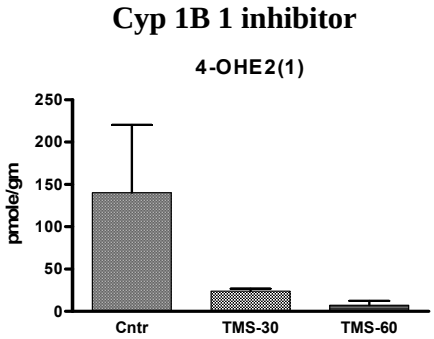


Figure 3. Levels of 4-OH estradiol (E_2) and estrogen (E_1) in breast tissue of aromatase over-expressing transgenic mice. Shown are basal levels and the levels detected during administration of the CYP 1B1 inhibitor, tetra-methoxy-stilbene (TMS) at 30 mg/kg and 60 mg/kg. CYP 1B1 catalyzes the conversion of estradiol and estrone to their 4-OH catecholestrogen metabolites. The inhibition of the 4-OH catecholestrogens provides evidence of the specificity and sensitivity of the assay used.

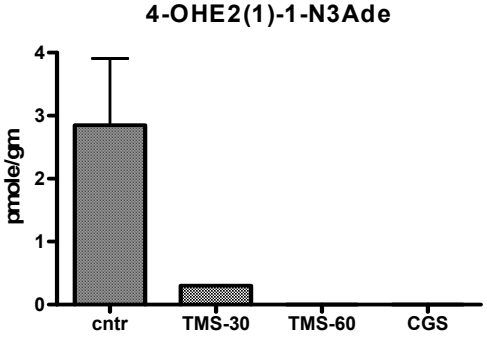


Figure 4. Levels of 4-OH-E_2/E_1-1-N3-adenine in breast tissue of aromatase overexpressing transgenic mice under basal conditions and during administration of TMS at the same doses as above.

C

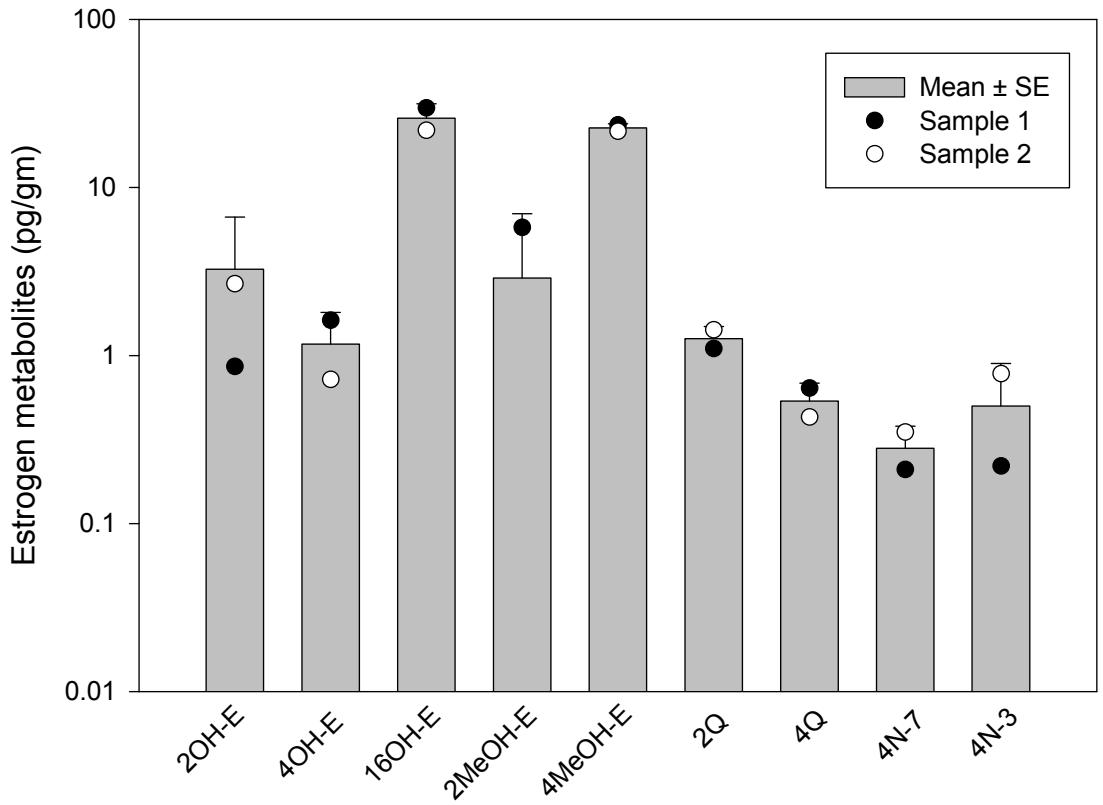


Figure 5. Levels of each of the estrogen metabolites in ERKO/Wnt-1 tumors as measured by LC-tandem mass spectrometry.

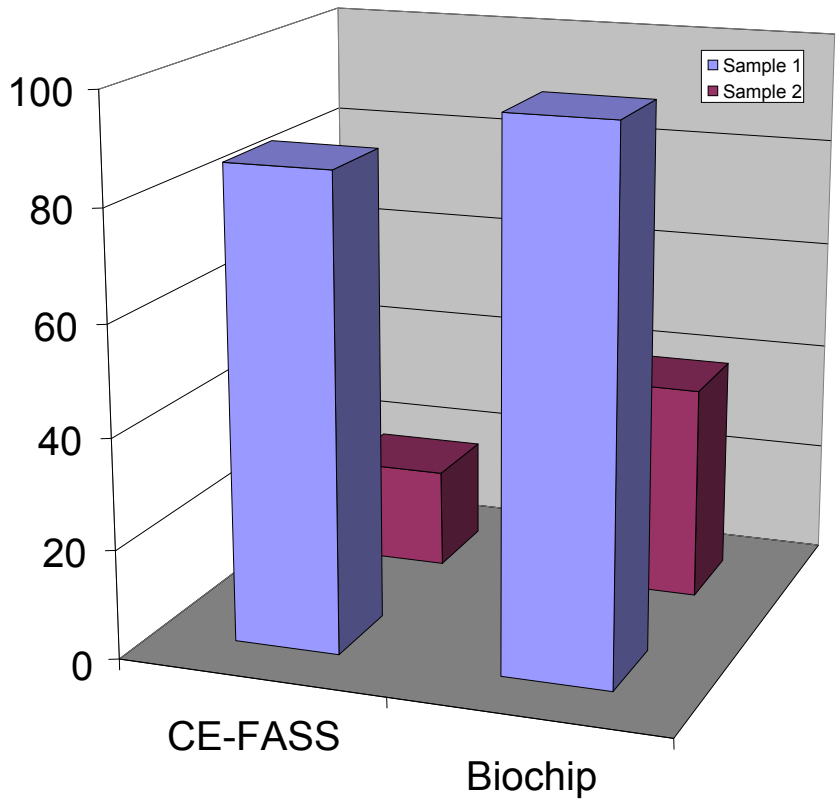


Figure 6. Verification of presence of 1-N3-adenine-estradiol in ERKO/Wnt-1 breast tumors using the CE-FASS and Biochip methods.

Complete elimination of effects of truncated ERα and ERβ. Prior studies had shown that our ERKO mice express an mRNA species that yields a 56 Kd truncated ERα message (32), whose translated protein should retain its DNA and ligand binding domains. However, we had found evidence of only minimal residual ER bio-activity with retention of 1-9% of ER binding activity in uterine cytosols and only 10-20% of stimulation of uterine weight. In our current study, western blot analysis in the ERKO/Wnt-1 animals detected only small amounts of truncated receptor protein. ERβ, while not found on RNase protection assays, could be also be detected by the more sensitive quantitative PCR methodology (33). Recognizing the need to eliminate the biologic effects of these possible residual receptors, we completely abrogated ERα and β function with the "pure antiestrogen" fulvestrant and examined the effect of E_2 under these conditions. Fulvestrant or vehicle was administered to castrate ERKO/Wnt-1 animals with E_2 clamped at 240 pg/ml. The tumors appeared at the same rate in the presence or absence of fulvestrant, providing evidence that the effect of E_2 was not mediated by a truncated ERα or low level ERβ (data not shown). A further strategy abrogated receptor mediated effects by the administration of 17α-OH-E2. This compound lacked ER mediated effects on uterine weight but is capable of forming potentially genotoxic compounds (34). 17α–OH estradiol induced tumors in the ERKO animals at a rate similar to that in animals with E_2 maintained at the same plasma level (i.e. 240 pg/ml) (data not shown) .

Bioassay of "clamped" E_2 on uterine weight. Our various strategies to examine the ER independent effects of E_2 critically depended upon complete blockade of any residual ER activity resulting from a truncated ERα or low level ERβ. Measurement of uterine weight provided a robust bioassay of the tissue effects of E_2 to determine if complete blockade was achieved. We measured uterine weight after at least two months of E_2 exposure under each experimental condition. Uterine weights in the intact (i.e. non-castrate) ER+ /Wnt-1 animals were 84 ± 12 mg (mean ± SE) and in the ER-/Wnt-1, 28 ± 3 mg. Ovariectomy (ovx) reduced uterine weights to 5 ± 0.9 and 4 ± 0.5 mg respectively in both the ER+ and ER- animals. This observation confirmed that the ERKO/Wnt-1 animals did in fact have biologically functional truncated ER alpha. (i.e. difference between intact and castrate ERKO). Administration of E_2 by the "clamp" method increased uterine weight to 164 ± 5 mg in the ER+/Wnt-1 animals and to 24 ± 3 mg in the ER-/Wnt-1. This blunted increase in uterine weight in ERKO animals represented only 18% responsiveness compared to ER+ animals and further confirmed the reduced biologic activity of the truncated E_1 receptor.

Since truncated ER was present and somewhat active, it appeared critically important to demonstrate that fulvestrant completely abrogated its activity. Notably, the administration of fulvestrant completely blocked the residual ER responsiveness in the ERKO/Wnt-1 animals since uterine weight fell to 7 ± 1 mg in the animals receiving this anti-estrogen plus 240 pg/ml E2. Fulvestrant also blocked uterine weight in the E_2 treated ER+/Wnt-1 animals by approximately the same percentage (i.e. 164 to 22 mg or 83% reduction) as in ERKO animals but the absolute reduction in uterine weight was less (22 mg). In aggregate, these data demonstrated that fulvestrant was capable of completely abrogating the effects of residual ER activity in ERKO animals.

We wished to confirm by bioassay that 17α-OH-E_2 also did not stimulate the uterus. At a level of 240 pg/ml this compound caused no increase in uterine weight (4 ± 0.5 mg) indicating its lack of uterotropic activity. As further proof of the minimal ER mediated effects of this compound, we tested its ability to stimulate transcription of endogenous and exogenous estrogen responsive genes and MCF-7 cell growth in vitro. The potency of 17α-OH E_2 on transcription of ERE-luciferase construct (exogenous reporter gene), on progesterone receptor synthesis (endogenous genes), and on cell growth was 1% or less than that of E_2 itself (data not shown).

D. Reportable Outcomes:

1. Song RX, Berstein LM, Santen RJ. RESPONSE: Re: Effect of Long-Term Estrogen Deprivation on Apoptotic Responses of Breast Cancer Cells to 17beta-Estradiol and The Two Faces of Janus: Sex Steroids as Mediators of Both Cell Proliferation and Cell Death. Journal of the National Cancer Institute. 94(15):1173-4, 2002.

2. Santen, R.J. Inhibition of aromatase: insights from recent studies. Steroids 68:559-567. 2003.

3. Yue, W., Santen, R.J., Wang, J-P., Li, Y., Verderame, M.F., Bocchinfuso, W.P., Korach, K.S., Devanesan, P., Todorovic, R., Rogan, E.G., and Cavalieri, E.L. Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis. Journal of Steroid Biochemistry and Molecular Biology 86:477-486, 2003.

4. Song, R-X., Barnes, C.J., Zheng, Z., Bao, Y., Kumar, R., and Santen, R.J. The role of SHC and insulin like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. Proceedings of the National Academy of Medicine USA, 101:2076-2081, 2004.

5. Come SE, Buzdar AU, Arteaga CL, Bissell MJ, Brown MA, Ellis MJ, Goss PE, Green JE, Ingle JN, Lee AV, Medina D, Nicholson RI, Santen RJ, Schiff R, Hart C. Proceedings of the Third International Conference on Recent Advances and Future Directions in EndocrineTherapy for Breast Cancer. Conference Summary Statement Clinical Cancer Research, 10:327s-330s 2004.

6. Zhang, Z., Kumar, R., Santen, R.J., Song, X.-D. The role of adapter protein Shc in estrogen non-genomic action. 69:523-529, Steroids, 2004.

7. Yue, W., Wang, J-P., Bocchinfuso, W., Korach, K., Devanesan, P., Rogan, E., Cavalieri, E., and Santen, R.J. Tamoxifen versus aromatase inhibitors for breast cancer prevention. Clinical Cancer Research 11:925s-930s, 2005.

8. Come SE. Buzdar AU. Ingle JN. Arteaga CL. Brodie AM. Colditz GA. Johnston SR. Kristensen VN. Lonning PE. McDonnell DP. Osborne CK. Russo J. Santen RJ. Yee D. Hart CS. Proceedings of the Fourth International Conference on Recent Advances and Future Directions in Endocrine Manipulation of Breast Cancer: conference summary statement. Clinical Cancer Research. 11(2 Pt 2):861s-4s, 2005.

9. Cavalieri, E., Chakravarti, D., Guttenplan, J., Hart, E., Ingle, J., Jankowiak, R., Muti, P., Rogan E., Russo, J., Santen, R., Sutter, T. Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. Biochimica et Biophysica Acta 1766:63-78, 2006.

Textbook Chapters

1. Santen, R.J. Endocrine-Responsive Cancer IN: Williams Textbook of Endocrinology, 10th edition, Larsen, R., Kronenberg, H., Melmed, S., and Polonsky, K. (ed) W.B. Saunders Company, Philadelphia, PA, pp 1797-1833, 2002.

2. Yue, W., More, G., Naftolin, F., Pauley, R., Shim, W-S., Harvey, H., and Santen, R.J. Use of aromatase inhibitors in breast cancer. IN: Endocrine Therapy of Breast Cancer. John Roberson, Robert Nicholson, and Daniel Hayes (eds), Taylor and Francis Group, London, UK, pp. 75-106, 2002.

3. Santen RJ, Yue W, Bocchinfuso W, Korach K, Wang J-P, Rogan E, Li Y, Cavalieri E, Russo J, Devanesan P, and Verderame M. Estradiol-induced carcinogenesis via formation of genotoxic metabolites, IN: Advances in Ednocrine Therapy of Breast Cancer: Proceedings of the 2003 Gleneagles Conference, Ingle, J.N. and Dowsett, M. (eds), Marcel Dekker, New York, NY, pp. 13-177, 2004.

4. Santen, R.J. Endocrinology of Breast and Endometrial Cancer IN: Yen and Jaffe's Reproductive Endocrinology, 5th ed., Jerome F. Strauss III and Robert L. Barbieri, Elsevier, Philadelphia, PA. 2004, pp. 787-809.

5. Russo, J., Santen, R.J. and Russo, I.H. Hormonal Control of Breast Development. IN: Endocrinology, 5th Edition, DeGroot, L.J. and Jameson, J.L., eds., Elsevier Saunders, Philadelphia, PA, 2006, pp. 3045-3056.

Manuscripts Accepted

1. The Breast Cancer Prevention Collaborative Group: Richard J. Santen, Norman F. Boyd, Rowan T. Chlebowski, Steven Cummings, John F. Cuzick, Mitch Dowsett, Douglas Easton, John F. Forbes, Tim Key, Susan E. Hankinson, Anthony Howell, and James Ingle. Critical assessment of risk factors for breast cancer: what should be used to develop a new breast cancer prediction model? In Press Endocrine related Cancer 2007

2. Park, H-Y., Aiyar, S.E., Fan, P., Wang, J., Yue, W., Okouneva, T., Cox, C., Jordan, M.A., Demers, L., Cho, H., Kim, S-H., Song, R.X-D., Santen, R.J. Effects of tetramethoxystilbene (TMS) on hormone resistant breast cancer cells: biological and biochemical mechanisms of action. (accepted for publication, May 1, 2007)

Reference List

(1) Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, et al. Cancer statistics, 2005. Ca: a Cancer Journal for Clinicians 55(1):10-30, 2005 Jan;-Feb.

(2) Wingo PA, Tong T, Bolden S. Cancer statistics, 1995.[erratum appears in CA Cancer J Clin 1995 Mar-Apr;45(2):127-8]. Ca: a Cancer Journal for Clinicians 45(1):8-30, 1995 Jan;-Feb.

(3) Elmore JG, Armstrong K, Lehman CD, Fletcher SW. Screening for breast cancer. [Review] [136 refs]. JAMA 293(10):1245-56, 2005 Mar 9.

(4) Cuzick J, International Breast Cancer Intervention Study. A brief review of the International Breast Cancer Intervention Study (IBIS), the other current breast cancer prevention trials, and proposals for future trials. Annals of the New York Academy of Sciences 949:123-33, 2001 Dec.

(5) Martin AM, Weber BL. Genetic and hormonal risk factors in breast cancer. Journal of the National Cancer Institute 92(14):1126-35, 2000 Jul.

(6) Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, et al. Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. New England Journal of Medicine 343(2):78-85, 2000 Jul 13.

(7) Thompson D, Easton D. The genetic epidemiology of breast cancer genes. Journal of Mammary Gland Biology & Neoplasia 9(3):221-36, 2004 Jul.

(8) Welcsh PL, King MC. BRCA1 and BRCA2 and the genetics of breast and ovarian cancer. [Review] [91 refs]. Human Molecular Genetics 10(7):705-13, 2001 Apr.

(9) Rebbeck TR, Lynch HT, Neuhausen SL, Narod SA, van't Veer L, Garber JE, et al. Prophylactic oophorectomy in carriers of BRCA1 or BRCA2 mutations.[see comment]. New England Journal of Medicine 346(21):1616-22, 2002 May 23.

(10) Clemons M, Goss P. Estrogen and the risk of breast cancer.[erratum appears in N Engl J Med 2001 Jun 7;344(23):1804]. [Review] [102 refs]. New England Journal of Medicine 344(4):276-85, 2001 Jan 25.

(11) Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. New England Journal of Medicine 354(3):270-82, 2006 Jan.

(12) Preston-Martin S, Pike MC, Ross RK, Henderson BE. Epidemiologic evidence for the increased cell proliferation model of carcinogenesis. Environmental Health Perspectives 101 Suppl 5:137-8, 1993 Dec.

(13) Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE. Increased cell division as a cause of human cancer. Cancer Research 50(23):7415-21, 1990 Dec 1.

(14) Yager JD. Endogenous estrogens as carcinogens through metabolic activation. [Review] [54 refs]. Journal of the National Cancer Institute Monographs (27):67-73, 2000.

(15) Cavalieri E, Chakravarti D, Guttenplan JB, Jankowiak R, Muti P, Rogan E, et al. Catechol Estrogen Quinones as Initiators of Breast and Other Human Cancers. Implications for Biomarkers of Susceptibility and Cancer Prevention. 2006.

(16) Jefcoate CR, Liehr JG, Santen RJ, Sutter TR, Yager JD, Yue W, et al. Tissue-specific synthesis and oxidative metabolism of estrogens. [Review] [149 refs]. Journal of the National Cancer Institute Monographs (27):95-112, 2000.

(17) Liehr JG. Is estradiol a genotoxic mutagenic carcinogen?. Endocrine Reviews 21(1):40-54, 2000 Feb.

(18) Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, et al. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene.[see comment]. Journal of the National Cancer Institute 96(23):1751-61, 2004 Dec 1.

(19) Santen RJ, Harvey HA. Use of aromatase inhibitors in breast carcinoma. [Review] [70 refs]. Endocrine-Related Cancer 6(1):75-92, 1999 Mar.

(20) Brueggemeier RW, Hackett JC, Diaz-Cruz ES. Aromatase inhibitors in the treatment of breast cancer. [Review] [166 refs]. Endocrine Reviews 26(3):331-45, 2005 May.

(21) Yue W, Wang JP, Li Y, Bocchinfuso WP, Korach KS, Devanesan PD, et al. Tamoxifen versus aromatase inhibitors for breast cancer prevention. [Review] [32 refs]. Clinical Cancer Research 11(2 Pt 2):925s-30s, 2005 Jan 15.

(22) Bocchinfuso WP, Hively WP, Couse JF, Varmus HE, Korach KS. A mouse mammary tumor virus-Wnt-1 transgene induces mammary gland hyperplasia and tumorigenesis in mice lacking estrogen receptor-alpha. Cancer Research 59(8):1869-76, 1999 Apr 15.

(23) Tsukamoto AS, Grosschedl R, Guzman RC, Parslow T, Varmus HE. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell 55(4):619-25, 1988 Nov 18.

(24) Korach KS, Couse JF, Curtis SW, Washburn TF, Lindzey J, Kimbro KS, et al. Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. [Review] [50 refs]. Recent Progress in Hormone Research 51:159-86; discussion 186-8, 1996.

(25) Deroo BJ, Korach KS. Estrogen receptors and human disease. [Review] [126 refs]. Journal of Clinical Investigation 116(3):561-70, 2006 Mar.

(26) Hewitt SC, Harrell JC, Korach KS. Lessons in estrogen biology from knockout and transgenic animals. [Review] [95 refs]. Annual Review of Physiology 67:285-308, 2005.

(27) Bocchinfuso WP, Lindzey JK, Hewitt SC, Clark JA, Myers PH, Cooper R, et al. Induction of mammary gland development in estrogen receptor-alpha knockout mice. Endocrinology 141(8):2982-94, 2000 Aug.

(28) Shim WS, Conaway M, Masamura S, Yue W, Wang JP, Kmar R, et al. Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. Endocrinology 141(1):396-405, 2000 Jan.

(29) Yue W, Santen RJ, Wang JP, Hamilton CJ, Demers LM. Aromatase within the breast. Endocrine-Related Cancer 6(2):157-64, 1999 Jun.

(30) Jeng MH, Shupnik MA, Bender TP, Westin EH, Bandyopadhyay D, Kumar R, et al. Estrogen receptor expression and function in long-term estrogen-deprived human breast cancer cells. Endocrinology 139(10):4164-74, 1998 Oct.

(31) Jeruss JS, Santiago JY, Woodruff TK. Localization of activin and inhibin subunits, receptors and SMADs in the mouse mammary gland. Molecular & Cellular Endocrinology 203;(1-2):185-96.

(32) Couse JF, Curtis SW, Washburn TF, Lindzey J, Golding TS, Lubahn DB, et al. Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene. Molecular Endocrinology 9(11):1441-54, 1995 Nov.

(33) Tekmal RR, Liu YG, Nair HB, Jones J, Perla RP, Lubahn DB, et al. Estrogen receptor alpha is required for mammary development and the induction of mammary hyperplasia and epigenetic alterations in the aromatase transgenic mice. Journal of Steroid Biochemistry & Molecular Biology 95(1-5):9-15, 2005 May.

(34) Merriam GR, MacLusky NJ, Johnson LA, Naftolin F. 2-hydroxyestradiol-17 alpha and 4-hydroxyestradiol-17 alpha, catechol estrogen analogs with reduced estrogen receptor affinity. Steroids 36(1):13-20, 1980 Jul.

(35) Cuzick J. Aromatase inhibitors in prevention--data from the ATAC (arimidex, tamoxifen alone or in combination) trial and the design of IBIS-II (the second International Breast Cancer Intervention Study). [Review] [10 refs]. Recent Results in Cancer Research 163:96-103; discussion 264-6, 2003.

(36) Ingle JN. Endocrine therapy trials of aromatase inhibitors for breast cancer in the adjuvant and prevention settings. [Review] [23 refs]. Clinical Cancer Research 11(2 Pt 2):900s-5s, 2005 Jan 15.

E. Conclusions

In summary, these studies provide proof of the principle that breast cancer development can be influenced by ER independent as well as ER dependent mechanisms and that these effects act in concert. Accordingly, this study provides mechanistic evidence in support of the use of aromatase inhibitors in preference to the anti-estrogens for prevention of breast cancer. Anti-estrogens primarily block receptor mediated pathways whereas the aromatase inhibitors block both receptor mediated and receptor independent effects of estradiol. Two current and one planned clinical trials are examining the aromatase inhibitors for prevention of breast cancer (35,36).

SPECIFIC AIM 5 – INGLE

A. Introduction

Globally, breast cancer remains a major problem for many women and is a concern for virtually all women. Much of the clinical research to date has involved large and very expensive clinical trials. The most recently reported is that of the Study of Tamoxifen and Raloxifene (STAR) that involved over 19,000 women accrued over six years with a reported cost of about $118,000,000. The current major efforts in prevention involve placebo-controlled trials of exemestane (MAP.3) involving almost 5,000 patients, and anastrazole (IBIS 2) that involves 6,000 women. It is clear that new approaches and paradigms are needed to identify biologically-based strategies for prevention of breast cancer. It would be of great value to identify biomarkers that would allow targeted studies involving smaller sample sizes at much lower cost.

B. Body

The approach taken in Specific Aim 5 has been to develop and conduct a prospective study aimed at determining levels of estrogen, catechol estrogen metabolites, catechol estrogen-glutathione conjugates, and most importantly, catechol estrogen-DNA adducts in women at high risk of developing breast cancer and in women with a personal history of breast cancer. The protocol that was developed at Mayo Clinic is entitled "Estrogen-DNA Adducts in Breast, Urine, and Serum as Biomarkers of Breast Cancer Risk" (protocol #19-2005).

To date material has been collected on 58 high risk women and 24 women with newly diagnosed breast cancer. An amendment has recently been approved by the Mayo Institutional Review Board to collect specimens (nipple aspirate fluid, urine, and serum) on low or average risk women. The amended protocol is appended and will be formally activated by the end of May 2007. The goal is to collect specimens on a total of 300 women (100 each from high-risk women, women with recently diagnosed breast cancer, and low or average risk women).

C. Key Research Accomplishments

Initial results have been developed from 12 high risk women and 17 women with a personal history of breast cancer from Mayo that have been combined with 4 average risk women from the University of Nebraska combined with 42 such women from Italy. Analysis of estrogen metabolites, conjugates, and depurinating DNA adducts in urine samples from these 46 healthy control women, 12 high-risk women, and 17 women with breast cancer showed that the levels of the ratios of depurinating DNA adducts to their respective estrogen metabolites and conjugates were significantly higher in high-risk women ($p<0.001$) and women with breast cancer ($p<0.001$) than control subjects. The high-risk and breast cancer groups were not significantly different ($p=0.62$). After adjusting for patient characteristics, these ratios were still significantly associated with

health status. These findings support the hypothesis that depurinating estrogen-DNA adducts can serve as potential biomarkers of risk of developing breast cancer.

D. Reportable Outcomes

The results in section C have been submitted for publication.

E. Conclusions

The results to date, as noted above, are highly supportive of the hypothesis that estrogen genotoxicity plays an important role in breast cancer development. Of particular importance is that depurinating estrogen-DNA adducts appear to biomarkers of risk and we are continuing the study of this concept with expansion of our clinical samples.

F. References

None to date

Mayo Clinic Cancer Center

Estrogen-DNA Adducts in Breast, Urine and Serum as Biomarkers of Breast Cancer Risk

Mayo Study Chair: Sandhya Pruthi, M.D.
Mayo Clinic
200 First Street, SW
Rochester, MN 55905
507/284–2511
507/284-5280 (FAX)

Document History	Implementation Date
Activation	May 20, 2005
Mayo Addendum 1	March 7, 2006
Mayo Addendum 2	March 22, 2006
Mayo Addendum 3	June 20, 2006
Mayo Addendum 4	January 22, 2007
Mayo Addendum 5	Pending Approvals

14 April, 2005

---

1. **Protocol Title:**
   Estrogen-DNA Adducts in Breast, Urine and Serum as Biomarkers of Breast Cancer Risk

2. **Phase:** Not applicable.

3. **Principal Investigator - Sandhya Pruthi, MD**
   Co- Investigator - James L. Ingle, MD
   Co-Investigator- Nicole Sandhu, MD
   Mayo Clinic, Rochester, MN
   Mayo Breast Diagnostic Clinic

   Collaboration and Investigators: Ercole Cavalieri, D.Sc., Professor, Eppley Institute for Research in Cancer, 986805 Nebraska Medical Center, Omaha, NE 68198-6805

   Eleanor Rogan, Ph.D., Professor, Eppley Institute for Research in Cancer, 986805 Nebraska Medical Center, Omaha, NE 68198-6805

4. **Location of Study:** Mayo Clinic, Rochester, MN

5. **Time Required to Complete:** 4/1/2005 – 4/2007 Two years

6. **Objectives:** We propose that a major carcinogenic risk for human breast cancer is associated with endogenous catechol estrogens (CE, both 2-CE and 4-CE), which can be activated to ultimate carcinogenic forms, namely quinones (CE-Q) [1,2]. Estrogens are oxidized to 4-CE primarily by cytochrome P450 (CYP) 1B1 [3]. An efficient protective mechanism that impedes oxidation of 4-CE to CE-3,4-Q is monomethylation at the 2-, 3- or 4-hydroxyl group catalyzed by catechol-O-methyltransferase (COMT). With elevated rates of CE synthesis and/or deficient methylation, significant quantities of 4-CE are available for oxidation to CE-3, 4-Q by peroxidases or cytochrome P-450. Covalent binding of CE-3, 4-Q to DNA leads to DNA damage that could initiate the cancer process [1, 2]. This damage is the formation of the depurinating DNA adducts, 4-hydroxyestradiol (estrone)-1-N7guanine and 4-hydroxyestradiol (estrone)-1-N3adenine. Such DNA adducts are released from DNA by breaking the bond between the purine base (adenine or guanine) and the deoxyribose, a process called “depurination”. The resulting “apurinic sites” in the DNA, gaps with no base, can cause mutations to occur at these sites. CE-Q also react with glutathione (GSH) to form conjugates that are present in tissues and excreted in urine as CE-GSH, CE-cysteine (Cys) and CE-*N*-acetylCys (CE-NAcCys) conjugates [4-6].
   With the purpose of supporting our hypothesis and developing bioassays for susceptibility to breast cancer, we plan to conduct a preliminary study in humans to determine the levels of CE, CE metabolites, CE-DNA adducts and CE conjugates in nipple aspirate fluid samples obtained from women at elevated risk for the development of breast cancer and women with breast cancer. CE, conjugates and adducts will be identified and quantified in the breast fluid by HPLC with electrochemical/mass spectrometric detection.
   The analyses of CE, conjugates and DNA adducts will be conducted under the supervision of Dr. Eleanor Rogan at UNMC, in a manner similar to the analyses of CE and CE conjugates

in breast tissue conducted previously [7]. Nipple aspirate fluid has been used in epidemiological studies by analyzing various biochemicals, including lactose, cholesterol, estrogens, androgens and related compounds for almost 20 years [8-10]. Recently Chatterton, et al., analyzed the levels of estradiol, estrone and estrone sulfate with improved sensitivity [11].

Drs. Cavalieri and Rogan's laboratory recently analyzed three additional nipple aspirate samples from healthy control women who have not been diagnosed with breast cancer. As seen in Figure 1, the 2-catechol estrogens (2-OHE_1 and 2-OHE_2) dominate the profile of estrogen metabolites in these samples, which would be expected from previous studies showing that in normal humans and animals the levels of 2-catechol estrogens are much higher than those of the 4-catechol estrogens. In addition, significant amounts of methylated catechol estrogens were detected in one sample, suggesting that this protective pathway was working. All three samples contained glutathione conjugates or their break-down products, the NAcCys conjugates. The presence of the glutathione or NAcCys conjugates suggests that DNA adducts are also formed by the catechol estrogen quinones.

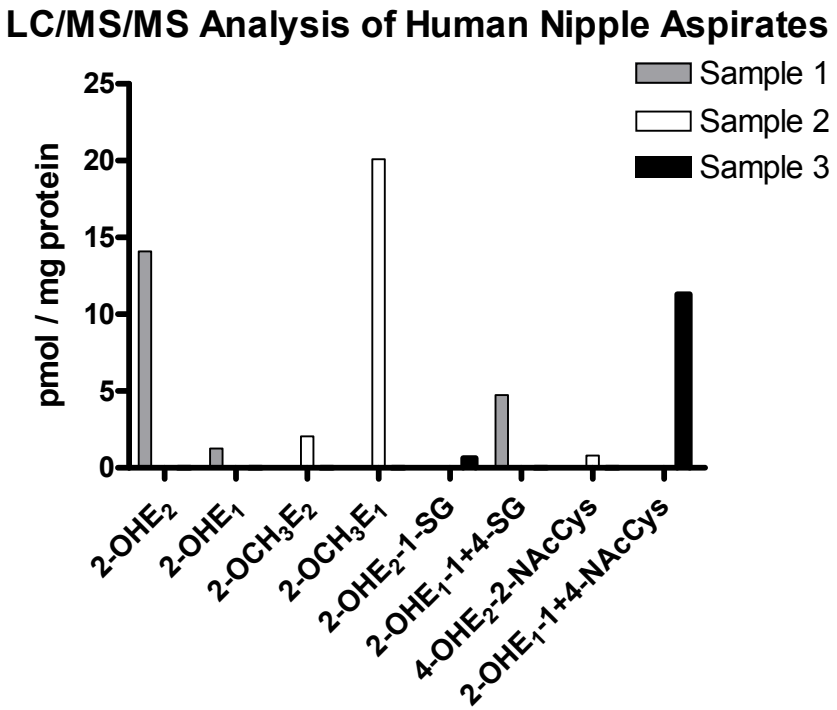


Figure 1

The proposed studies will yield valuable new information and insights in a relatively short time-frame. The analysis of estrogen metabolites, estrogen conjugates and estrogen-DNA adducts by LC/MS/MS is rapid, and sample preparation for nipple aspirate fluid is minimal (ultrafiltration to remove cells and cellular debris). The information acquired on levels of estrogen metabolites, estrogen conjugates and estrogen-DNA adducts in breast fluid will be novel, as these compounds have never before been measured in breast fluid. They should provide us with novel insights into the formation and metabolism of estrogens in the breast, the possible association with development of breast cancer and expression of selected enzymes in breast tissue. Because the subjects can be recruited and the collection of samples

completed within 12 to 18 months and the analyses can be completed soon thereafter, we expect this project to yield significant scientific progress within two years.

Add 1 Similar potential estrogen biomarkers have also been observed in urine samples.

Add 3 New research analyzing estrogen biomarkers in serum samples has shown that estrogen compounds can be identified in serum. These samples may provide a more extensive picture of estrogen-associated biomarkers because we know that these metabolites, conjugates and depurinating DNA adducts are excreted in both urine and feces. It is postulated that serum-based samples may be more useful in predicting cancer susceptibility.

Using the results of this study, future prospective studies can be planned where nipple aspirate samples taken from women before and after treatment with Arimidex or Tamoxifen will provide completely new information and insights into the effects of these drugs on estrogen metabolism in the breast. Other future studies include being able to determine the utility of these various estrogen derivatives as biomarkers for risk of developing breast cancer.

7. Study Population:

a. The target population is adult women, ages 19 to 70 years of age. In 2003, there were approximately 2000 new and 3000 established patient visits in the Breast Diagnostic Clinic (BDC) and Breast Cancer Clinic (BCC) Our new patients include those seen in the BDC with new breast concerns and those at seeking counseling regarding a family history or at high risk for developing breast cancer, as well as those women seen in the BCC with a new diagnosis of invasive or non-invasive breast cancer. Our established patients include those with a history of breast cancer, atypical hyperplasia or lobular carcinoma in situ (LCIS) who are at high risk for the development of breast cancer, and women returning for follow-up of breast concerns.

b. The collaborative study will include women who are at elevated risk for the development of breast cancer determined by the Gail Model, a personal history of atypical hyperplasia or LCIS, or a known deleterious BRCA 1 or BRCA 2 gene mutation, women newly diagnosed with invasive or non-invasive breast cancer and women who are low or average risk (control group). The Gail Model, a validated tool used to estimate a woman's risk of breast cancer, incorporates five significant prognostic indicators of risk for breast cancer: age, age at menarche, number of pregnancies, number of first degree relatives with breast cancer, number of previous breast biopsies and a history of atypia on prior breast biopsy. A 5 year risk score of $\geq$1.66 % is and lifetime risk of $\geq$ 20 % are considered high risk. The tool however has some limitations as it can underestimate risk for women with multiple affected relatives and those relatives diagnosed at a < age 50 years who may be BRCA 1 or 2 gene mutation carriers. Women at high-risk are advised regarding various risk-reduction strategies such as chemoprevention with tamoxifen and even prophylactic mastectomy. Tamoxifen, a selective estrogen receptor blocker, is an approved therapy for the reduction of breast carcinoma risk. It has been shown to reduce the risk of the development of breast cancer by 49% in the Breast Cancer Prevention Trial (BCPT-P1). However, many women who are candidates for this drug choose not to take it because of side-effects which include exacerbation of menopausal symptoms and risk of thromboembolic events and endometrial carcinoma. It is becoming more important for us to be able to provide women with an accurate risk estimate which can

Add 3 assist in decision-making regarding which option to pursue for risk reduction. The study of nipple aspirate fluid, urine and serum for analysis of estrogen metabolites hopefully will be able to more accurately identify women at high risk where specific modalities for risk-reduction can potentially offer the best outcome.

Potential participants for all 3 groups (high risk - based on the Gail Model score, atypical hyperplasia, history of LCIS, or BRCA ½ gene mutation carriers, newly diagnosed with

breast cancer and low or average risk) will be identified by the physicians/ providers who work in the two breast clinics. Once identified, the participant will meet with the study coordinator who will discuss the study, review the consent form and obtain consent from those eligible and wanting to participate in the study.

Add 1 The number we expect to accrue who meet eligibility criteria and provide urine and serum samples will be 300 women over 2 years. We plan to accrue 100 women who are at high risk, 100 women newly diagnosed with early breast cancer and 100 women who

Add 3 are low or average risk (this group would serve as the control group).

Nipple Aspirate Procedure:

The collection of nipple aspirate fluid will be offered to women who are at high risk, BRCA ½ gene mutation carriers or newly diagnosed with breast cancer. The nipple

Add 1 aspirate fluid collected from the consenting participants will then be sent to Dr. Rogan at the Eppley Institute for Research in Cancer in Nebraska.

Of the 200 women who are eligible for the nipple aspirate procedure and consent to the study, we know that that there will be women from whom we are unable to obtain nipple aspirate fluid and they will be designated as a non-yielder (approximately ½). Also, we expect that some will decline to have the nipple aspirate procedure.

Women who consent to the nipple aspirate procedure will receive $25.00 study remuneration regardless of outcome (yielder vs. non-yielder).

Urine Sample Collection:

Add 1 Each participating woman will be asked to give a urine sample. If the woman chooses to have the nipple aspirate procedure performed, she will ideally be asked to provide a morning spot urine sample on the same or next day as the procedure. The urine samples will also be analyzed by ultraperformance liquid chromatography monitored by tandem mass spectrometry for the estrogen metabolites, conjugates and depurinating DNA adducts. The urine will need to be prepared with 100 mg of ascorbic acid per 50 ml sample. The ascorbic acid needs to be added before freezing. The urine samples will

Add 3 then be sent to Dr. Rogan.

Serum Sample Collection:

Eligible women will be asked to provide a single serum sample along with the urine

Add 3 sample. The serum would be collected on the same day as the urine collection and/or the nipple aspirate fluid procedure.

Participant charts will be reviewed to obtain information about age, race, general health, any endocrine disorders, history of cancer, estrogen and progesterone receptor status, and Her 2/neu status (for patients newly diagnosed with breast cancer), menopausal status, and reproductive history, history of breast disease and medication use. The participant's history of smoking and alcohol consumption will also be obtained. Such information is be relevant because alcohol and some components of smoke can induce higher levels of certain enzymes, in particular the cytochrome P450 enzyme that are involved in estrogen metabolism. Subjects may be asked to provide clarification of information or information needed that is not available from their medical records.

c. There are no enrollment restrictions based on race or ethnic origin.

Inclusion Criteria

All Groups

- Women between the age of 19 and 70 years of age

Group 1 – High Risk

- At least one of the following:
  - Gail model 5 year score of ≥1.66 % (the Gail Model has been developed only for use in women 35 years or older)
  - Gail Model lifetime risk estimate of ≥20 % (in women younger than 35 years the 5 year risk is often very low despite significant risk factors)
  - Known deleterious BRCA 1 or 2 gene mutation carrier
  - History of lobular carcinoma in-situ or atypical ductal or lobular hyperplasia (Gail model score is not necessary in women who carry these diagnoses). The nipple aspirate fluid would be obtained from the unaffected breast.

Group 2 – Newly diagnosed:

- Either of the following:
  - Women newly diagnosed (within 30 calendar days of diagnosis) with Stages I and II (node-negative and node-positive) breast cancer. The nipple aspirate fluid will be obtained from the unaffected breast
  - Women with newly diagnosed DCIS (Stage 0) from the unaffected breast

Group 3 - Control

- Low or average risk (Gail Model 5 year risk < 1.66 % or lifetime risk < 20 %) – control group

Exclusion Criteria

All Groups

- Women treated with chemotherapeutic agents for breast or other cancers
- Women with advanced breast cancer
- SERM use (Tamoxifen or Raloxifene) or Aromatase Inhibitor use
- Estrogen or other hormone use currently or in the past 3 months
- Oral contraceptive use in the past 3 months

Add 3

8. Protocol Design

This will be a prospective study.

a. Subject Identification: To maintain confidentiality, subjects will be identified by a code number based on the sample number and sample acquisition date and no patient identifiers will be sent to the Eppley Institute. All patient identifier information will remain at Mayo Clinic.

b. Review of Records: Yes, patient medical charts will be reviewed.

c. Recruitment Process:

Participants at average /low risk for breast cancer, those with an elevated risk for breast cancer, and those who are newly diagnosed with breast cancer will be recruited from the BDC and BCC at the Mayo Clinic in Rochester, MN.

Another control group of healthy women will be accrued at University of Nebraska Medical Center.

d. Informed Consent Process: When potential patients are identified they will be informed about this study by the study coordinator who will explain the study, consent form. Eligible participants are invited to participate by allowing a nipple aspirate fluid sample

to be collected for use in the study. The participant will be given the option to provide a urine and serum sample in addition to the nipple aspirate fluid sample, or she can provide only urine and serum samples. The potential participant will be given the written informed consent document and will be given enough time to read and understand it. The study coordinator or investigators will be able to obtain consent.

When the process of informed consent has been completed, the investigator will again be available to answer any questions before the participant signs the consent form. After the participant has signed the consent form the nipple aspiration procedure will be scheduled if agreed to and the urine and serum samples will be obtained.

Add 3

Participants who are at low/average risk for developing breast cancer will not be asked to provide the nipple aspirate fluid sample. These patients will be consented with a separate consent form.

e. Subject Assignment:

Eligible participants include: 1) women at increased risk for the development of breast cancer and have a Gail model 5 year score of $\geq$1.66 % OR lifetime risk estimate of $\geq$20 % 2) personal history of atypical hyperplasia or LCIS 3) newly diagnosed with breast cancer (within 30 calendar days) with Stages I and II breast cancer (node-negative and node-positive) 4) newly diagnosed with DCIS (Stage 0) and 5) women who are carriers of a BRCA 1 or BRCA 2 gene mutation. These women may or may not have a Gail model score of > 1.66 %.

A control group will include women who are low or average risk for the development of breast cancer. These women have a 5 year Gail model score of <1.66 %. The population will be identified by the physicians/providers evaluating women presenting with a breast concern.

f. Subject Screening Procedures:

Women who are evaluated in the BDC and BCC who are eligible participants include: 1) women at increased risk for the development of breast cancer with a Gail model 5 year score of $\geq$1.66 % or lifetime risk estimate of $\geq$20 % 2) women with a personal history of atypical hyperplasia or LCIS (a Gail Model score is not necessary if an individual is diagnosed with a history of atypia) 3) women who are known BRCA 1 or BRCA 2 mutation carriers 4) women newly diagnosed with breast cancer (within 30 calendar days) with Stages I and II breast cancer (node-negative and node-positive) 5) newly diagnosed DCIS (Stage 0) and 6) women at low/average risk of breast cancer with a 5 year Gail score of <1.66 %.

Women with advanced breast cancer or other disease that may profoundly alter their estrogen metabolism will not be included. Women treated with chemotherapeutic agents for breast or other cancers will also be excluded.

g. Data Collection Procedures:

A research study coordinator will be involved in the reviewing the participant's chart and data collection**.** A participant's medical chart will be reviewed to obtain information about age, race, age at menarche, number of pregnancies, and age at first live birth, number of first degree relatives with breast cancer, number of previous breast biopsies and history of atypia or LCIS on prior breast biopsy, general health, endocrine disorders, menstrual status, history of cancer or prior breast disease, estrogen and progesterone receptor status of tissue, Her 2 neu status in patients recently diagnosed with breast cancer, tobacco, and alcohol use. Most of this information can be obtained most easily from the medical record chart.

Add 1

Once the participant has consented to the study they will be scheduled for a nipple aspirate procedure to be performed by a health care provider at the Breast Clinic, who will process the fluid for transport to the Eppley Institute in Nebraska.

Collection of nipple aspirate fluid samples will be carried out in the using the First Cyte Breast Aspirator (Cytyc Health Corporation). Although the utility of the analytical data obtained from nipple aspirate samples is not yet established, we think that we are establishing a core set of data from human [11] and animal [12-14] studies that will enable us and others to interpret the profiles of estrogen compounds in the nipple aspirate fluid samples in terms of risk of developing breast cancer.

Add 3

The area of the skin around the nipple will be washed to reduce the possibility of infection. EMLA, an anesthetic cream, will be applied to the breast/areolar region that is to be sampled. The breast will then be warmed for 5-10 min with a heating pad and then gently massaged by the participant for 2 min. The area of the skin around the nipple will be washed to reduce the possibility of infection. An aspirator will then be centered directly over the nipple and areolar region to elicit the nipple aspirate fluid. Aspiration will be achieved by applying gentle suction using a 35 cc syringe connected to the aspirator, a technique similar to using a breast pump for lactation. The gentle suction tiny amounts of fluid from the ducts to the surface of the nipple for collection in a glass capillary tube. The amount of nipple fluid collected will be approximately 10-30 ul.

Following collection, the fluid will be chilled in ice until stored at the –80 EC prior to sending to Dr. Rogan in Nebraska. Dr. Rogan's team will analyze the nipple aspirate fluid per protocol. This fluid will first be passed through a filter to remove cells and cell debris. The material collected on the filter will be used to prepare RNA for analysis of enzyme expression by the Molecular Biology Core. The nipple aspirate fluid will be analyzed for 31 estrogen metabolites, conjugates and depurinating DNA adducts by HPLC with electrochemical and mass spectrometric detectors.

From the experience of others, we expect 10-30 ul of nipple fluid will be obtained from each subject [7-10] who is a yiedler. The minimum amount of nipple fluid needed for analysis is 5 ul. The analysis will be normalized to total protein concentration. Our experience to date with biological samples makes us confident that we will accomplish successful analyze of the majority of the estrogen compounds in the nipple aspirate using our HPLC with the mass spectrometry detector. Additional nipple aspirate fluid obtained will be stored at Mayo Clinic for future studies that may be of value**.**

Previous studies of breast tissue [11] and breast fluid [15] suggest that the analysis of estrogen compounds in the breast fluid will be very informative. Based on our previous results [11], we expect to find that women with breast cancer have relatively higher levels of estrogens, more 4-CE than 2-CE, relatively lower levels of methoxy CE and higher levels of CE-GSH conjugates compared to women without breast cancer. We expect to use these data (and other data we are collecting) to develop profiles of biomarkers for women at high risk of breast cancer or protected from breast cancer. Although, the absolute levels of these compounds will vary, we expect that relative values of the various biomarkers will be very informative.

Add 1

The urine samples will also be analyzed by ultraperformance liquid chromatography monitored by tandem mass spectrometry for the estrogen metabolites, conjugates and depurinating DNA adducts. The urine will need to be prepared with 50 mg of ascorbic acid per 50 ml sample. The ascorbic acid needs to be added before freezing. A total of four 10ml aliquots of urine sample will be shipped to Dr. Rogan and one 10ml aliquot will be stored at Mayo Clinic Rochester.

Add 3, 4

Add 3 The serum samples will need to be prepared with 1 mg of ascorbic acid per 1 ml of serum. The ascorbic acid needs to be added before freezing the sample at -80°C. 20 ml of blood will be collected for each sample in order to obtain an end product of 8 ml of serum. The samples will be processed at room temperature for optimum result. The serum will be divided into four-2 ml aliquots. Two aliquots will be shipped to Dr. Rogan and two aliquots will be stored at Mayo Clinic Rochester. Samples will be batched and shipped on dry ice.

All samples (including nipple aspirate fluid, urine and serum) are to be shipped by courier to the following address:

Eleanor Rogan, Ph.D.
Eppley Research Institute, ESH 6031
University of Nebraska Medical Center
668 South 41st Street
Omaha, NE 68105
(402) 559-4095

h. Clinical Assessments: None will be conducted for this study.

i. Research Interventions: First, the participant will be asked to participate in the study and sign an informed consent document. Second, the participant will undergo the nipple aspiration procedure at a scheduled time. This procedure is described in detail in Section 8.f. above. The participant may also opt to have both urine and serum samples collected taken in addition to the nipple aspiration procedure at this time, or choose to complete the urine and serum samples only. One day after (unless the aspiration is performed on the eve of a weekend or holiday, in which case this may take up to 3 days) the nipple aspiration procedure, the study coordinator or health care provider will call the participant to determine whether any problems have arisen. Assuming that none have arisen, that will be the last event the participant will experience as part of this study.

Add 1

Add 3

j. Data Analysis:

Add 3 For all participants, characteristics including race, body mass index, age at menarche, menopausal status, age at menopause if applicable, smoking history, alcohol consumption, pregnancy history including age at each pregnancy, and age at first live birth, lactation, history of benign breast disease, hysterectomy and disease type including hormone receptor status and Her2/neu status (for breast cancer patients) will be collected. Our initial analyses of urine samples have not shown differences in the levels of estrogen-DNA adducts between pre- and post-menopausal women. We will have both pre- and post-menopausal women in this study and will learn whether this preliminary finding holds true in the larger data set. All hypothesis tests will be two-sided and conducted at the 0.05 level of significance. Participant characteristics will be summarized using descriptive statistics.

<u>Statistical considerations for Group 1</u>

The comparisons of interest are the levels of estrogens, catechol estrogen metabolites, catechol estrogen-GSH conjugates and catechol estrogen-DNA adducts (N3Ade and N7Gua) among healthy women, high risk women and women with breast cancer. The three groups will be age -matched (19-34, 35-50, 51-70). Analysis of variance (ANOVA) will be used to test whether there is a significant difference in the mean analytes among the groups. If ANOVA indicates a significant difference in between the means, further analysis will be conducted using Tukey's pairwise comparison procedure to control for multiple testing. The distribution of the patient characteristics described above among

the three groups will be examined. Multivariate regression will be used to adjust for relevant patient characteristics that do not appear to be balanced among the groups.

Our first study of breast samples (~1 g each) obtained from breast biopsies, which included 49 control women (18 with normal tissue and 31 with fibrocystic changes) and 28 with breast carcinoma, yielded statistically significant differences between cases and controls in the levels of 4-$OHE_1(E_2)$ ($p< 0.01$), and $E_1(E_2)$-Q conjugates ($p< 0.003$) [8], although the analyses were much less sensitive at that time. These results strongly suggest that the analyses of nipple aspirate fluid samples will provide even more significant data. We therefore anticipate a medium effect size of 0.20 [40]. These data suggest a difference of approximately 0.64 standard deviations between breast cancer cases and healthy controls. Assuming that the means for healthy controls, high risk and breast cancer participants are approximately equally spaced in this range, a 0.05 level of significance and 80% power, 80 subjects with nipple aspirate fluid available for analysis in each group are needed [40]. A total of 100 subjects in each of the three groups will provide approximately 80% power (at the 0.05 level of significance) to detect an effect size of 0.18 in the mean analytes obtained from urine and serum samples.

We estimate an accrual of 100 low or average risk women over the 2 year period. This will be the control group. These women will be asked to provide a urine and serum sample only.

9. Risks/Benefits Assessment

a. Risks: The potential risks from this procedure include possible pain associated with the procedure. Participants will be advised that the results from this study will be combined with the results of other studies to learn what factors are significant in the development of breast cancer, and these results cannot be used by themselves to predict risk of developing breast cancer.

Add 1 To address possible physical and/or emotional risks, at the time of the procedure the study coordinator will provide subjects with telephone numbers if they have problems or concerns, and she will contact the subjects by telephone the day after the procedure to inquire if there are an symptoms after the procedure. If a subject is having problems or concerns, Dr. Pruthi or Dr. Sandhu will be informed and will arrange for the participant to receive appropriate medical care as soon as possible.

b. Benefits to the subject: There will be no direct benefit to the subjects from the analyses of estrogen compounds.

Add 3 **c. Compensation:** Participants will receive $25.00 remuneration for their participation in the nipple aspirate fluid collection component of the study.

10. Reporting of Serious of Unexpected Adverse Events: A serious adverse event would be an undesirable experience associated with undergoing nipple aspiration that resulted in death, a life-threatening condition, hospitalization, disability or intervention to prevent permanent impairment or damage. Nipple aspiration has been conducted for 20 years [7-10]. Thus, the occurrence of serious adverse events is highly unlikely and the procedure is very similar to using a breast pump for lactation.

11. Description of Protocol Drugs or Devices: Not applicable.

a. Disposition of Data: Data will be stored in the Eppley Institute (offices 6006 and 6015, Eppley Science Hall) in locked cabinets and in computer files protected by passwords. The information about a subject will be stripped of its identifiers by the staff member

maintaining our database and assigned a code number based on sample number and date of acquisition, which will allow us to refer back to the medical record, through the nurse coordinator, if needed. The code breaker document is kept in Dr. Rogan's laboratory in paper form in a locked file cabinet in room 6015 ESH, the office of Ms Sheila Higginbotham, Research Technologist II, who maintains all our human subjects' records. Ms Higginbotham has the key to the cabinet. The code breaker document is kept electronically in Ms Higginbotham's password-protected human subjects' folder on the Eppley Institute Local Area Network. Data coded to protect subjects' identities will be used internally by members of the research group only for statistical analysis of the results of the determinations of estrogen compounds in the nipple aspirate fluid samples. One of the strengths of our proposal is the ability to use subject information (obtained from patient records at Mayo Clinic, Rochester, MN) on age, race, smoking history, alcohol consumption, menopausal status, reproductive history, hormone receptors, etc., in analyzing the results of the study. Reports and publications resulting from this study will not contain any information that identifies subjects.

12. Modification of the Protocol: Any modifications to the protocol and/or consent form will be submitted to our IRB and Cancer Center Clinical Research Administrative Committee for approval.

13. Departure from the Protocol: Any deviations from the protocol, which are not expected, will be reported to our IRB and Cancer Center Clinical Research Administrative Committee for approval.

14. Medical Care for Research-Related Injuries:

To address any concerns at the time of the procedure, the study coordinator will provide subjects with telephone numbers to call. The RN in the GCRC will contact the subjects by telephone a day or so after the procedure to inquire about whether they are having any medical concerns after the procedure.

15. Literature Cited:

1. 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.. Molecular origin of cancer: Catechol estrogen-3,4-quinones as endogenous tumor initiators. *Proc. Natl. Acad. Sci. USA*, 94: 10937-10942, 1997.
2. Cavalieri, E.L., and Rogan, E.G. The key role of endogenous catechol estrogen-3,4-quinones in tumor initiation. In: *The Role of Catechol Quinones in Cellular Toxicity* (C.R. Creveling, Ed.), F.P. Graham Pub. Co., New York, 247-260, 2000.
3. Hayes, C.L., Spink, D.C., Spink, B.C., Cao, J.Q., Walker, N.J., and Sutter, T.R. 17β-Estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *Proc. Natl. Acad. Sci. USA*, 93: 9776-9781, 1996.
4. Cao, K., Devanesan, P.D., Ramanathan, R., Gross, M.L., Rogan, E.G. and Cavalieri, E.L. Covalent binding of catechol estrogens to glutathione catalyzed by horseradish peroxidase, lactoperoxidase or rat liver microsomes. *Chem. Res. Toxicol.*, 11: 917-924, 1998.
5. Devanesan, P., Todorovic, R., Zhao, J., Gross, M.L., Rogan, E.G., and Cavalieri, E.L. Catechol estrogen conjugates and adducts in the kidney of male Syrian golden hamsters treated with 4-hydroxyestradiol: Potential biomarkers for estrogen-induced cancer. *Carcinogenesis*, 22: 489-497, 2001.
6. Todorovic, R., Devanesan, P., Higginbotham, S., Zhao, J., Gross, M.L., Rogan, E.G., and Cavalieri, E.L. Analysis of potential biomarkers of estrogen-initiated cancer in the urine of Syrian golden hamsters treated with 4-hydroxyestradiol. *Carcinogenesis*, 22: 905-911, 2001.

7. Rogan, E.G., Badawi, A. F., Devanesan, P.D., Meza, J.L., Edney, J.A., West, W.W., Higginbotham, S.M., and Cavalieri, E.L. Relative imbalances in estrogen metabolism and conjugation in breast tissue of women with carcinoma: Potential biomarkers of susceptibility to cancer. *Carcinogenesis*, 24: 697-702, 2003.
8. Petrakis, J.L., Lim, M.L., Miike, R., Lee, R.E., Morris, M., Lee, L., and Mason, L. Nipple aspirate fluids in adult nonlactating women-lactose content, cationic Na+, K+, Na+/K+ ratio, and coloration. *Breast Cancer Res. Treat.*, 13: 71-78, 1989.
9. Wrensch, M.R., Petrakis, N.L., Gruenke, L.D., Miike, R., Ernster, V.L., King, E.E., Hauck, W.W., Craig, J.C., and Goodson, W.H. 3rd. Breast fluid cholesterol and cholesterol beta-epoxide concentrations in women with benign breast disease. *Cancer Res.*, 49: 2168-2174, 1989.
10. Petrakis, N.L. Nipple aspirate fluid in epidemiologic studies of breast disease. *Epidemiol. Rev.*, 15: 188-195, 1993.
11. Chatterton, R.T., Jr., Geiger, A.S., Khan, S.A., Helenowski, I.B., Jovanovic, B.D., and Gann, P.H. Variation in estradiol, estradiol precursors, and estrogen-related products in nipple aspirate fluid from normal premenopausal women. *Cancer Epidemiol. Biomarkers Prev.*, 13: 928-935, 2004.
12. Cavalieri, E.L., Kumar, S., Todorovic, R., Higginbotham, S., Badawi, A.F., and Rogan, E.G. Imbalance of estrogen homeostasis in kidney and liver of hamsters treated with estradiol: implications for estrogen-induced initiation of renal tumors. *Chem. Res. Toxicol.*, 14: 1041-1050, 2001.
13. Devanesan, P., Santen, R.J., Bocchinfuso, W.P., Korach, K.S., Rogan, E.G., and Cavalieri, E. L. Catechol estrogen metabolites and conjugates in mammary tumors and hyperplastic tissue from estrogen receptor-α knock-out (ERKO)/Wnt-1 mice: implications for initiation of mammary tumors. *Carcinogenesis*, 22: 1573-1576, 2001.
14. Cavalieri, E.L., Devanesan, P., Bosland, M.C., Badawi, A.F., and Rogan, E.G. Catechol estrogen metabolites and conjugates in different regions of the prostate of Noble rats treated with 4-hydroxyestradiol: implications for estrogen-induced initiation of prostate cancer. *Carcinogenesis*, 23: 329-333, 2002.
15. Castagnetta, L. A., Granata, O. M., Arcuri, F. P., Polito, L. M., Rosati, F., and Cartoni, G. P. Gas chromatography/mass spectrometry of catechol estrogens. *Steroids*, 57: 437-443, 1992.

MOLECULAR BIOLOGY CORE – SUTTER

A. INTRODUCTION

The molecular biology core of this BCCOE brings high throughput molecular analyses to the specific aims of this center. The primary capacity of this core is high throughput gene expression analysis facilitated by Affymetrix GeneChip technology. In addition to massively parallel analysis of gene expression, new technologies, released in 2005, permitted the expansion of these facilities into high resolution analysis of chromosome structure. As determined at the December 6, 2004 meeting of the BCCOE held in Washington, DC, the efforts of this core was focused on Specific Aim 2 (Russo) of the center: to determine the effects of estrogen and its metabolites on the progressive steps of neoplastic transformation of human breast epithelial cells (HBECs) and to determine whether the neoplastic phenotypes and genotypes thus induced can be abrogated by known and new preventive agents. In addition, Dr. Sutter continued his seminal work on CYP1B1, the enzyme responsible for production of the 4-hydroxyestradiol metabolite.

B. BODY

B-i. Methods and procedures

B.i.a. Cell Lines, DNA and RNA

The cell lines and the malignant transformation protocol are shown in Fig. 1 and described previously. In this work, three individual samples of each cell line were analyzed as independent replicates. For comparison to the previous work we report the current and (previous) cell sample designations: MCF10F samples 1,2 3 (MCF-10F 1,2,3); trMCF samples 1,2 3 (E_2-70 nM 1,2,3); bsMCF (C5 1,2,3) and caMCF (L1, L4, L8). The designation E_2-70 nM referred to treatment conditions resulting in cell transformation; C5 referred to the position of the well in the selection chamber; L1, L4 and L8 referred to the cell line derived from the tumor of C5 cells in animal 1 (L1) and so forth. For isolation of the bcMCF cell lines, bsMCF cells were plated at low density and observed under the microscope. Individual colonies were isolated using cloning rings, selective trypsinization and plating, giving rise to six clones designated Clones A, B, C, F, H, and I. bcMCF clones A, B and F were analyzed in this study. Furthermore, the Clone A cell line of bcMCF was tested for its tumorigenic capacity in 45 day old female SCID mice, which were obtained from the FCCC animal care facility, as previously described. Animals were housed four to a cage and maintained in a laminar flow rack at 72 ^{0}F with a 12h light/dark cycle. They received water and food ad libitium. The cells were injected into the mammary fat pad of the abdominal region of the mice at a concentration of 10-15 x 10^6 cells suspended in 0.1 ml of sterile phosphate buffered saline. The animals were palpated twice a week for detection of tumor development and were followed for up to six months post injection. Animals were killed by carbon dioxide inhalation. Each animal was autopsied, carefully examined for identification of visceral metastasis and palpable tumors were dissected from the skin (1). High molecular weight genomic DNA and total cellular RNA were isolated from three individual samples of each cell line: MCF10F, trMCF, bsMCF, bcMCF, and caMCF. For DNA, cells were treated with lysis buffer containing 20 mM Tris-CL, pH 8.0, 100 mM NaCl, 25 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulphate, and 200 μg/mL proteinase K for 15 min at 65 ^{o}C

with gentle agitation. The samples were cooled on ice and treated with 100 μg/mL RNase A at 37 ^{o}C for 30 min. The samples were extracted once with buffered phenol, and again with chloroform:isoamyl alcohol (24:1). The aqueous layer was adjusted to 0.75 M ammonium acetate and the DNA was precipitated by the addition of 2.5 volumes of 100% ethanol. The precipitate was washed with 70% ethanol, dried and dissolved in sterile water. Total cellular RNA was isolated using the TRIZOL (Life Technologies, Gaithersburg, MD) modification of the guanidinium thiocyanate procedure. The concentration and quality of the DNA and RNA was determined spectrophotometrically and by capillary gel electrophoresis (Agilent 2100 Bioanalyzer, Palo Alto, CA).

B-i-b. Genotyping and microarray assays

ffymetrix 100k Single Nucleotide Polymorphism (SNP) mapping was performed using the combined Xba I (Mapping50K_Xba240) Hind III (Mapping50K_Hind240) GeneChip mapping Array set according to the manufacturer's recommended procedures (Affymetrix, Santa Clara, CA) with the following modifications. The time of the restriction endonuclease digestions was increased to 6 hr at 37 ^{o}C, and the ligation reaction was carried out overnight at 16 ^{o}C. Using this 100k SNP set, one obtains allele information at a mean intermarker distance of 23.6 kb and median intermarker distance of 8.5 kb. The average heterozygosity of each SNP is 0.30. Gene expression microarray analysis was performed using the Affymetrix HG-U133_Plus_2 Array, measuring more than 47,000 transcripts. Eight μg of total RNA was used in the cDNA synthesis reaction. After hybridization, the chips were washed and scanned on the GeneChip Scanner 3000 (Affymetrix). The genotype calls (heterozygous or homozygous) were determined using the Affymetrix GTYPE v4.0; the P(Present)- or A(Absent)-calls of the probes in the gene expression chips were determined using the Affymetrix GCOS v1.4. The microarray data have been submitted to the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus repository (series accession no. GSE5116.

B-i-c. Data analysis

The chromosome copy number changes and LOH were determined using dChip (2). The .CEL files of Mapping50K_Hind240 and Mapping50K_Xba240 chips and their corresponding .TXT files containing the SNP genotype calls were put into dChip to calculate the intensities of probes. The human genome release v17 was used to provide the genome information files (refGene and cytoBand files) that were used for the SNP data analysis in dChip. The output files containing the SNP intensities and SNP genotype calls were merged together for the 100k SNP analysis. The MCF10F cell line served as diploid reference for detection of copy number changes. The genotype of the MCF10F cell line was also interrogated using the Affymetrix CNAT v3.0, in order to detect potential aneuploidy. Genomic smoothed analysis (GSA) with 0.5Mbp distance was used to delineate the copy number change and LOH. The intensities of probe sets in the HG-U133_Plus_2 Genechips were calculated by dChip software using the Perfect-match/Mismatch difference model after invariant-set normalization. A gene is considered expressed in the group of interest if the gene is "Present" in all 3 samples of that group. Differentially expressed genes were identified by pairwise comparison using MCF10F as the reference. The significance level was $p < 0.05$ in an unpaired t-test of the log transformed expression values. To identify and extract the expression data for probesets corresponding to

genes located within specific regions of individual chromosomes, the Entrez Gene ID and corresponding location in Mb were used to search the annotation file of the HG-U133_Plus_2 Genechip. To integrate the Entrez Gene ID and associated gene with the human genome map, we used the GeneLoc tool of the Weismann Institute of Science.

B-i-d. Immunohistochemistry

The purified serum IgG was used at a concentration of either 10 μg/mL (CYP19) or 10 μg/mL (CYP1B1). Non-specific staining was assessed using normal rabbit serum (Sigma) at a dilution of 1:5000. For the tissue microarray analysis, paraffin-embeded human breast cancer (invasive ductal carcinoma) IMH-36460 samples for cancer metastasis and normal breast tissue were obtained as tissue microarrays from Imgenex (San Diego, CA, USA). The slides were deparaffinized in xylene (twice for 4 min) followed by washing in 100% ethanol (4 min), 95% ethanol (2 min), 80% ethanol (15 s) and distilled water as described previously (3) using diaminobenzidine as the chromagen. The slides were washed with distilled water for 1 min and counterstained for 1 min with hematoxilin.

B-i-e. Enzyme kinetics assays of recombinant human CYP1B1

The recombinant human CYP1B1 protein was expressed in S. cerevisiae as described previously (4). The microsome preparation was evaluated by measuring the enzymatic activity as described before (5). The inhibition kinetics of CYP1B1 was determined in a range expected to produce 30 to 90 percent inhibition. A fixed substrate concentration and varying inhibitor concentrations were used. An IC_{50} value was determined at the point where 50% inhibition of the enzyme's catalytic activity occurred. The E2 hydroxylation assay was performed with the addition of inhibitor and has been previously described in detail (5). Inhibition was calculated as percent of product formation compared to the corresponding control (enzyme-substrate reaction) without the inhibitors.

B-ii. Results

B-ii-a. Malignant Cell Transformation by Estogens

Epithelial to Mesenchymal Transition in Human Epithelial Cells Transformed by 17-beta-estradiol.

Yong Huang, [1] Sandra Fernandez, [2] Shirlean Goodwin, [1] Patricia A. Russo, [2] Irma Russo, [2] Thomas R. Sutter, [1] and Jose Russo [2,*]

Abstract

The estrogen-dependence of breast cancer has long been recognized, however, the role of 17β-estradiol (E2) in cancer initiation was not known until we demonstrated that it induces complete neoplastic transformation of the human breast epithelial cells MCF-10F. E2-treatment of MCF-10F cells progressively induced high colony efficiency and loss of ductulogenesis in early transformed (trMCF) cells, invasiveness in a Matrigel invasion chamber (bsMCF and bcMCF),

and carcinoma formation in SCID mice (caMCF). These phenotypes correlated with gene dysregulation during the progression of the transformation phenomenon. The highest number of dysregulated genes was observed in caMCF cells, being slightly lower in bcMCFcells, and lowest in trMCF cells. This order was consistent with the extent of chromosome aberrations (caMCF > bcMCF >> > trMCF). Chromosomal amplifications were found in 1p36.12-pter, 5q21.1-qter and 13q21.31-qter. Losses of the complete chromosome 4 and of 8p11.21-23.1 were found only in tumorigenic cells. In tumor-derived cell lines, additional losses were found in 3p12.1-14.1, 9p22.1-pter and 18q11.21-qter. Functional profiling of dysregulated genes revealed progressive changes in the integrin signaling pathway, inhibition of apoptosis, acquisition of tumorigenic cell surface markers and epithelial to mesenchymal transition. In both, bcMCF and caMCF cells, the levels of E-cadherin, EMA, and various keratins were low and CD44E/CD24 were negative, whereas SNAI2 (CDH1 repressor), vimentin, S100A4, FN1, HRAS and TGFβ1, and CD44H were high. The phenotypic and genomic changes triggered by estrogen exposure that lead normal cells to tumorigenesis confirm the role of this steroid hormone in cancer initiation.

Progressive loss of CST6 expression in a model of malignant cell transformation of human breast epithelial cells.

Sandra V. Fernandez[1], Yong Huang[2], Irma H Russo[1], Thomas R. Sutter[2], and Jose Russo[1].

Abstract

Cystatin E/M (CST6) is a potent inhibitor of endogenous mammalian lysosomal cysteine proteases, including cathepsin B and cathepsin L which promote tumor growth, invasion and metastasis through degradation of extracellular connective matrices and endothelial cell growth-directed activities. In the present work we demonstrate that CST6 is silenced by methylation during the early stages of 17 ˜ estradiol neoplastic transformation of the human breast epithelial cells MCF10F. In the in vitro-in vivo model of breast cancer induced by estrogen, different stages in the breast tumor progression are represented being the MCF-10F, the normal stage; trMCF, transformed but non tumorigenic; bsMCF, the invasive stage and tumorigenic in heterologous host ; and the more advanced tumorigenic stage represented by caMCF cells . Levels of cystatin E/M (CST6) mRNA were down-regulated in trMCF, bsMCF and caMCF cells. The invasive bsMCF cells have very low expression of CST6 and higher levels of expression of cathepsin C and cathepsin L-like 3, whereas the caMCF cells showed low levels of CST6 and increase in cathepsin C, cathepsin B and cathepsin L. We show that 5-aza-dC but not trichostatin was able to increase CST6 expression in MCF-10F, trMCF and bsMCF cells, indicating that CST6 expression is regulated, in part, by DNA methylation. Epigenetic control of this gene appears as an early event in the process of estrogen-induced malignant cell transformation of human MCF-10F cells.

Work in Progress
Russo/Sutter labs

Studies in Dr. Russo's laboratory demonstrated that treatment of transformed and matrigel selected cell with hCG could reverse or inhibit the phenotypes of cell transformation and invasion. Microarray analysis performed in the Sutter lab has revealed underlying gene

expression mechanisms that correspond to these observed phenotypes. This important collaboration is the first work to understand molecular mechanisms for chemoprevention of malignant cell transformation mediated by estrogen.

B-ii-b. 17-beta-estradiol metabolism and Cytochrome P4501B1

Regioselective 2-hydroxylation of 17β-estradiol by rat cytochrome P4501B1
Mostafizur Rahman[a,b], Carrie Hayes Sutter[a], Gary L. Emmert[b] and Thomas R. Sutter[a,b*]

Abstract

Previous work demonstrated that human cytochrome P4501B1 (CYP1B1) forms predominantly 4-hydroxyestradiol (4-OHE2), a metabolite which is carcinogenic in animal models. Here, we present results from kinetic studies characterizing the formation of 4-OHE2 and 2-hydroxyestradiol (2-OHE2) by rat CYP1B1 using 17β-estradiol (E2) as a substrate. K_m and K_{cat} values were estimated using the Michaelis-Menten equation. For rat CYP1B1, the apparent K_m values for the formation of 4-OHE2 and 2-OHE2 were 0.61 ± 0.23 and 1.84 ± 0.73 μM; the turnover numbers (K_{cat}) were 0.23 ± 0.02 and 0.46 ± 0.05 pmol/min/pmol P450; and the catalytic efficiencies (K_{cat}/K_m) were 0.37 and 0.25, respectively. For human CYP1B1, the apparent K_m values for the formation of 4-OHE2 and 2-OHE2 were 1.22 ± 0.25 and 1.10 ± 0.26; the turnover numbers were 1.23 ± 0.06 and 0.33 ± 0.02; and the catalytic efficiencies were 1.0 and 0.30, respectively. The turnover number ratio of 4- to 2-hydroxylation was 3.7 for human CYP1B1 and 0.5 for rat CYP1B1. These results indicate that although rat CYP1B1 is a low K_m E2 hydroxylase, its product ratio, unlike the human enzyme, favors 2-hydroxylation. The K_i values of the inhibitor 2,4,3′,5′-tetramethoxystilbene (TMS) for E2 4- and 2-hydroxylation by rat CYP1B1 were 0.69 and 0.78 μM, respectively. The K_i values of 7,8-benzoflavone (α-NF) for E2 4- and 2-hydroxylation by rat CYP1B1 were 0.01 and 0.02 μM, respectively. The knowledge gained from this study will support the rational design of CYP1B1 inhibitors and clarify results of CYP1B1 related carcinogenesis studies performed in rats.

Co-expression of CYP19 and CYP1B1 in tumor epithelial cells of invasive ductal carcinoma
Mostafizur Rahman [1, 2, 3], Sigurd F. Lax [4], Carrie Hayes Sutter [1, 3], Gary L. Emmert [2], Jose Russo [5], William R. Miller [6], Richard J. Santen [7], and Thomas R. Sutter [1, 2, 3, *]

Abstract

A cytochrome P45019 (CYP19, aromatase) polypeptide expressed as a hexahistidine-tagged fusion protein in E coli was purified and used to produce a polyclonal antibody in rabbit. Immunoblot analysis showed that this antibody was very specific and capable of detecting human CYP19 protein. Immunohistochemical analyses of CYP19 and CYP1B1 in a panel of 29 cases of invasive ductal carcinoma of the breast, showed epithelial cell staining for CYP19 in 76% of samples and for Cyp1B1 in 97%. Since CYP1B1 and CYP19 were co-expressed in breast cancer and since both enzymes are efficient estrogen hydroxylases, we investigated whether CYP1B1 expression may affect the disposition of Aromatase inhibitors (AIs). To do this, we determined the inhibition properties against CYP1B1-catalyzed hydroxylation of 17β-

estradiol (E_2) by a series of AIs including three steroidal inhibitors; formestane, exemestane, and androstenedione, and five non-steroidal inhibitors; aminoglutethimide, fadrozole, anastrozole, letrozole, and vorozole. Of the eight compounds tested, only vorozole exhibited inhibition of CYP1B1 activity with IC_{50} values of 17 and 21 μM for 4-OHE_2 and 2-hydroxyestradiol (2-OHE_2), respectively. The estimated K_i values of vorozole for E_2 4- and 2-hydroxylation were 7.26 and 6.84 μM, respectively. Spectrophotometric studies showed that vorazole is a type II inhibitior of CYP1B1. None of the other seven AIs tested showed significant inhibition towards CYP1B1 activity and the IC_{50} values for 4-OHE_2 and 2-OHE_2 were all greater than 100 μM. This study shows that with the exception of vorozole, the aromatase inhibitors are selective for CYP19 relative to CYP1B1. Thus, although both CYP19 and CYP1B1 are expressed in a high percentage of breast cancers, CYP1B1 does not appear to be an important determinant of the disposition or metabolism of the aromatase inhibitors tested.

Genetic variation of Cytochrome P450 1B1 (CYP1B1) and risk of breast cancer among Polish women.
Gaudet MM, Chanock S, Lissowska J, Berndt SI, Yang XR, Peplonska B, Brinton LA, Welch R, Yeager M, Bardin-Mikolajczak A, Sherman ME, Sutter TR, Garcia-Closas M.

Abstract

Four single nucleotide polymorphisms (SNPs) in CYP1B1 (Ex2 + 143 C > G, Ex2 +356 G > T, Ex3 + 251 G > C, Ex3 + 315 A > G) cause amino acid changes (R48G, A119S, L432V and N453S, respectively) and are associated with increased formation of catechol estrogens; however, epidemiologic evidence only weakly supports an association between these variants and breast cancer risk. Because genetic variability conferring increased susceptibility could exist beyond these putative functional variants, we comprehensively examined the common genetic variability within CYP1B1. A total of eight haplotype-tagging (ht)SNPs (including Ex3 + 315 A > G), in addition to two putatively functional SNPs (Ex2 + 143 C > G and Ex3 + 251 G > C), were selected and genotyped in a large case-control study of Polish women (1995 cases and 2296 controls). Haplotypes were estimated using the expectation-maximization algorithm, and overall differences in the haplotype distribution between cases and controls were assessed using a global score test. We also evaluated levels of tumor CYP1B1 protein expression in a subset of 841 cases by immunohistochemistry, and their association with genetic variants. In the Polish population, we observed two linkage disequilibrium (LD)-defined blocks. Neither haplotypes (global P-value of 0.99 and 0.67 for each block of LD, respectively), nor individual SNPs (including three putatively functional SNPs) were associated with breast cancer risk. CYP1B1 was expressed in most tumor tissues (98%), and the level of expression was not related to the studied genetic variants. We found little evidence for modification of the estimated effect of haplotypes or individual SNPs by age, family history of breast cancer, or tumor hormone receptor status.
The present study provides strong evidence against the existence of a substantial overall association between common genetic variation in CYP1B1 and breast cancer risk.

Hormonal markers in breast cancer: co-expression, relationship with pathologic characteristics and risk factor associations in a population-based study

Xiaohong R. Yang(1), Ruth M. Pfeiffer(1), Montserrat Garcia-Closas(1), David L.Rimm(2), Jolanta Lissowska(3), Louise A.Brinton(1), Beata Peplonska(4), Stephen M.Hewitt(5), Richard Cartun(6), Daniza Mandich(6), Hiro Sasano(7), Dean B. Evans(8), Thomas R. Sutter(9), Mark E.Sherman(1)

Abstract

The objective of this study was to evaluate co-expression patterns of markers related to hormone pathways in breast cancer tissue and their relationship with pathologic characteristics and risk factors. We evaluated immunohistochemical expression of 17 markers in tissue microarrays prepared from 842 invasive breast carcinomas collected in a population-based case-control study conducted in Poland. Based on marker correlations, factor analysis identified four major co-expression patterns (factors): the "nuclear receptor factor" (ER-α, PR, androgen receptor, cyclin D1, and aromatase); the "estrogen metabolism / ER-β factor" (ER-β, peroxisome proliferators-activated receptor, steroid sulfatase, estrogen sulfonotransferase , and cytochrome P450 1B1; the "HER-2 factor" (HER-2, E-cadherin, cyclooxygenase-2, aromatase, STS; and the "proliferation factor" (cytokeratin 5, cytokeratin 5/6, EGFR, P53). In contrast with other factors, the estrogen metabolism / ER-β factor did not correspond to previously defined molecular subtypes by global gene expression studies. High scores for this factor were associated with high tumor grade (P-heterogeneity =0.02), younger age at menarche (P-heterogeneity = 0.04), lower current BMI among premenopausal women (P-heterogeneity = 0.01), and older age at menopause (P-heterogeneity = 0.04). High scores for the proliferation factor were also associated with early menarche (P-heterogeneity < 0.0001), and in contrast to the estrogen metabolism / ER-β factor, higher current BMI among premenopausal women (P-heterogeneity = 0.03). Our results suggest that analysis of biologically relevant markers may reveal associations with pathologic characteristics and risk factors that overlap with and add to relationships discovered by analysis of molecular subtypes defined by expression profiling.

D. REPORTABLE OUTCOMES

D-i. in press

1. Rahman, M.M., Sutter, C.H., Emmert, G.L., and Sutter, T.R. 2006. Regioselective 2-hydroxylation of 17β-estradiol by rat cytochrome P4501B1. Toxicol. Appl. Pharmacol., 216: 469-478.

2. Gaudet, M.M., Chanock, S., Lissowska, J., Berndt, S.I., Yang, X.R., Peplonska, B., Brinton, L.A., Welch, R., Yeager, M., Bardin-Mikolajczak, A., Sherman, M.E., Sutter, T.R., and Garcia-Closas, M. 2006. Genetic variation of cytochrome P450 1B1 (CYP1B1) and risk of breast cancer among Polish women. Pharmacogenetics and Genomics, 16: 547-553.

3. Cavalieri, E., Chakravarti, D., Guttenplan, J., Hart, E., Ingle, J., Jankowiak, R., Muti, P., Rogan, E., Russo, J., Santen, R. and Sutter, T. 2006. Catechol estrogen quinones as

initiators of breast and other human cancers: Implications for biomarkers of susceptibility and cancer prevention. BBA Reviews in Cancer, 1760: 63-78.

D-ii. submitted

4. Yong Huang, [1] Sandra Fernandez, [2] Shirlean Goodwin, [1] Patricia A. Russo, [2] Irma Russo, [2] Thomas R. Sutter, [1] and Jose Russo [2, *] 2007. Epithelial to Mesenchymal Transition in Human Epithelial Cells Transformed by 17-beta-estradiol. Submitted

5. Sandra V. Fernandez[1], Yong Huang[2], Irma H Russo[1], Thomas R. Sutter[2], and Jose Russo[1]. 2007. Progressive loss of CST6 expression in a model of malignant cell transformation of human breast epithelial cells. Submitted

D-ii. in preparation

6. Mostafizur Rahman [1, 2, 3], Sigurd F. Lax [4], Carrie Hayes Sutter [1, 3], Gary L. Emmert [2], Jose Russo [5], William R. Miller [6], Richard J. Santen [7], and Thomas R. Sutter [1, 2, 3,] 2007. Co-expression of CYP19 and CYP1B1 in tumor epithelial cells of invasive ductal carcinoma.

7. Xiaohong R. Yang(1), Ruth M. Pfeiffer(1), Montserrat Garcia-Closas(1), David L.Rimm(2), Jolanta Lissowska(3), Louise A.Brinton(1), Beata Peplonska(4), Stephen M.Hewitt(5), Richard Cartun(6), Daniza Mandich(6), Hiro Sasano(7), Dean B. Evans(8), Thomas R. Sutter(9), Mark E.Sherman(1) 2007. Hormonal markers in breast cancer: co-expression, relationship with pathologic characteristics and risk factor associations in a population-based study

E. CONCLUSIONS

We have continued to elaborate on our important findings of the role of CYP1B1 in estrogen hydroxylation and the risk of developing breast cancer. During this period, we have made contributions to the areas of comparative metabolism, aromatase inhibition, genetic predisposition and tumor classification. In our genomic analysis of the cell transformation model established by the Russo laboratory, we identified important molecular events leading to the expression of tumorigenic markers and epithelial-mesenchymal transition (EMT). EMT is an important cellular determinant of invasiveness and metastasis. By identifying these characteristics occurring during malignant cell transformation by estrogen in the ER-negative cell line MCF-10F, it identifies a new and essential cell model for understanding the especially aggressive characteristics of ER-negative tumors.

F. REFERENCES

1. 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.

2. Lin, M, Wei, LJ, Sellers, WR, Lieberfarb, M, Wong, WH and Li C (2004) dChipSNP: significance curve and clustering of SNP-array-based loss-of-heterozygosity data. Bioinformatics 20: 1233-1240.

3. Walker NJ, Crofts FG, Li Y, et al. Induction and localization of cytochrome antibodies raised to histidine-tagged fusion proteins produced and purified from P450 1B1 (CYP1B1) protein in the livers of TCDD-treated rats: detection using polyclonal bacteria. Carcinogenesis 1998;19:395-402.

4. Hayes CL, Spink DC, Spink BC, Cao JQ, Walker NJ, Sutter TR. 17β-Estradiol hydroxylation catalyzed by human cytochrome P450 1B1. Proc Natl Acad Sci USA 1996;93:9776-81.

5. Rahman M, Sutter CH, Emmert GL, Sutter TR. Regioselective 2-hydroxylation of 17β-estradiol by rat cytochrome P4501B1. Toxicol Appl Pharmacol 2006;216:469-78.

ANALYTICAL CORE – ROGAN

A. Introduction

The Analytical Core provides consistent analytical power to the research projects so that estrogen metabolites, estrogen conjugates and estrogen-DNA adducts can be identified and quantified with the most sensitivity and reliability. The HPLC with multi-channel electrochemical detection enables detection of 34 metabolites and conjugates at the picomole level in one run. A second set-up is used exclusively to analyze the 6 depurinating catechol estrogen-DNA adducts, which need a different elution buffer to achieve separation. Our newer ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) instrumentation has increased the sensitivity of our analyses to the femtomole level and provides confirmation of structures.

B. Body

B-i. Methods and Procedures

The Analytical Core uses both HPLC with electrochemical detection and UPLC/MS/MS to analyze estrogen metabolites, conjugates and depurinating DNA adducts. A variety of types of samples are analyzed. These include *in vitro* reaction mixtures, human and animal tissue extracts, cell culture medium extracts, and human fluids, such as urine, serum and nipple aspirate fluid. Depending on the sample, preparation for analysis is as simple as filtration through a 5,000-molecular weight filter, or as complex as grinding minced tissue in liquid nitrogen, incubation with glucuronidase/sulfatase, passage through a Sep-Pak column to extract desired analytes, and filtration through a 5,000-molecular weight filter.

B-ii. Results

In the past year we have used our Acquity UPLC (Waters, Inc), which is coupled with a MicroMass QuattroMicro tandem mass spectrometer, to separate and analyze 34 estrogen metabolites and GSH conjugates [and their breakdown products containing cysteine (Cys) and *N*-acetylcysteine (NAcCys)] and 6 depurinating DNA adducts by UPLC/MS/MS.

In the past year we primarily analyzed human samples, including nipple aspirate fluid from women with and without breast cancer, urine from women with and without breast cancer and at high risk of breast cancer [1], and serum from women without breast cancer. These results are the first demonstration that estrogen-DNA adducts in urine are potential biomarkers for risk of developing breast cancer.

We continued to analyze medium from MCF-10F cells incubated with E_2 in Specific Aim 2. We detected estrogen metabolites, estrogen conjugates and the depurinating estrogen-DNA adducts 4-OHE_1(E_2)-1-N3Ade and 4-OHE_1(E_2)-1-N7Gua formed by MCF-10F cells. These results are described in several publications [2,3].

We analyzed mammary tissue and serum from ERKO/Wnt-1 mice and aromatase-transfected mice from Dr. Santen's laboratory (Specific Aim 4). We detected estrogen metabolites, conjugates and depurinating DNA adducts in the mammary tissue and serum from ovariectomized ERKO/Wnt-1 mice in a dose-related manner, depending on the dose of E_2 implanted into the mice. These results are described more completely in Specific Aim 4.

C. Key Research Accomplishments

We have established a procedure with the Acquity UPLC/QuattroMicro MS/MS to analyze 40 estrogen metabolites, GSH conjugates and depurinating DNA adducts with high sensitivity and selectivity.

We have successfully analyzed human nipple aspirate fluid and detected estrogen-DNA adducts in samples from women with breast cancer, but not in samples from women without breast cancer.

We have successfully analyzed human urine samples from women with and without breast cancer and at high risk for breast cancer and found that the level of depurinating estrogen-DNA adducts in urine from women with breast cancer and at high risk for breast cancer is significantly higher than the level of adducts in urine from healthy control women ($p<0.001$).

We have successfully analyzed human serum samples from healthy control women and found baseline levels of estrogen metabolites, conjugates and depurinating DNA adducts.

D. Reportable Outcomes

a. Publications

1. Yue, W., Santen, R.J., Wang, J-P., Li, Y., Verderame, M.F., Bocchinfuso, W.P., Korach, K.S., Devanesan, P., Todorovic, R., Rogan, E.G. and Cavalieri, E.L. Genotoxic metabolites of estradiol in breast: Potential mechanism of estradiol induced carcinogenesis. J. Steroid Biochem. Mol. Biol., 86:477-486, 2003.

2. Rogan, E.G. and Cavalieri, E.L. The role of specific estrogen metabolites in the initiation of breast and other human cancers. Int. J. Cancer Prev., 1:243-249, 2004.

3. Yue, W., Wang, J.-P., Li, Y., Bocchinfuso, W., Korach, K., Devanesan, P., Rogan, E., Cavalieri, E. and Santen, R.J. Tamoxifen versus aromatase inhibitors for breast cancer prevention. Clin. Cancer Res., 11:925s-930s, 2005.

4. Singh, S., Chakravarti, D., Edney, J.A., Hollins, R.R., Johnson, P.J., West, W.W., Higginbotham, S.M., Cavalieri, E.L. and Rogan, E.G. Relative imbalances in the expression of estrogen-metabolizing enzymes in the breast tissue of women with breast carcinoma. Oncology Reports, 14:1091-1096, 2005.

5. Markushin, Y., Kapke, P., Saeed, M., Zhang, H., Dawoud, A., Rogan, E.G., Cavalieri, E.L. and Jankowiak, R. Development of monoclonal antibodies to 4-hydroxyestrogen-2-*N*-acetylcysteine conjugates: Immunoaffinity and spectroscopic studies. Chem. Res. Toxicol., 18:1520-1527, 2005.

6. Zahid, M., Kohli, E., Saeed, M., Rogan, E. and Cavalieri, E. The greater reactivity of estradiol-3,4-quinone versus estradiol-2,3-quinone with DNA in the formation of depurinating adducts. Implications for tumor-initiating activity. Chem. Res. Toxicol., 19:164-172, 2006.

7. Zhao, Z., Kosinska, W., Khmelnitsky, M., Cavalieri, E.L., Rogan, E.G., Chakravarti, D., Sacks, P. and Guttenplan, J.B. Mutagenic activity of 4-hydroxyestradiol, but not 2-hydroxyestradiol, in BB rat2 embryonic cells, and the mutational spectrum of 4-hydoxyestradiol. Chem. Res. Toxicol., 19:475-479, 2006.

8. Markushin, Y., Gaikwad, N., Zhang, H., Kapke, P., Rogan, E.G., Cavalieri, E.L., Trock, B.J., Pavlovich, C. and Jankowiak, R. Potential biomarker for early risk assessment of prostate cancer. Prostate, 66:1565-1571, 2006.

9. Saeed, M., Rogan, E., Fernandez, S.V., Sheriff, F., Russo, J. and Cavalieri, E. Formation of depurinating N3Adenine and N7Guanine adducts by MCF-10F cells cultured in the presence of 4-hydroxyestradiol. Int. J. Cancer, 120:1821-1824, 2007.

10. Zhang, Y., Gaikwad, N.W., Olson, K., Zahid, M., Cavalieri, E.L. and Rogan, E.G. Cytochrome P450 isoforms catalyze formation of catechol estrogen quinones that react with DNA. Metabolism, in press, 2007.

11. Lu, F., Zahid, M., Saeed, M., Cavalieri, E.L. and Rogan, E.G. Estrogen metabolism and formation of estrogen-DNA adducts in estradiol-treated MCF-10F cells. The effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induction and catechol-*O*-methyltransferase inhibition. J. Steroid Biochem. Mol. Biol., doi 10.1016/jsbmb.2006.12.102, in press, 2007.

12. Gaikwad, N.W., Rogan, E.G. and Cavalieri, E.L. Evidence for NQO1-catalyzed reduction of estrogen *ortho*-quinones. Mass spectrometric determination of enzyme-substrate complex. Free. Radic. Biol. Med., in press, 2007.

13. Gaikwad, N.W., Muti, P., Meza, J., Yang, L., Pruthi, S., Ingle, J., Rogan, E.G. and Cavalieri, E.L. The molecular etiology of breast cancer and biomarkers of risk. Submitted, 2007.

b. Reviews and Book Chapters

1. Cavalieri, E., Rogan, E. and Chakravarti, D. The role of endogenous catechol quinones in the initiation of cancer and neurodegenerative diseases. In: Methods in Enzymology, Quinones and Quinone Enzymes, Part B (H. Sies & L. Packer, Eds.), Vol. 382, Elsevier, Duesseldorf, Germany, 293-319, 2004.

2. Santen, R.J., Yue, W., Bocchinfuso, W., Korach, K., Wang, J.-P., Rogan, E.G., Li, Y., Cavalieri, E., Russo, J., Devanesan, P. and Verderame, M. Estradiol-induced carcinogenesis via formation of genotoxic metabolites. In: Advances in Endocrine Therapy of Breast Cancer, (J.N. Ingle & M. Dowsett, Eds.), Marcel Dekker, New York, 163-177, 2004.

3. Rogan, E.G. and Cavalieri, E.L. Estrogen metabolites, conjugates and DNA adducts: Possible biomarkers for risk of breast, prostate and other human cancers, In: Adv. Clin. Chem., (G.S. Makowski, G. Nowacki & K.-J. Hsiao, Eds.) 38:135-149, 2004.

4. Cavalieri, E.L. and Rogan, E.G. A unifying mechanism in the initiation of cancer and other diseases by catechol quinones, Ann. NY Acad. Sci., 1028:247-257, 2004.

5. Cavalieri, E., Chakravarti, D., Guttenplan, J., Hart, E., Ingle, J., Jankowiak, R., Muti, P., Rogan, E., Russo, J., Santen, R. and Sutter, T. Catechol estrogen quinones as initiators of breast and other human cancers. Implications for biomarkers of susceptibility and cancer prevention. BBA-Reviews on Cancer, 1766:63-78, 2006.

6. Cavalieri, E. and Rogan, E. Catechol quinones of estrogen in the initiation of breast, prostate, and other human cancers. Ann. NY Acad. Sci., 1089:286-301, 2006.

c. Invited Presentations and Abstracts

1. Rogan, E.G., Singh, S., Chakravarti, D., Higginbotham, S. and Cavalieri, E.L. Differential expression of estrogen-metabolizing enzymes in four quadrants of human breast. Proc. Amer. Assoc. Cancer Res., 44:307, 2003.

2. Cavalieri, E., Kohli, E., Zahid, M. and Rogan, E. Greater reactivity of estradiol-3,4-quinone *vs* estradiol-2,3-quinone with DNA in the formation of depurinating DNA adducts. Proc. Amer. Assoc. Cancer Res., 44:180, 2003.

3. Chakravarti, D., Mailander, P.C., Higginbotham, S., Cavalieri, E.L. and Rogan, E.G. The catechol estrogen-3,4-quinone metabolites induces mutations in the mammary gland of ACI rats. Proc. Amer. Assoc. Cancer Res., 44:180, 2003.

4. Cavalieri, E., Saeed, M., Gunselman, S. and Rogan, E. Depurination of adenine and guanine adducts occurs in DNA, but only very slowly in RNA. Proc. Amer. Assoc. Cancer Res., 44:1324, 2003.

5. Cavalieri, E. Unifying mechanism in the etiology of cancer and neurodegenerative diseases. Prevention is a reality at hand. Eppley Cancer Center Grand Rounds, UNMC, Omaha, NE, November 10, 2003.

6. Gunselman, S.J., Saeed, M., Higginbotham, S., Rogan, E.G. and Cavalieri, E.L. Formation of the depurinating N3adenine and N7guanine adducts by reaction of DNA with hexestrol-3′,4′-+quinone or enzyme-activated 3′-hydroxyhexestrol. Implications for a unifying mechanism of tumor initiation by natural and synthetic estrogens. Proc. Amer. Assoc. Cancer Res., 45:1564, 2004.

7. Saeed, M., Zahid, M., Gunselman, S.J., Rogan, E. and Cavalieri, E. Slow loss of deoxyribose from the N7deoxyguanosine adducts of estradiol-3,4-quinone and hexestrol-3′-4′-quinone. Implications for mutagenic activity. Proc. Amer. Assoc. Cancer Res., 45:1555, 2004.

8. Zhang, Y., Schoomaker, S., Cavalieri, E. and Rogan, E. Metabolism of estradiol by cytochrome P450 in rat liver microsomes to form quinones that bind to DNA. Proc. Amer. Assoc. Cancer Res., 45:1886, 2004.

9. Cavalieri, E. and Rogan, E. Catechol quinones in the initiation of cancer and other diseases: A target for prevention. Keystone Symposia Meeting, Hormonal Regulation of Tumorigenesis, Monterey, California, February 20-25, 2005.

10. Yue, W., Wang, J.-P., Fan, P., Rogan, E., and Cavalieri, E. Estradiol can cause breast cancer through a non-receptor mediated mechanism in ERα knockout/Wnt-1 transgenic mice. Proc. Amer. Assoc. Cancer Res., 46:2096, 2005.

11. Cavalieri, E.L. and Rogan, E.G. The etiology of breast cancer. Prevention is now the solution. Era of Hope, Department of Defense Breast Cancer Research Meeting, Philadelphia, PA, June 8-11, 2005

12. Cavalieri, E.L., Saeed, M. and Rogan, E. The mechanism of tumor initiation by the human carcinogen diethylstilbestrol is similar to that of natural estrogens. Era of Hope, Department of Defense Breast Cancer Research Meeting, Philadelphia, PA, June 8-11, 2005

13. Rogan, E.G. and Cavalieri, E.L. The role of estrogen metabolism in the initiation of human breast cancer. Era of Hope, Department of Defense Breast Cancer Research Meeting, Philadelphia, PA, June 8-11, 2005

14. Yue, W., Wang, J.-P., Fan, P., Rogan, E., Cavalieri, E., and Santen, R. Estradiol can cause breast cancer through a non-receptor mediated mechanism in ERα knockout/Wnt-1 transgenic mice. Era of Hope, Department of Defense Breast Cancer Research Meeting, Philadelphia, PA, June 8-11, 2005

15. Cavalieri, E., Russo, J., Guttenplan, J., Santen, R., Sutter, T., Rogan, E., Ingle, J., and Hart, E. Estrogen-induced depurination of DNA: A novel target for breast cancer prevention. Center for Excellence Meeting, Department of Defense Breast Cancer Research Program, Baltimore, MD, October 6-7, 2005.

16. Gaikwad, N.W., Markushin, Y., Saeed, M., Jankowiak, R., Trock, B.J., Rogan, E.G. and Cavalieri, E.L. The depurinating 4-hydroxyestrone(estradiol)-1-N3Ade DNA adducts as biomarkers of prostate cancer risk. Proc. Amer. Assoc. Cancer Res., 47:78, 2006.

17. Gaikwad, N.W., Cavalieri, E.L. and Rogan, E.G. NQO1-catalyzed reduction of estradiol-3,4-quinone. Implications for tumor initiation by estrogens. Proc. Amer. Assoc. Cancer Res., 47:445, 2006.

18. Lu, F., Cavalieri, E.L. and Rogan, E.G. TCDD-induced expression of cytochrome P450 enzymes and formation of estrogen metabolites in human breast epithelial MCF-10F cells. Proc. Amer. Assoc. Cancer Res., 47:445, 2006.

19. Rogan, E.G., Zhang, Y., Gaikwad, N.W., Olson, K., Zahid, M. and Cavalieri, E.L. Cytochrome P450 isoforms catalyze oxidation of catechol estrogens to their quinones, which react with DNA. Proc. Amer. Assoc. Cancer Res., 47:446, 2006.

20. Zahid, M., Saeed, M., Olson, K., Gaikwad, N., Rogan, E., Cavalieri, E. Inhibition of the formation of the N3Ade and N7Gua depurinating adducts after reaction of estradiol-3,4-quinone or lactoperoxidase-oxidized 4-hydroxyestradiol with DNA. Proc. Amer. Assoc. Cancer Res., 47:446, 2006.

21. Saeed, M., Rogan, Ed., Cavalieri, E., Sheriff, F., Fernandez, S. and Russo, J. Formation of the DNA depurinating N3Ade and N7Gua adducts of 4-hydroxyestradiol by MCF-10F cells cultured with the carcinogenic estrogen metabolite 4-hydroxyestradiol. Proc. Amer. Assoc. Cancer Res., 47:447, 2006.

22. Singh, S., Zahid, M., Gaikwad, N., Chakravarti, D., Cavalieri, E.L. and Rogan, E.G. The effect of NAD(P)H:quinone oxidoreductase 1 polymorphisms on estrogen metabolism in relation to initiation of breast cancer. Proc. Amer. Assoc. Cancer Res., 47:835, 2006.

23. Cavalieri, E. Symposium Co-Chairman, Special Topic Symposium, Catechol Estrogen Quinones as Initiators of Breast and Other Human Cancers, 81st Annual Meeting "Science and the Next Generation" AAAS-SWARM Meeting, Tulsa, OK, April 5-8, 2006

24. Rogan, E. Estrogen Metabolism in Human Breast Cancer, AAAS-SWARM Division Annual Meeting, Science and the Next Generation, Tulsa, OK, April 5-8, 2006.

25. Rogan, E. Xenoestrogens, Biotransformation, and Differential Risks for Breast Cancer, The 13th International Symposium on Functional Medicine, Managing Biotransformation: The Metabolic, Genomic, and Detoxification Balance Points, Tampa, FL, April 19-22, 2006.

26. Cavalieri, E. Catechol quinones of estrogens and dopamine in the initiation of cancer and neurodegenerative diseases. 132nd Advanced Course on Estrogens and Human Diseases, Erice, Italy, May 15-21, 2006.

27. Rogan, E. The role of estrogen metabolism in the initiation of breast, prostate and other human cancers, The Michigan Society of Toxicology Fall Meeting, East Lansing, MI, November 3, 2006.

28. Rogan, E., The Role of Estrogen Metabolism in the Initiation of Breast, Prostate and Other Human Cancers, CAMExpo West, Los Angeles, CA, November 10-12, 2006

29. Rogan, E., Estrogen-DNA Adducts in the Etiology and Prevention of Breast and Prostate Cancer, Functional Medicine of Colorado, Boulder, CO, February 7, 2007

E. Conclusions

The Analytical Core has successfully implemented UPLC/MS/MS to detect estrogen metabolites, GSH conjugates and depurinating DNA adducts in human nipple aspirate fluid and urine from women with and without breast cancer and at high risk of developing breast cancer. The depurinating DNA adducts are present at higher levels in women with breast cancer and at high risk for breast cancer, compared to healthy control women. We are also analyzing estrogen

metabolites, GSH conjugates and depurinating DNA adducts in human serum from healthy control women and will compare the levels with those in serum from women with breast cancer.

The Analytical Core is interacting with the other investigators in the Breast Cancer Center of Excellence to analyze a variety of samples for estrogen metabolites, estrogen GSH conjugates and depurinating estrogen-DNA adducts.

F. References

1. Gaikwad, N.W., Muti, P., Meza, J., Yang, L., Pruthi, S., Ingle, J., Rogan, E.G. and Cavalieri, E.L. The molecular etiology of breast cancer and biomarkers of risk. Submitted, 2007.

2. Saeed, M., Rogan, E., Sheriff, F., Fernandez, S., Russo, J. and Cavalieri, E. Formation of depurinating N3adenine and N7guanine adducts by MCF-10F cells cultured in the presence of 4-hydroxyestradiol. Int. J. Cancer, submitted, 2006.

3. Lu, F., Zahid, M., Saeed, M., Cavalieri, E.L. and Rogan, E.G. Estrogen metabolism and formation of estrogen-DNA adducts in estradiol-treated MCF-10F cells. The effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induction and catechol-*O*-methyltransferase inhibition. J. Steroid Biochem. Mol. Biol., doi 10.1016/jsbmb.2006.12.102, in press, 2007.

ADVOCACY CORE - HART

Advocates have been and continue to be an integral part of this DoD COE grant, providing input into the specific aims of the grant as well as specific advocacy issues related to consent documents, pilot study design and implementation, and funding opportunities.

Specific projects outlined in the COE Advocacy Core:

Web-based model reference tool
The decision has been made to leave the web-based model reference tool under the auspices of the National Library of Medicine and remain available for consultation and input if they decide to incorporate our web-based tool as a part of their public information search tools.

Consumer Guide to Involvement in Basic Research
The consumer guide has been published (see attached) and is available for upload to organizational websites as desired. This has been a labor of great satisfaction to the advocates of this COE and it is hoped that it will prove beneficial and interest more consumers in becoming involved in basic scientific research.

Article for publication based on current research
An article for publication continues to be in progress.

We continue to be very excited about the progress that is being made in prevention of cancer through this grant and the extraordinary findings outlined in this progress report. It is a privilege to be a part of such innovative research with these outstanding researchers who work collaborative and congenially and both welcome and value the participation of the advocates in this grant.

PARTNERS IN RESEARCH
ADVOCATES & SCIENTISTS

Elizabeth A. Hart

Margaret J. Borwhat

PARTNERS IN RESEARCH

Advocates & Scientists

Advocate's Guide

Elizabeth A. Hart – HART INTERNATIONAL

Partnerships Advancing Women's Health Research.

Margaret J. Borwhat – Women's Cancer Advocacy Network (WCAN)

Published by HART INTERNATIONAL

Dallas, Texas

Grateful acknowledgement is made to DoD COE Grant: DAMD 17-03-1-0229

TABLE OF CONTENTS

Introduction

As members of the advocacy community, we are passionately committed to advancing breast cancer research in partnership with scientists in the field. We each have a vital role in eradicating breast cancer and one day, preventing the disease. Scientists have the education in the basic sciences and laboratory techniques, which enable them to conduct basic, translational and clinical research. Advocates, survivors and family members, on the other hand, are the end recipients of the research and the human laboratory to confirm whether the disease hypothesis was indeed correct and the proposed prevention or treatment effective. The two roles are complementary, each informing the other and the focus of this guide to aid advocates who are interested in participation in research activities, specifically basic sciences, to find the avenue that best suits their particular interests. It is not meant to be an exhaustive guide, but a guide to spur the advocate to become involved and the scientist to seek that involvement if he or she has not already done so.

In the age of limited resources to fund research, it is vital for the general public to understand the necessity for basic scientific research and to be willing to fund such research. The more the public understands, the better the chance of increasing funding levels and resources to continue to fight breast cancer and one day prevent it. It is our hope that this brief guide will induce interest among the advocacy community and entice advocates who are reticent to become involved in basic scientific research to take the plunge. The rewards are enormous for the advocate, the scientists and research in general.

Chapter 1

Requisites of Participation

Involvement in basic scientific research for the advocate generally occurs when one has had an experience with cancer at close range, either personally, a family member, or a close friend. The experience is often overwhelming, frightening, sad, frustrating, helpless and hopeful, to name but a few of the emotions that one traverses during the cancer process. Over 136 cancer organizations are listed in the Office of Liaison Activities at the National Cancer Institute alone, helping people in their respective communities find access to care, support in living out the cancer experience, raising money for research, sitting on local, regional, national, and international committees that address research, policy, survivorship, quality of life, cultural competency, prevention, detection, risk and many other important issues.

If one's interest is in participating in basic research – often referred to as bench research – it helps to have some rudimentary knowledge of biology, chemistry, anatomy and physiology. Do not worry if you don't have an understanding of all of these subjects, one will suffice to begin with. The key issue is exposure to a level of science so that you can understand some basic scientific concepts. A strong foundation in science is the goal. If you do not have these requisites in your educational background, invade the local library and READ, READ, READ. Better yet, audit some classes. Ask questions about EVERYTHING you do not understand. The more in-depth exposure you have to scientific concepts, the greater will be your understanding. It does not matter if you don't understand everything that is between the pages on a first read. However, THE MORE YOU READ, THE GREATER YOUR UNDERSTANDING! Take notes; look up words you don't understand. Attend special lectures open to the public at local cancer institutes or community cancer centers, and develop a relationship with a laboratory scientist whom you respect and of whom you may ask questions. Attend scientific meetings and lectures if you are able to do so. Most medical schools have ongoing CME (continuing medical education) conferences for basic, translational, and clinical researchers, treating physicians, nurses and other medical professionals. Become involved in a cancer organization that funds basic laboratory research. A number of these organizations host lectures given by their respective grant recipients and most of the lectures are given with a lay audience in mind. However, always try to understand the basic science rather than rely on lay "translations". If you do this, you will be adding another piece of information to your growing foundation of scientific knowledge.

There are several professional journals that one can subscribe to: *JNCI (Journal of the National Cancer Institute), Oncology, New England Journal of Medicine, Nature, American Journal of Human Genetics, Cancer, Endocrinology*, etc., plus popular press – *Scientific American*, etc. Many of these can be accessed online. The Library of Medicine can be accessed online (www.nlm.nih.gov) and has thousands of journals, articles and health related information. It is easy to access and easy to use.

Chapter 2

Resources

Accessing organizations that are involved in cancer research is quite easy in this day and age of websites and computers. There is a plethora of cancer organizations of which one can become a part. In addition, the Department of Defense (DoD) Congressionally Mandated Research Program includes advocates from cancer organizations in its research initiatives during the peer review process and on its Integration Panels. Members are selected based on criteria developed by DoD and a prerequisite is that one must be associated with a constituency so that multiple points of view are represented, not just one's personal cancer experience. Training is provided and participation welcomed. They have an extensive database of organizations and participants in their cancer program initiatives. Their website is: http://cdmrp.army.mil.

As mentioned earlier, the National Cancer Institute's Office of Liaison Activities has a listing of over 135 organizations whose missions are cancer related. You may begin by contacting one of the organizations listed either at the national level or in the local community in which you live, assuming that it is an affiliate of the national organization. Consumer Advocates in Research and Related Activities' (CARRA) website is http://la.cancer.gov/carra. Some of the major organizations one might begin with, several of which have local affiliates, are:

American Cancer Society (ACS); www.cancer.org

National Breast Cancer Coalition (NBCC); www.nbcc.org

National Coalition of Cancer Survivorship (NCCS); www.canceradvocacy.org

Susan G. Komen Breast Cancer Foundation; www.komen.org

Y-Me National Breast Cancer Organization; www.y-me.org

Investigate and find the one that best suits your interests and needs. Those that actually fund basic research have in some instances included advocates in the peer review process for grant applications, both nationally and locally if the local affiliate awards grants to researchers working in local institutional laboratories.

The Food and Drug Administration includes advocates as consumer representatives on Agency advisory committees to represent the consumer perspective on issues and actions before the various committees; serve as liaison between the committee and interested consumers, associations, coalitions, and consumer organizations; and facilitate dialogue with advisory committees on scientific issues that affect consumers. Consumer representatives must be able to analyze scientific data, understand research design, discuss benefits and risks, and evaluate the safety and efficacy of products under review (www.fda.gov). Consumer representatives must have an affiliation with or be an active participant in a community-based organization and can provide testimony before FDA committees.

Chapter 3

Avenues to Serve

There are many avenues in which an advocate can provide valuable service in the research arena:

Locally: Begin by becoming involved with a cancer organization that funds basic research. The Susan G. Komen Breast Cancer Foundation is dedicated to eradicating breast cancer as a life threatening disease and funds basic, translational and clinical research at the national and international levels. Many of the grant recipients, nationally, carry out their work in local communities that have teaching/ research based medical centers. Many of these are designated by the National Cancer Institute as Comprehensive Cancer Centers and must meet rigorous criteria to be so designated. Become involved in the grant making process and team up with one of the grant recipients to learn about the specific research that he/she is investigating. Visit the laboratory; talk with the research associates about the scope of the research being conducted. Ask questions about their understanding of how this will impact cancer treatment or prevention. How soon will the particular research be available for utilization at the bedside? Ask them to explain any aspects that you do not understand. It has been the experience of the authors that scientists are enthusiastically willing to talk about their research and will often give you background reading from published papers. Once you have established your credibility as an informed and knowledgeable advocate, begin to give input from your perspective. Position yourself as an advocate resource, providing your thoughts and perspective. Take every opportunity presented to participate and do not be shy about requesting participation.

Nationally: Once involved at the local level, it is much easier to become involved at the national level in a variety of capacities. You may represent your organization as a representative to a variety of research symposia. National organizations are often invited to participate in government, pharmaceutical and foundation symposia related to breast cancer research as well as other issues related to the cancer experience. This is an opportunity for you to add to your basic scientific knowledge as well as keeping your ear to the ground for other opportunities for which you may volunteer your organization and yourself. The Department of Defense Congressionally Directed Medical Research Programs' Breast Cancer Research Program utilizes advocates from a large database of breast cancer organizations to participate in a two-tiered process of basic, translational and clinical research scientific review. A larger group of advocates is utilized in the peer review process; the first tier, and offer valuable input from the advocates' perspective of the science being presented. Advocates must be members of an organization involved in the breast cancer arena and undergo orientation and training to serve. Advocates who serve on the Integration Panel, the second tier of review, are generally leaders in their respective organizations and are selected based on experience, expertise and national representation. It is important to note that one experience leads to another and another and so on. We must stress here that any representation of your specific organization must be backed up with substantive knowledge and an ability to articulate issues of significance in breast cancer research in a thoughtful, objective, firm, yet respectful way.

Internationally: There is ever increasing interest from other countries in the model of advocate involvement in breast cancer research and indeed, international symposia that address current issues. Your organization may be included in one of these symposia – either as a panelist, presenter or workshop leader – among the many opportunities. Again, the stress is upon credible, substantive articulation of issues.

Chapter 4

Participation as a Partner

Participation as a partner in research with scientists requires objectivity and a very strong sense of self. One must ascribe to the belief that the advocate's contribution to the scientific research process is critical and has a major impact upon national research directions and how that research is carried out. Participation is not about you and your specific experience with cancer, although such experience informs your contribution. It is about breast cancer for the general population. How quickly can we prevent or eliminate this disease?

Advocates become involved, as stated earlier, because breast cancer has touched their lives in a significant way. So much so, that they are compelled to "do something" to eradicate the disease or better yet, to prevent it. The avenue to eradication of current disease and prevention is initially through basic research. Advocates lend an air of urgency to the research at hand. The saving of lives depends upon the speed with which new discoveries are translated into clinical practice. Advocates posing a question or contributing an observation during scientific presentations underscores the need to stay focused on the goal. Extraneous avenues that have little contributory value, though fascinating, are less appealing. There are limited resources to get to the goal and it behooves everyone involved in the scientific process to use those resources as wisely as possible.

It is important to be knowledgeable regarding the research upon which you wish to make a comment and how that research will be carried out in the clinical setting. Is the research going to translate into a clinical trial? How soon? What are the ramifications for the participants of the trial? Is there drug development involved, a biologic, or is the research leading to a non-toxic or less invasive form of treatment or prevention? Are adequate patient safeguards being considered as the research progresses? Will patients be willing to participate in a trial if there are substantial biological side effects? Are informed consent documents considered early on in translating basic research to the clinical setting? Are they adequate to protect participants from harm?

As you can see from the comments in the previous paragraph, it is important to focus upon the end treatment early in laboratory research. Safe and effective treatments that work wonders in animal models will not always translate into the human cell environment. It may mean beginning again with a new hypothesis and scrapping years of work because the original hypothesis has not been borne out in the laboratory. That is to be expected in basic research. Research findings advance the body of knowledge from which others may draw. Serendipitous events occur in the laboratory! A scientist working in a specific area may observe an unusual outcome in an experiment, note it, repeat it, and eureka, a scientific breakthrough is announced! All of these occurrences in the laboratory are necessary in order to arrive at the juncture for a clinical trial to be contemplated, designed and conducted.

Chapter 5

Rewards

Diving into basic breast cancer research has extraordinary rewards. It is an opportunity for you as an advocate to learn, to provide the advocates' perspective and to ultimately make a difference in the lives of those who are diagnosed with breast cancer. You, and/or your family member who may have experienced cancer personally will have a particular sensitivity to cancer research findings. Currently there is tremendous interest in finding a treatment or prevention that is non-toxic, one that does not have the plethora of side effects of today's standard treatments.

Prevention is more and more at the forefront of basic scientific research. Imagine interrupting the initiation of cancer with a non-toxic pill. Research is ongoing to do just that! One of the authors is an advocate on a grant looking at estrogen carcinogenesis, how to interrupt the estrogen metabolic pathway leading to cancer.

Both have authored books and/or articles in breast cancer research and treatment, participated in national and international panels, and are invited presenters at government hearings, and symposia, have given testimony before congressional bodies, served on research peer review panels, committees of Cooperative Research Groups such as CALGB, NSABP etc.. Each has had the opportunity to share her experience with cancer and the necessity of funding basic research with an intensity that will translate the findings in the laboratory to treatment at the bedside. Advocates help provide the focus and generate the impetus to move forward rapidly.

Advocates who are very involved with organizations focused on research and knowledgeable about current research are valuable resources in anticipating consumer issues in translating basic research into the clinical setting. They are adept at identifying issues in research design, informed consent documents, public communications, side effects, and recruitment to name but a few. Many have been participants in clinical trials and have come to appreciate the tremendous effort and dedication of scientists in solving the mysteries of this disease. They have indeed worked in partnership and value that partnership! One of the most heartbreaking experiences is to sit at the bedside of a loved one who has fought a valiant fight only to lose the battle just short of some magnificent discovery that, in the not too distant future, translates to treatment. It is too late for them, and such incidents further fuel the urgency that advocates feel in unlocking the keys to preventing cancer.

The ultimate reward for both scientists and advocates will be the prevention of cancer. Imagine cancer relegated to the category of preventable disease! It is on the horizon, and thus all scientific, advocate, and funding efforts must be focused to that end.

Chapter 6

Shared Experience

The authors have found different avenues to serve as an advocate and each has included a brief personal profile in hope that it will serve as a motivator to become involved. In addition more extensive vitae are included in the appendix to further demonstrate the variety of opportunities for involvement. Remember, what is required is the sincere interest and persistence!

Elizabeth A. Hart, President & CEO

HART INTERNATIONAL-Partnerships Advancing Women's Health Research

Multiple members of my family have died of cancer, the majority of whom were diagnosed with breast cancer. However, there were other cancers as well. Briefly, my two female first cousins – breast cancers (dead); mother-breast cancer (dead); sister-breast cancer (dead); oldest brother-throat and lung cancer (dead); brother-breast, kidney, colon, and liver cancer (survivor); and father-prostate cancer (dead). Both my daughter and I have had breast biopsies that fortunately to date have been benign. When my mother was diagnosed in 1984, it was the same year I became involved in the Susan G. Komen Breast Cancer Foundation and subsequently became Vice Chairman of Grants and finally Chairman and CEO. I was enormously interested in the research that Komen funded and along with a task force restructured the grant making process into a peer-reviewed system that allowed the organization to fund cutting edge research. The more I talked with scientists and learned about the avenues of research they were pursuing, the more I felt that the only way to eradicate the disease was to find out how cancer was initiated, proliferated, and metastasized. Research had the best chance to answer the questions that resonated each time I learned of a new diagnosis of cancer in my family and close friends. I immersed myself in laboratory breast cancer research, and have subsequently come to believe that cancer can be prevented. Research is more and more focused upon prevention initiatives. I am currently a participant with scientists on a Department of Defense Center of Excellence Grant that may well lead to prevention of breast cancer. The path I have traveled as an advocate has brought me through many of the finest research laboratories in the country and in contact with brilliant minds that I know hold to key to the prevention of this disease. I have lost many friends and family members along the way, some of them researchers, but their deaths have not been in vain. Each has added to the store of knowledge that will one day totally obliterate this disease. It cannot come too soon!

Elizabeth Hart can be reached via e-mail at ehart@hart-international.com

HART INTERNATIONAL was formed in 1995 by Ms. Hart specifically to work in partnership with scientists to advance women's health research, particularly breast cancer research at the basic scientific level.

Margaret Borwhat, Co-Founder & President

Women's Cancer Advocacy Network (WCAN)

As a survivor of cancer and currently battling a recurrence, I am indebted to basic research for offering hope for survival. Having been involved in the cancer arena since 1993 as a advocate, I focused attention on legal and ethical issues for breast cancer patients, serving in a variety of leadership roles culminating in the co- founding of WCAN, Women's Cancer Advocacy Network in 1997 and serving as its President. It has been my passion to work with breast cancer researchers providing the advocate's perspective and helping facilitate the translation of basic research into the clinical setting, looking particularly at ethical issues of treatment impacting the lives of patients. WCAN is a source of accurate information and advocacy related to issues in breast cancer, and training.

Margaret Borwhat can be reached via e-mail at mborwhat@wcan.org

.

APPENDIX

CURRICULUM VITAE

ELIZABETH A. HART, PRESIDENT & CEO

HART INTERNATIONAL

Brigham & Women's Hospital School of Nursing	Diploma	1963	Nursing	Boston, MA
George Washington University	BA	1971	Psych/Eng	Washington, DC
LeTourneau University		1999	MBA studies	Dallas, TX

PARTIAL LISTING

Ms. Hart is from a family with 5 first degree relatives with cancer: breast, prostate, kidney, throat & lung, colon and liver

1995-Present President & CEO, HART INTERNATIONAL Mission: Develop Partnerships Advancing Women's Health Research in the area of cancer. Key focus is to facilitate/develop partnerships among advocates, organizations and funding sources to advance basic scientific research.

2001-Present Appointed to Consumer Advocates in Research and Related Activities (CARRA) of the National Cancer Institute as Mentor. Reviewed and scored applications on Study Sections. Participants serve in a variety of capacities: setting research priorities, evaluating and developing information for public dissemination, identifying gaps in research and evaluating clinical trials from the patient advocate's perspective

2000-2003 Member, Data Safety & Monitoring Committee-International Breast MRI Consortium

1997-1999 National Cancer Policy Board – Initial Board Member- Identifying policy issues significant to cancer research and treatment on a national level. Providing expertise for publication and dissemination of board findings.

1996-Present Member and Cancer Advocate to Cancer Cube – Collaborative Group of Scientists working on the etiology of breast & prostate cancer, variety of disciplines from sixteen institutions across the country. Responsible for advocate perspective into research agenda.

1994 Program Committee – Special Conference Industries Coalition Against Cancer

1993, Feb. 4 Testimony before the Institute of Medicine's Committee to advise the Department of Defense Breast Cancer Research Program

1993 Chairman, Komen Alliance: Komen Foundation, UT Southwestern Medical Center and Baylor University, a consortium working together on breast cancer research and clinical practice

1993-1994 National Chairman for Susan G. Komen Breast Cancer Foundation/NCI Regional Breast Cancer Leadership Summits – Major and Minor Summits to reach expanded and more diverse populations, with particular emphasis on minority populations

1993-1997 Consultant to Department of Defense (DoD Congressionally Directed Medical Research Programs (CDMRP) U.S. Army Breast Cancer Research Program (BCRP) providing expertise in program design, development and execution, review of proposals in multiple disciplines, service on initial Integration Panel, and subcommittees.

1995-1996 Member-Executive Secretaries Liaison Subcommittee, DoD BCRP

1994-1998 Member-Consumer Evaluation Subcommittee and Writing Group, DoD BCRP

1994-1995 Member-Executive Committee- Integration Panel, DoD BCRP

1995-2000 National Action Plan on Breast Cancer – Ensure Consumer Involvement Working Group

1995 CALGB Ad Hoc Committee on Policy for Genetic Research in Clinical Cancer Trial Patients

1995 NSABP Breast Cancer Prevention Trial Patient Advisory Committee

1995 NSABP BCPT Subcommittee for Clinical Center Performance Evaluation

1995 NCI SPORE Special Review Committee

1995 Industries Coalition Against Cancer Symposium – Panel Chairman –*Tying Cancer Education, Prevention, Screening to an Overall Wellness Program. April 1, 1995*

1995 RDOG Data Safety and Monitoring Board

1995, Feb.14 Testimony before FDA's Oncologic Drug Advisory Committee – "Consumers on ODAC"

1994-1995 Chairman & CEO, Susan G. Komen Breast Cancer Foundation – responsible for overall functioning of a national/international non profit organization with multiple affiliates whose mission is to eradicate breast cancer as a life threatening disease through research, education, treatment and screening. Under Hart

1994 NSABP BCPT Steering Committee

1994, June 7 Testimony before FDA's Oncologic Drug Advisory Committee "Resume Breast Cancer Prevention Trial"

1994 Program Committee and Dorothy Height Lifetime Achievement Awards Selection Committee – 5th Biennial Symposium on Minorities, the Medically Underserved & Cancer "Cultural Diversity, Public Policy and Survivorship"

1993 Chairman Elect – Board of Directors, Chairman National Advisory Board, Susan G. Komen Breast Cancer Foundation

1993, Oct.14 HHS Secretary's Conference to Establish a National Action Plan on Breast Cancer – Basic Research Working Group

1993, Oct. 21 Testimony before the Board of Scientific Counselors, Division of Cancer Prevention and Control National Cancer Institute – Screening Mammography

1993, Mar. 18 Testimony before the President's Cancer Panel's Special Commission on Breast Cancer - Komen/NCI Breast Cancer Leadership Summits

1991-1992 National Co-Chairman Regional Breast Cancer Leadership Summits – Co-chaired with member of the National Cancer Advisory Board/NCI, a series of breast cancer summits across the country targeting the leadership of corporations, health care providers and minority populations to increase breast cancer awareness and education efforts

PUBLICATIONS, MEDIA & SYPOSIA, HONORS (Partial Listing)

2006 Cavalieri, Ercole; Chakravarti, Dhubajyoti; Guttenplan, Joseph; **Hart, Elizabeth**; Ingle, James; Jankowiak, Ryszard; Muti, Paola; Rogan, Eleanor; Russo, Jose; Santen, Richard; and Sutter, Thomas. *Catechol Estrogen Quinones as Initiators of Breast and Other Human Cancers. Implications for Biomarkers of Susceptibility and Cancer Prevention.* Review. Biochimica et Biophysica Acta 1766:63-78, 2006.

2002 Andejeski, Yvonne, M.D., Erica S. Breslau, Ph.D., M.P.H., **Elizabeth Hart, R.N**., Ngina Lythcott, Dr. P.H., Linda Alexander, Ph. D., Irene Rich, D.N.Sc.. Isabelle Bisceglio, Ph.D., Helene S. Smith, Ph.D., Fran M. Visco, Esq., and the U.S. Army Medical research and Materiel Command Fiscal Year 1995 Breast Cancer Research Program Integration Panel. *Benefits and Drawbacks of Including Consumer Reviewers in the Scientific Merit Review of Breast Cancer Research.* Journal of Women's Health & Gender-Based Medicine, Volume 11, Number 2, 2002.

2000 **Hart, Elizabeth**, *Chapter 10 – Hope for Prevention: Perspective of the Cancer Advocate.* Journal of the National Institute Monograph, Number 27, pages 157-159. 2000.

1999 National Cancer Policy Board – *Ensuring Quality Cancer Care.* National Academy Press, Washington, DC.

1998 Panelist – Women's Forum – "Menopause: Treatment Options for Women Surviving Breast Cancer or Concerned about Estrogen Replacement Therapy: June 23, 1998, Oschner Clinic, New Orleans, LA. Sponsored by the Hormone Foundation.

1998 Participant –Press Conference, "Menopause: Treatment Options for Women Surviving Breast Cancer or Concerned About Estrogen Replacement Therapy," The Endocrine Society 80th Annual Meeting, June 24, 1998; New Orleans, LA.

1998 Chairperson-Advocates' Perspective Panel – International Symposium, *Estrogens as Endogenous Carcinogens in the Breast and Prostate.* March 16-17, 1998. McLean, VA.

1999-1997 Member, Technical Program Committee, "Era of Hope" Symposium, USAMRMC Breast Cancer Research Program, October 31-November 4, 1997

1995 Association of Community Cancer Centers' 21st Annual Meeting *ReVisioning Oncology.* Speaker and-Panelist, High Tech and Patient Advocacy Panel, "State of the Art Medicine?"

1996 University of Texas Southwestern Medical Center: Speaker, Breast Care: Advances & Update for Primary Care Physicians: A Didactic Skills Symposium, "Physician-Patient: Partners in Health", July 15, 1995

1995 Executive Producer "For Women's Lives: Dialogues on Breast Cancer" PBS film aired in Spring of 1996. Finalist for Best of Category – American Medical Association's International Film Competition, November 1996

1994 Opinion Editorial, "Peace dividend shows up in medicine", Dallas Morning News, November 14,

1994 -July Participant "New Frontiers in Breast Cancer Imaging and Early Detection" Symposium sponsored by the Office of Women's Health, NCI, and the Congressional Caucus For Women's Issues, Washington, D.C.

CURRICULUM VITAE

MARGARET J. BORWHAT, PRESIDENT

WOMAN'S CANCER ADVOCACY NETWORK

Russell Sage College	B.A.	1975	Psychology	New York
University of Maine at Orono	Masters Studies	1976	Social Psychology	New York
University of New York, Albany	MA/Ph.D.Studies (Completed Coursework)	1981	Social Psychology	New York

PARTIAL LISTING

CONTINUING EDUCATION

University of Virginia 1991-93, completed all courses required to sit for the CPA Exam. Research in International Accounting. 1995, Course in Strategic Planning for Non-profits.

Center for Clinical Bioethics, Georgetown University Medical Center, March 2000. Completed 14-hour workshop, Research Ethics: Institutional Review Board Responsibilities and Related Competencies.

Public Responsibilities in Medicine and Research (PRIM&R) at Tufts University School of Medicine, May 18-19, 2001. Completed 16-hour workshop, Promoting Responsible Conduct of Research: Policies, Challenges, and Opportunities.

Kennedy Institute of Ethics, Georgetown University, June 5-10, 2001. Completed Intensive Bioethics Course, XXVII, an in-depth study of major topics and contemporary challenges in health care and research ethics.

RELAVANT ADVOCACY WORK AND ORGANIZATION EXPERIENCE

Virginia Breast Cancer Foundation (VBCF) President, June 199-96, Vice President 1994-95, Legislative Chair 1993-95, Board Member 1993 -1997.

National Breast Cancer Coalition (NBCC) 1993-95, State Coordinator, served as liaison between NBCC and BVCF. Board Member, 1995, Completed Project Lead, 1996 – training program on the science of breast cancer for advocates.

Hospice of the Piedmont, Charlottesville.VA 1995-96 Volunteer

National Patient Advocate Foundation, June 1996 to June 1999, Legislative Liaison, Researcher, and Board Member. Researched 50 states' legislative bills and resolutions related to cancer and health care insurance. Formulated and helped implement legislative initiatives, conducted research, developed web page, and assisted in fundraising, establishing and maintaining databases.

Patient Advocate Foundation, June 1996 to June 1999. Board of Directors. Provided education and pro bono

legal counseling to cancer patients concerning managed care, insurance, and financial issues.

Legal Information Network for Cancer (LINC) T.C. Williams School of Law, University of Richmond, October 1997 to December 1998, Advisory Board, an organization of volunteers committed to helping patients and their families resolve non-medical issues arising from the diagnosis or treatment of cancer.

JAUNT January 1998 to 2000, Secretary, Board of Directors and Finance Committee, Virginia's Planning District Ten, providing services for the general public, human service agency clients, the elderly and people with disabilities.

Women's Cancer Advocacy Network (WCAN) 1997 to present, Co-founder and President, work with researchers and clinicians to provide consumer advocate perspective and to facilitate translation of research to clinical setting. Served on Advocate Core for SPORE projects and development of educational training modules for advocates. Public speaking and outreach, e.g., Breast Cancer Patient's Perspective presented at Virginia Commonwealth University for social work graduate students; and Internet Support Group Participation presented at the University of Virginia for medical staff and public. Development and distribution of materials for advocates including Department of Defense Breast Cancer Research Program Meeting, Era of Hope summary.

CONSULTATION AND SPECIAL PROJECTS

Consumer Reviewer of Grant Proposals for Department of Defense Breast Cancer Research Program 1995, Supplemental Grants, 1996; Endocrinology panel, and review of process; 1998, Molecular genetics panel.

Commonwealth of Virginia General Assembly Study Group on Patient Information 1996, Provided consumer perspective on legislative study group regarding informed consent and patient information.

Workshop Conference on Treatment of Estrogen Deficiency Symptoms in Women Surviving Breast Cancer. Served as patient advocate in the organization and participation of consumer advocates at an international conference held in September, 1997.

Georgetown University, Lombardi Cancer Center (LCC) June 1996 to present. Consumer advocate for Department of Defense Research and Specialized Program of Research Excellence (SPORE) in Breast Cancer Conducted at LCC.

Cancer and Leukemia Group B (CALGB) January 1999-2003, Consumer advocate on the Breast Committee, Patient Issues Committee, Clinical Economics Subcommittee, and Patient Advocate Working Group.

National Action Plan on Breast Cancer, May 1999, Consumer Involvement Working Group member studying existing consumer representation in a variety of breast cancer research and programs, and identifying areas for increased involvement.

Cancer Genetics Network (CGN, Bioethics Working Group, June 1999-2003, National Cancer Institute

Sponsored infrastructure to support genetics research. Contributed article for Mid-Atlantic Cancer Genetics Network, Spring 2000, and the Northwest Cancer Genetics Network, Spring/Summer, 2000.

Susan G. Komen Breast Cancer Foundation 1999, 2000, and 2001, Consumer reviewer for grant proposals, tumor biology section. Selected "Local Hero" for Komen's Drive for the Cure.

Office of Liaison Activities, March 2000, appointed to serve on the Advocate Committee to the Working Group on Liaison to Advocacy and Voluntary Organizations.

National Human Research Protections Advisory Committee (HHRPAC), Third Parties Working Group January 2001 to present. Provides advice and recommendations to the Secretary of HHS, Assistant Secretary for Health (ASH), the Director, Office for Human Research Protections (OHRP), and other departmental officials on issues and topics pertaining to the protection of human research subjects.

National Human Research Protections Advisory Committee (NHRPAC) January 2001 to September 2002. Appointed to serve a three-year term, member of Third Party/Genetics Working Group, and Informed Consent Working Group. Provides advice and recommendations to the Secretary of HHS, Assistant Secretary for Health (ASH), the Director, Office for Human Research Protections (OHRP), and other departmental officials on issues and topics pertaining to the protection of human research subjects.

National Cancer Institute Central Institutional Review Board 2003-2005, IRB review of NCI sponsored multi-site trials at the national level before the protocol is distributed to local investigators.

Cancer Biomedical Informatics Grid, caBIG, patient advocate, 2004-2005, a voluntary network or grid connecting individuals and institutions to enable the sharing of data and tools, creating a World Wide Web of cancer research under the leadership of National Cancer Institute's Center for Bioinformatics.

PUBLICATIONS

Menopause: Treatment Options for Women Surviving Breast Cancer or Concerned about Estrogen Replacement Therapy. Santen, Richard J., M.D.; **Borwhat, Margaret**, and Gleason, Sarah, The Hormone Foundation, 1998. Revised 1999, *Managing Menopause: A Change for the Better.*

"Managing accrual in cooperative group clinical trials:, Demmy, TL, Yasco, JM, Collyar, DE, Katz, ML, Krasnov, CL, **Borwhat, MJ**, Battershell, A, George, SL. *Journal of Clinical Oncology.* August 1, 2004, 2997-3002.

Partnerships Advancing Women's Health Research