// KU ScholarWorks

Novel Strategies in Cancer Prevention and Fertility Preservation with Tamoxifen

Item Type	Dissertation
Authors	Ting, Alison
Publisher	University of Kansas
Rights	This item is protected by copyright and unless otherwise specified the copyright of this thesis/dissertation is held by the author.
Download date	2024-08-25 23:28:09
Link to Item	https://hdl.handle.net/1808/5389

NOVEL STRATEGIES IN CANCER PREVENTION AND FERTILITY PRESERVATION WITH TAMOXIFEN

BY

C2009
Alison Yuh Fen Ting

B.A., University of Kansas, 2001

Submitted to the graduate degree program in Molecular and Integrative Physiology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

____
Chairperson

Committee members ______________________________

____

____

____

Date Defended: **March 5th, 2009**

The Dissertation Committee for Alison Yuh Fen Ting certifies
that this is the approved version of the following dissertation:

NOVEL STRATEGIES IN CANCER PREVENTION AND FERTILITY PRESERVATION WITH TAMOXIFEN

Committee:

____
Chairperson

____

____

____

____

Date approved:________________________

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor Dr. Brian Petroff. I want to thank him for continuous support and patience throughout my Ph.D. training. He is always making sure that I am well trained in every aspect for becoming a good scientist from lab techniques, writing manuscript and grant and preparing presentations. Next, I would like to acknowledge the members of my Ph.D. dissertation committee, Drs. Christenson, Guo, Li, and Kimler. I appreciate their guidance, advises and great insights for my Ph.D. research projects and future career development. I would also like to thank the Department of Molecular and Integrative Physiology, especially Dr. Cheney and Imig for providing the best environment possible for graduate training. I would like to thank members of the Petroff lab, especially Kelli Valdez for her advice and encouragement, and for being a great friend. Also, I want to thank Sara for all her support. I would like to thank Dr. Albertini and members of his lab who have become a great help to me especially during the second part of my Ph.D. research studies. I would also like to thank Dr. Carol Fabian and members of the breast cancer prevention center for all their support and help. Last but not least, I would like thank all my family and friends. My mom and dad for their care, love and support. I would like to thank my brother Jay and his wife Annie and all the members of my families in Taiwan for their support and being proud of me. Lastly, I would like to thank Wohaib for being a great partner for his encouragement and support not only intellectually but also emotionally.

TABLE OF CONTENTS

Acknowledgements..……………………………………………………………….…….3

Table of Contents……………………………………………………………………...4

Abbreviations...………………………………………………………………………..7

Abstract………………………………………………………………………….....10

Chapter I: Introduction

Breast and ovarian cancer and their risk factors……………………………..13

Histopathology of breast cancer…………………………………………….....14

Current breast cancer chemoprevention……………………………………...15

Histopathology of ovarian cancer……………………………………………16

Current ovarian cancer chemoprevention………………..…………………..18

Dual cancer prevention………………………………………………………19

Animal models for breast and ovarian cancer……………….……………..……20

Common biomarkers for breast and ovarian cancer…………………………21

Breast stem cells in breast cancer progression………………………………22

Current breast stem cell markers……………………………………………….23

Tamoxifen…………………………………………………………………....24

Fertility in female cancer patients……..……………………………………..25

Current options for fertility preservation…………………………………….26

Animal models for the study of chemical-induced ovarian toxicity…………27

Specific aims of this work……………………………………………………28

Chapter II: Characterization of a Preclinical Model of Simultaneous Mammary and Ovarian Cancer Progression

Title…..……………………………………………………………………31

Abstract……………………………………………………………………32

Introduction…………………………………………………………………..33

Material and Methods………………………………………………………..34

Results………………………………………………………………………..38

Discussion……………………………………………………………………40

Tables………………………………………………………………………...45

Figures…..……………………………………..…………...…..……..46

Chapter III: Tamoxifen Prevents Premalignant Canges of Mammary but not Ovarian Cancer in Rrats at High Risk for Both Diseases

Title…..……………………………………………………………………50

Abstract……………………………………………………………………51

Introduction…………………………………………………………………..52

Material and Methods………………………………………………………..54

Results………………………………………………………………………..59

Discussion……………………………………………………………………62

Tables………………………………………………………………………...67

Figures………………………………………………………………………..69

Chapter IV: Tamoxifen as a molecular shield to protect the ovary against DMBA and cyclophosphamide-induced follicle loss in the rat

Title…..……………………………………………………………………74

Abstract……………………………………………………………………75

Introduction……………………………………………………………..77

Material and Methods…………………………………………………..80

Results…………………………………………………………………..84

Discussion…………………………………………………………………86

Figures…………………………………………………………………..91

Chapter V: Overall Conclusions and Discussion…………………..…..…104

References……………………………………………………………………..115

ABBREVIATIONS

4HC = 4-hydroxtycyclophosphamide

4HT = 4-hydroxytamoxifen

ALDH-1 = aldehyde dehydrogenase-1

BCRP-1 = breast cancer resistance protein-1

BRCA 1/2 = breast cancer 1/2 gene

BSA = bovine serum albumin

CA-125 = cancer antigen-125

CARC = carcinogens-treated animals

CHOP = cyclophosphamide + doxorubicin + prednisone + vincristine

CMF = cyclophosphamide + methotrexate + fluorouracil

CONT = vehicle-treated animals

COX-2 = cyclooxygenase-2

Cy = cyclophosphamide

DCIS = ductal carcinoma *in situ*

DAB = diaminobenzidine

DMBA = 7, 12 dimethylbenza[α]anthracene

DMEM/F-12 = Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (1:1)

DMSO = Dimethyl sulfoxide

E_2 = 17β-estradiol

EGCG = epigallocatechin 3-gallate

EOC = epithelial ovarian cancer

ER = estrogen receptor

FSH = follicular stimulating hormone

GnRH = gonadotropin releasing hormone

hCG = human chorionic gonadotropin

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

H&E = hematoxylin and eosin

HER-2/neu = human Epidermal growth factor Receptor 2

IOD = Integrated Optical Density

LCIS = lobular carcinoma in situ

LH = luteinizing hormone

MAC = mammary adenocarcinoma

MEM EAA = Minimal Essential Medium Essential Amino Acids

MEM NEAA = Minimal Essential Medium Non-essential Amino acids

MMTV = Mouse mammary tumor virus

MNU = N-Nitroso-N-methylurea

MRECM1 = Modified Rat Embryo Culture Medium

NSAIDs = Non-steroidal anti-inflammatory drugs

PFA = paraformaldehyde

PR = progesterone receptor

RR = relative risk

SERMs = selective estrogen receptor modulators

TAM = tamoxifen

TBST = Tris-Buffer Saline with Tween-20

Tris = tris(hydroxymethyl)aminomethane

ABSTRACT

Women at high risk for breast cancer are often also at high risk for ovarian cancer, reflecting similar risk factors and suggesting intertwined disease pathways and common prevention targets. A novel strategy to overcome obstacles in preventing ovarian neoplasia (low incidence, lack of specific disease markers, and difficulties in tissue sampling), the deadliest gynecologic cancer, may be to develop a prevention strategy that targets breast and ovarian cancer simultaneously. Tamoxifen, a selective estrogen receptor modulator, reduces hormone responsive breast cancer risk by 50% but its effects on risk of ovarian cancer, also hormonal responsive, are unclear. The goals of this work were to 1) develop and characterize a preclinical model of concurrent breast and ovarian cancer and 2) use this dual cancer model to examine the efficacy of tamoxifen to prevent both breast and ovarian cancer. Mammary carcinogens [7,12-dimethylbenz[α]anthracene (DMBA), N-methyl-N-nitrosourea and estradiol (E_2)] were tested separately in combination with local ovarian DMBA administration to determine the best combined treatment to induce mammary and ovarian cancer concurrently and effectively in the rat. Results showed that systemic E_2 and ovarian DMBA promoted the highest incidence of dysplasia in the mammary gland and ovary and elevated levels of mammary Ki-67 and cyclooxygenase 2 (COX-2) mimicking the human disease. Next, the ability of tamoxifen to prevent mammary and ovarian cancer simultaneously was evaluated. Tamoxifen which inhibited mammary carcinogenesis and normalized levels of Ki-67 and COX-2, had no effect on (neither accelerated nor inhibited) ovarian cancer progression. In addition,

carcinogen treatment increased levels of stem cell markers, Oct-4 and aldehyde dehydrogenase-1, in the mammary gland; interestingly, this expansion was not reversed by tamoxifen. Intriguingly, while examining ovaries from this study, we serendipitously discovered an apparent protective effect of tamoxifen against DMBA-induced follicular destruction and this effect was further investigated. Chemotherapy and environmental toxicants (e.g. DMBA) deplete ovarian follicles and often lead to accelerated ovarian aging and premature ovarian failure; however, there is no established treatment that can protect the ovary from these toxic insults. *In vivo*, rats were treated with tamoxifen and DMBA or cyclophosphamide (the most ovotoxic chemotherapy) and total numbers of follicles in the ovary were determined. *In vitro*, ovarian organ culture and oocyte culture were carried out to examine local effects of tamoxifen on DMBA-induced follicle loss and doxorubicin-induced oocyte fragmentation, respectively. We demonstrated for the first time that tamoxifen protects ovarian follicles against not only DMBA- but also chemotherapy (cyclophosphamide and doxorubicin)-induced ovarian damage. Clinically, tamoxifen has already been tested for safe use as an adjuvant therapy for several cancers; therefore, if translated into clinical use, these results may have immediate impact on options for fertility preservation and quality of life in young female cancer patients undergoing chemotherapy. The long term goals of this work are to 1) use the dual cancer model to screen for promising agents that decrease risks for both breast and ovarian cancer and 2) examine the mechanism by which tamoxifen inhibits toxicant-induced ovarian follicle loss.

CHAPTER I

INTRODUCTION

Breast and ovarian cancer and their risk factors:

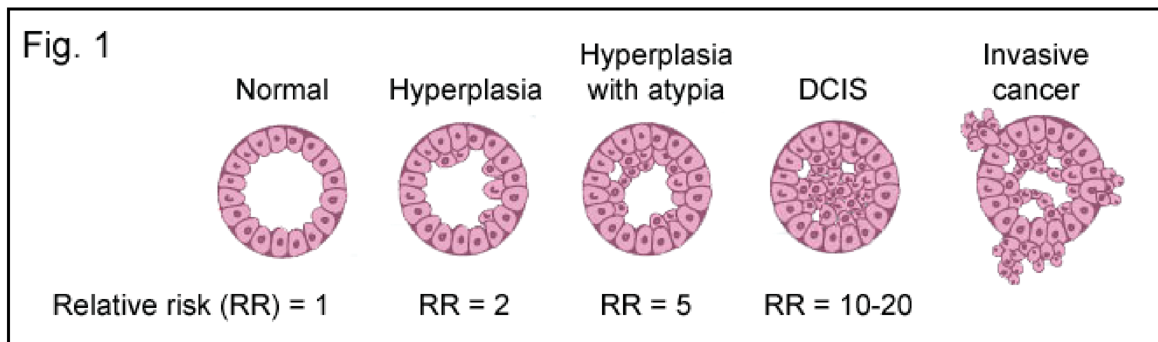
Breast cancer affects 1 in 8 women in the United States and causes over 40,000 deaths annually [1]. Ovarian cancer affects only 1 in 70 women; however, it results in almost half as many fatalities as those of breast cancer and is the leading cause of death for all gynecological malignancies [1]. This high mortality rate of ovarian cancer is largely due to a lack of diagnostic tools for detection during treatable stages, indicating the need for prevention strategies. However, ovarian cancer prevention trials are seldomly attempted due to low incidence, lack of disease biomarkers and difficulties in sampling the ovary [2]. Interestingly, women at high risk for breast cancer are often also at high risk for ovarian cancer [3], reflecting common risk factors and intertwined disease pathways [4,5].

Over 90% of all breast and ovarian cancer cases are sporadic while 5-10% are familial caused by germline mutation in BRCA1 or BRCA2 genes [4,6]. BRCA1/2 mutations increase lifetime risks of breast and ovarian cancer from 12% and 1.4% to approximately 60-80% and 20-40%, respectively [7-10]. Nulliparity and prolonged lifetime exposure to estrogen (i.e. early menarche and late menopause as well as hormone replacement therapy) are associated with elevated risks for both breast and ovarian cancer, while early age at first childbirth and multiple pregnancies decrease their incidences [11-13]. Oral contraceptive usage has been shown to have a protective role against ovarian cancer but with a less clear role in breast cancer risk [14-16]. The similarity between risk factors for breast and ovarian cancer suggest their interdependent disease pathways and possible common prevention targets. A

logical and feasible approach to ovarian cancer chemoprevention may be the development of breast cancer prevention drugs that simultaneously decrease ovarian cancer risk.

Histopathology of breast cancer:

Ductal carcinoma, the most common type of breast cancer, is derived from the epithelial lining of milk ducts and accounts for approximately 80% of all breast cancer cases in women [17,18]. Lobular carcinoma is derived from alveoli and represents the majority of non-ductal breast carcinomas [17,18]. Other types of breast cancer, while less common, include mucinous (3%; mucus-producing cells), medullary (2%; central breast tissue) and tubular (2%; ductal cells) [19]. Early changes of ductal carcinoma include cellular hyperplasia, hyperplasia with atypia and carcinoma *in situ* and each of these histological stages is associated with an elevated risk for breast cancer (Figure 1) [20-25].



Current breast cancer chemoprevention:

The average woman has a 12% lifetime risk for breast cancer and currently about a 75-80% chance of cure once the cancer has developed, due largely to early detection as well as advances in prevention therapy [26]. Indeed, breast cancer chemoprevention trials are facilitated by minimally invasive techniques to sample breast tissue and the availability of a number of surrogate biomarkers to evaluate prevention drugs in phase II trials, as well as the high incidence of this disease in phase III trials [27-29]. Candidates for breast cancer prevention include selective estrogen receptor modulators (SERMs), aromatase inhibitors, COX-2 inhibitors, retinoids, and natural compounds such as resveratrol, epigallocatechin-3-gallate (EGCG, found in green tea), etc. A list of detailed current clinical trials for breast cancer prevention is summarized in table I.

Table I: Examples of current clinical trials for breast cancer prevention [30]

Prevention agent tested	**Drug class**	**Phase**	**Patient population**
Exemestane	Aromatase inhibitor	III	Postmenopausal
Dietary soy	Natural compound	III	Premenopausal
Letrozole	Aromatase inhibitor	III	Postmenopausal
hCG analog	hCG	III	Age 30-75
Deslorelin	GnRH analog	II	Age 21-48
Grape Seed Extract	Natural compound	I	Age 40-75
Green Tea Extract (EGCG)	Natural compound	I	Age 21-65
White Button Mushroom Extract	Natural compound	I	Age 21 and over

Currently, the SERMs tamoxifen and raloxifene have been shown to reduce breast cancer risk by roughly 50% and are the only approved agents for use in high risk women to prevent breast cancer. Following results of the National Surgical Adjuvant Breast and Bowel Project P2 trial, it appears likely that tamoxifen and

raloxifene will be the mainstay for breast cancer prevention for pre- and postmenopausal women for at least the next decade [31]. However, despite some known effects of SERMs on ovarian function in premenopausal females [32,33], little attention has been paid to the possible ovarian effects of breast cancer chemoprevention and potential risk for ovarian cancer.

Histopathology of ovarian cancer:

Classification of ovarian cancer is determined by the cell type of tumor origin (Table II). Epithelial ovarian cancer (EOC) is the most common type of ovarian cancer and accounts for 90% of all cases. EOC is derived from the surface epithelium consisting of a single cell layer that covers the entire ovary (Fig. 2). Based on their tissue types, ovarian epithelial tumors can be further divided into a number of subtypes with serous EOC being the most prevalent (Table III). The etiology of ovarian cancer is still unknown; however, studies have associated its cause with "incessant ovulation."

Table II: Histologic classification of ovarian cancer

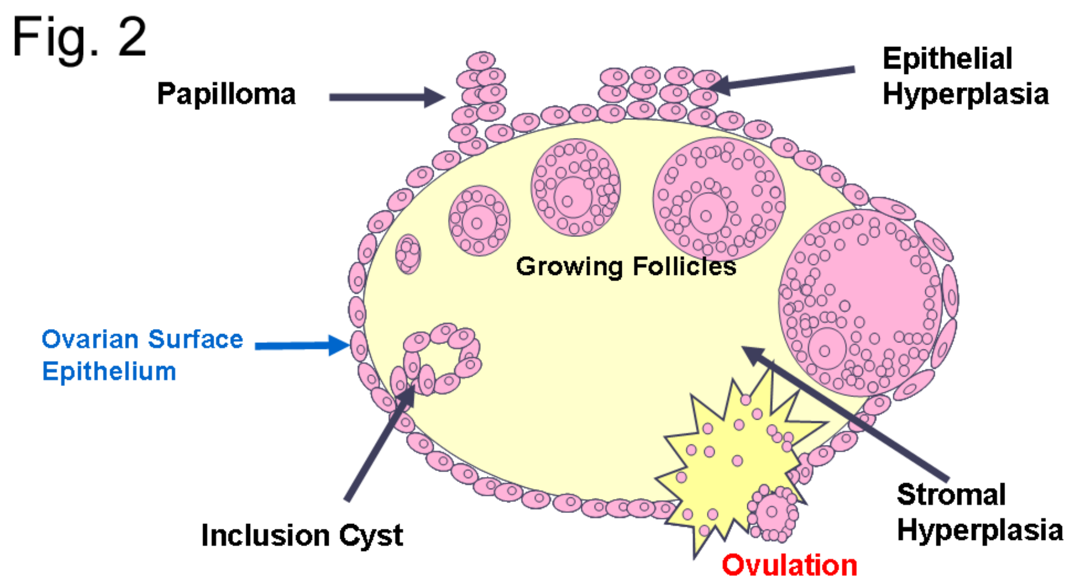
Ovarian cancer types	**Tumor origin**	**Prevalence**
Epithelial ovarian tumor	Surface epithelial	85-90%
Sex cord-stromal tumor	Germ cells	5-7%
Germ cell tumor	Granulosa/Theca	5%

Table III: Different types of epithelial ovarian cancer (EOC)

EOC subtypes	**Tumor cell type**	**Prevalence**
Serous	Fallopian tube epithelium	40%
Endometrioid	Proliferative endometrium	20%
Mucinous	GI tract or endocervical epithelium	1%
Clear cell	Gestational endometrium	6%
Borderline	Mixed	10-15%
Undifferentiated	Poorly differentiated	15%

This hypothesis states that constant proliferation and DNA repair at the ovulatory rupture site may be an initiating factor for common ovarian cancers, reflecting preneoplastic changes of the ovary, such as surface epithelial hyperplasia and formation of surface papilloma and epithelial inclusion cysts (Fig. 2) [34-36].

Studies have also suggested the involvement of gonadotropins in ovarian cancer etiology [37]. The "gonadotropin hypothesis" reasons that atypical entrapment of ovarian surface epithelium (inclusion cysts or epithelial invagination) within the ovarian stroma will lead to proliferation and malignant transformation under the influence of excessive follicle stimulating hormone (FSH) and luteinizing hormone (LH) [38,39]. Other factors such as p53 mutation, estrogen, progesterone and androgen levels have also been associated with ovarian cancer risk [38]. While several hypotheses exist for the cause of ovarian cancer, the etiology is most likely a combination of multiple factors.



Current ovarian cancer chemoprevention:

A woman's lifetime risk of developing ovarian cancer is less than 2%, but it is the deadliest gynecological cancer with 5 year survival of less than 25% for Stages III and IV where over 75% of cases are detected [40,41]. This is primarily because ovarian cancer usually remains clinically silent and inaccessible diagnostically until it is beyond treatable stages, suggesting a need for prevention strategies.

Apart from surgically removing the ovary, past candidates for ovarian cancer prevention include oral contraceptives, fenretinide and COX inhibitors. Oral contraceptives have been shown to reduce risk of sporadic and familial ovarian cancer especially for longer duration of use [42]. The preventive effect of oral contraceptive use is, in part, due to inhibition of ovulation, a risk factor for ovarian cancer [43-46]. Fenretinide, a vitamin A derivative, was found to decrease ovarian cancer risk as a secondary effect in a breast cancer prevention trial [47,48]. COX-1/2 are often overexpressed in ovarian tumors [49-52], and COX inhibitors were shown to be associated with a reduction in ovarian cancer risk in a pooled study examining the link between non-steroidal anti-inflammatory drugs (NSAIDs) and cancer [53,54]. However, the use of some COX inhibitors raises concerns including increased risk of heart attack and stroke and questions their use for cancer prevention [55]. Effects of SERMs on ovarian function and pathology have been reported [32,56]; in fact, both tamoxifen and raloxifene promote abnormal ovarian function (i.e. irregular menstrual cycle) and follicular cyst formation in the ovary, but their effect on ovarian cancer risk are still controversial [32,33,57,58].

Research in ovarian cancer prevention is sparse due in large part to a low incidence of the disease, delayed diagnosis, lack of ovarian cancer specific biomarkers, and difficulty in accessing ovarian tissue, leading to difficulties in conducting clinical trials even with promising preventive agents available. A potential strategy for ovarian cancer prevention may be to develop prevention strategies targeting more common cancers, with selection of drugs that also protect against ovarian cancer.

Dual cancer prevention:

Breast and ovarian cancer have similar risk factors such as estrogen exposure, ovulation, nulliparity, obesity, family history as well as BRCA1/2 mutations and women at increased risk for one of these cancers are often also at risk for the other suggesting intertwined disease pathways and possible common targets for prevention [4,59]. Therefore, difficulties in preventing ovarian neoplasia may be overcome by a prevention strategy that targets breast and ovarian cancer simultaneously. Indeed, successful human ovarian cancer chemoprevention has only been demonstrated incidentally during the course of breast cancer prevention trials (i.e. fenretinide); although results from this study showed a moderate preventive effect in premenopausal women while an opposite trend (elevated risk) in postmenopausal women [47,48]. Very few compounds have been tested for dual action against both cancers due in part to the lack of appropriate animal models that develop both diseases. Although the effects of chemoprevention drugs can be tested separately in

preclinical models, this approach ignores the intertwined etiologies of breast and ovarian cancer as well as potential synergism between effects in the breast and ovary of prevention drugs.

Animal models for breast and ovarian cancer:

Rats are the most frequently used animal model for breast cancer chemoprevention studies due to their shorter life span, plentiful tissue, and lack of the mouse mammary tumor virus (MMTV) which causes mammary tumors by a different etiology than the human disease. Most common and well-established mammary cancer models used for prevention studies involve tumor induction by chemical carcinogens 7,12-dimethylbenz[α]anthracene (DMBA), and N-methyl-N-nitrosourea (MNU) and the hormonal carcinogen E_2 [60-63]. Common features of these models include high incidence of tumor induction, organ site specificity, tumors of ductal origin, and tumors that are hormone-dependent and have similar histology as the human disease. As demonstrated by Li and colleagues [62], one major advantage of using E_2 as a mammary carcinogen over chemical carcinogens is that estrogen-induced tumors are highly aneuploid as is human breast cancer. No commonly used rat model of EOC exists but one promising method is the direct application of DMBA to the ovarian surface epithelium, resulting in an approximate 30-50% occurrence of EOC following 12 months of treatment [64-66].

Common biomarkers for breast and ovarian cancer:

Surrogate end point biomarkers vary with risk and response to prevention drugs [67]. Common surrogate end point biomarkers for breast cancer include hormone receptors (ER and progesterone receptor, PR), Ki-67, human epidermal growth factor receptor-2 (HER-2), and COX-2 [29,68-71]. While specific biomarkers for ovarian cancer are still being identified, currently, levels of serum cancer antigen-125 (CA-125), COX-1/2, ER, PR, and Ki-67 are used to track disease progress, however, without enough sensitivity and specificity for early diagnoses [72-76].

The estrogen, progesterone and ER pathway plays an important role in normal development as well as the pathophysiology of the breast and ovary. Therefore, serum levels of estrogen and progesterone and their local receptor expression are critical in risk assessment, therapy and prognosis of breast and ovarian cancer [68,77,78]. Ki-67 is a nuclear antigen expressed by proliferating cells in all active phases (G1, S, G2 and M) of the cell cycle and absent in resting (G_0 phase) cells [70]. It is used routinely as a proliferation marker to measure human tumor growth [29,69].

COX-2 expression is induced by growth factors as well as pro-inflammatory cytokines and elevated during the growth of various tumors [79]. In the breast, COX-2 expression increases aromatase production by which cells resulting in an elevated local estrogen concentration. Overexpression of COX-2 is found in proliferative breast disease (hyperplasia), ductal carcinoma in situ/lobular carcinoma in situ (DCIS/LCIS), and invasive breast cancer [80]. In the ovary, COX-2 is involved in normal female reproductive functions such as ovulation, implantation and

decidualization. However, COX-2 overexpression is often associated with ovarian neoplasia [49,50,81]. HER-2/neu belongs to the epidermal growth factor receptor family and is associated with cell growth and proliferation. Overexpression of HER-2 is found in 15-30% of breast cancer cases and corresponds to a higher nuclear grade and aneuploidy as well as a poorer prognosis of the disease [82,83].

CA-125, a serum biomarker, is useful for monitoring response to chemotherapy and detecting disease recurrence [84,85]. However, its low specificity and sensitivity have limited its use primarily to disease monitoring and it is usually used only in parallel with other cancer biomarkers [86]. Currently, identification of additional biomarkers specific to breast and ovarian cancer are still a top priority for early detection.

Breast stem cells in breast cancer progression:

The size of the stem cell pool in the breast has been suggested as an indicator of poor clinical outcome for breast cancer [87]. The existence of self-renewing, pluripotent stem cells have been demonstrated both in human breasts and rodent mammary glands [88,89]. In animal models, transplantation of enriched mammary stem cells into cleared mammary fat-pads causes regeneration of an entire functional gland [90-92]. In the human breast, stem cells can be isolated and are characterized by their ability to divide and form mammospheres in culture as well as to reconstruct functionally normal breast tissues in a rodent model [88,93]. Studies have suggested that breast cancers are likely to emerge from inherited or acquired mutations in these

breast stem cells [94-96]. Deregulation of cancer stem cells results in tumor growth and regrowth of refractory cancer cells following primary treatment. Inability of chemotherapy agents to destroy deregulated stem cell populations has been suggested to be the reason for cancer recurrence, tumor regrowth and poor clinical outcome, indicating a need for new treatment strategies targeting stem cells [97].

Current breast stem cell markers:

Potential markers for breast stem cells include Hoechst dye exclusion, protein expression of breast cancer resistance protein-1 (BCRP-1, membrane transporter responsible for Hoechst dye efflux), Oct-4, survivin and aldehyde dehydrogenase-1 (ALDH-1). BCRP-1 belongs to a family of drug-efflux protein pumps (ABC transporters) present in stem cells and is suggested to cause drug resistance in many types of cancer including breast [98,99]. Oct-4 is a transcriptional factor expressed by early embryo cells and germ cells and has been used to identify pluripotent cell populations [100,101]. Additionally, ALDH-1, an enzyme that is required for the conversion of retinol to retinoic acids, is highly enriched in hematopoietic stem cells and recently, researchers have suggested its presence in breast stem cells as well [102]. In the current study, expression of stem cell markers was examined during mammary carcinogenesis and in response to cancer chemoprevention therapy with tamoxifen.

Tamoxifen:

Tamoxifen, a selective estrogen receptor modulator (SERM), binds estrogen receptors competitively with endogenous estrogen and promotes estrogenic or anti-estrogenic activities in a tissue specific manner [103]. In the breast, tamoxifen blocks cell proliferation, has been shown to cause tumor regression and prevent tumor formation, and is the most widely prescribed drug for treatment and prevention of hormone-dependent breast cancer [104]. Tamoxifen also antagonizes the effect of estrogen in the brain while agonizes estrogen's action in the bone, uterus and cardiovascular system. In the ovary, the role of tamoxifen is far less understood due to cellular complexity of the ovary. In premenopausal women, tamoxifen has been suggested to promote abnormal ovarian function and cyst formation but its effect on ovarian cancer risk is still unclear [32,105].

Tamoxifen and other SERMs have a complex and only partially understood mechanism of tissue specific activation or inhibition of estrogen signaling [106]. This tissue specificity of SERMs is dependent upon 1) levels of endogenous estrogens, 2) local concentrations of ER α and β and their ratio, 3) estrogen receptor polymorphisms, 4) availability of ER coactivators (for estrogenic actions) or corepressors (for anti-estrogenic actions), and 5) activation of classical (or genomic) pathways or non-classical (dependent upon protein-protein interactions between ER complex and AP-1) pathways [106]. The impact of SERMs on ovarian estrogen signaling is particularly complex since any direct ovarian action is coupled with altered gonadotropin stimuli and estrogen feedback system in premenopausal females

[107]. In cycling rats, SERMs increases gonadotropin secretions due to loss of hypothalamic estrogen feedback signaling and elevates local estrogen production in the ovary [108].

A single study examined the effect of prednisone or prednisone + tamoxifen in addition to standard chemotherapy on reproductive function in premenopausal breast cancer patients [109]. While, this study did not investigate the effect of tamoxifen on ovarian cancer risk, it showed that interestingly, the addition of tamoxifen was associated with a delayed amenorrhea; this is an interesting observation considering that amenorrhea is a common side effect of standard chemotherapy.

Fertility in Female Cancer Patients:

More than 100,000 women under the age of 45 are diagnosed with cancer each year in the United States. Standard cancer chemotherapy and radiation therapy often cause immediate or eventual premature menopause and infertility [110,111]. Incidence of ovarian failure following cancer treatment varies widely with age and chemotherapy regimen, with advancing age and dose intensity and regimens including alkylating agents, particularly cyclophosphamide, predisposing to ovarian damage and infertility [112,113]. On average, 50-80% of patients receiving chemotherapy experience prolonged or permanent amenorrhea post-treatment [112,114]. Previously, this infertility and iatrogenic menopause was viewed by physicians and patients as an acceptable cost of curative chemotherapy and radiation regimens. However, with early diagnosis, higher survival rate [115], and increased age for child bearing [116],

cancer therapy-induced infertility has become a major survivorship issue. The impact of cancer chemotherapy on reproductive health and fertility has only recently been appreciated and strategies to prevent such reproductive toxicity are urgently needed.

Current options for fertility preservation:

Embryo cryopreservation prior to cancer treatment is currently the only approved option for these patients, however, this technique has limitations. Cryopreservation of embryos does not preserve ovarian function, requires several weeks to months, must be completed prior to cancer therapy, is applicable only to patients with a current partner and often fails due to poor embryo quality [117]. Experimental options for fertility preservation include ovarian or oocytic cryopreservation and GnRH agonist therapy [118,119]. Oocyte freezing also delays treatment and has little success to date due to fragility of the oocytic meiotic spindle and formation of ice crystals during freezing [117,120]. Ovarian tissue cryopreservation is surgically invasive, carries risk for possible cancer cell transmission, and causes ischemic damage to the ovary leading to poor oocyte viability [120]. Hypothalamic suppression with GnRH agonists is used in an effort to decrease the ovarian toxicity from cancer therapy by inducing a putatively protective hypogonadotropic hypogonadism [121]. GnRH agonist was shown to decrease cyclophosphamide-induced ovarian toxicity in non-human primates [122]. Blumenfeld and colleagues have documented chemoprotective effects of GnRH suppression against infertility in women following chemotherapy for lupus and lymphoma [123,124]. However, other

groups have not observed benefit from this controversial protocol [125]. In addition, treatment with GnRH agonists does not protect the ovary from radiation-induced ovotoxicity [126] and the interaction of GnRH modulation with chemotherapy in cancer cells is unknown. Additional fertility preservation options for cancer patients are still needed.

Animal models for the study of chemical-induced ovarian toxicity:

Two of the most extensively studied ovotoxic agents in animal models are DMBA and cyclophosphamide. DMBA, a polycyclic aromatic hydrocarbon, is commonly used in rodent models to induce a variety of cancers [64,127]. In addition to being a carcinogen, DMBA also destroys follicular reserve and causes ovarian failure [128,129]. While the mechanism by which DMBA targets and destroys follicles in the ovary is not entirely understood, many investigators have suggested that apoptosis plays a critical role [129,130]. Cyclophosphamide, also known as Cytoxan, is an alkylating agent that is commonly used as a part of standard regimen therapy for many malignancies including lymphomas, leukemia, and solid tumors [131,132]. Cyclophosphamide is a prodrug that is converted in the liver into active metabolites, mainly 4-hydroxycyclophosphamide, which is further converted into its ultimate toxicant, phosphoramide mustard, that forms adduct with DNA leading to cell death [133]. Cyclophosphamide is considered to be the most ovotoxic cancer drug resulting in a high rate of ovarian failure in cancer patients [110,134]. In rodents, cyclophosphamide causes gonadal damage and sterility in a dose-, duration-, species-,

and strain- dependent manner [135,136]. Doxorubicin, also known as Adriamycin, intercalates with DNA and inhibits helicases and topoisomerases and is another frequently studied chemotherapeutic agent known for its ovotoxicity [137]. Similar to DMBA-induced ovarian toxicity, cyclophosphamide and doxorubicin have been shown to target the ovary directly and cause atresia [138]. However, while some studies have shown that DMBA and doxorubicin delete ovarian follicles by elevating Bax protein and apoptosis in oocytes [129,137]; others have suggested that cyclophosphamide-induced follicle depletion is driven by apoptosis in oocyte supporting cells [138].

Specific aims of this work:

Women at high risk for breast cancer are often also at high risk for ovarian cancer [3], reflecting similar risk factors and suggesting intertwined disease pathways and common prevention targets [4]. A novel strategy to overcome obstacles in preventing ovarian cancer, the deadliest gynecologic malignancy, may be to develop a strategy that will simultaneously decrease risks for both breast and ovarian cancer. Very few compounds have been tested for dual action against both cancers due in part to the lack of appropriate animal models that develop both diseases. Tamoxifen, a SERM, reduces breast cancer risk by almost 50% but its effects on ovarian cancer risk are still unclear. In testing tamoxifen for ovarian cancer prevention, we found that tamoxifen inhibits ovotoxicant-induced follicle loss in the ovary. Chemotherapy and environmental toxicants such as DMBA deplete ovarian follicles leading to

accelerated ovarian aging and premature ovarian failure. To date, there is no established method that protects the ovary from the ovotoxic effect of environmental toxicants and chemotherapy. **Objectives** of this study were to 1) develop and characterize a preclinical model that exhibits concurrent mammary and ovarian cancer, 2) test the effect of tamoxifen on the progression towards mammary and ovarian cancer using the dual cancer model, and 3) investigate the protective effect of tamoxifen against ovotoxic agents including DMBA and chemotherapy. These objectives were investigated through the following aims:

Aim 1: Develop and characterize a rat model of simultaneous breast and ovarian cancer suitable for dual cancer prevention studies

In this aim, three established mammary cancer carcinogen models (DMBA, MNU and E_2) were combined with local ovarian DMBA administration to induce progression to mammary and ovarian cancer concurrently in the rat. Tissue histology, degree of dysplasia, cellular proliferation status (Ki-67), hormone receptor levels (ER) and COX-2 expression were examined to validate this dual cancer model.

Aim 2: Determine the effect of tamoxifen on ovarian cancer using a rat model of simultaneous breast and ovarian cancer progression

Tamoxifen is a proven prevention drug for ER+ mammary cancer, but its effect on ovarian cancer is not clear. In this aim, we evaluated the possibility of tamoxifen as a dual-target prevention agent using the dual cancer model. End point biomarkers

including dysplasia score, Ki-67, ER, COX-2 and putative stem cell markers (Oct-4 and ALDH-1) were examined.

Aim 3: Investigate the protective effect of tamoxifen against ovotoxic agents including DMBA and chemotherapy drugs

In vivo and *in vitro* studies were conducted to study the effect of tamoxifen against ovarian follicle loss caused by DMBA and chemotherapy agents including cyclophosphamide and doxorubicin. *In vivo*, female Sprague Dawley rats were treated with tamoxifen and DMBA or cyclophosphamide. The effect of tamoxifen on rescuing ovarian follicles from DMBA or cyclophosphamide was examined by determining the number of follicles in the ovary. *In vitro*, ovarian organ culture was performed to investigate local effects of tamoxifen on follicle numbers of DMBA-treated ovaries. Also, effects of tamoxifen on oocyte directly were examined by culturing in the presence of doxorubicin.

The **long term goals** of this work are to 1) use the dual cancer model to screen for promising agents that decrease risks for both breast and ovarian cancer and 2) examine the mechanism by which tamoxifen inhibits toxicant-induced ovarian follicle loss.

Chapter II

Characterization of a Preclinical Model of Simultaneous Breast and Ovarian Cancer Progression

This work has been published: Alison Y. Ting, Bruce F. Kimler, Carol J. Fabian and Brian K. Petroff. Carcinogenesis 2007; 28(1):130-5.

Abstract

Women at increased risk for breast cancer are often also at increased risk for ovarian cancer, reflecting common risk factors and intertwined etiologies for both diseases. Unlike breast cancer prevention, primary ovarian cancer prevention has been impractical due to the low incidence, lack of risk and response biomarkers and difficulties in sampling ovarian tissue. Challenges in the development of ovarian cancer prevention drugs, however, may be circumvented through the development of breast cancer prevention strategies that simultaneously decrease ovarian cancer. In the present study, three commonly used mammary cancer carcinogen models [7,12-dimethylbenz[α]anthracene (DMBA), *N*-methyl-*N*-nitrosourea (MNU) and estradiol (E_2)] were combined with local ovarian DMBA administration to induce progression to mammary and ovarian cancer concurrently in the rat. Animals were treated for three or six months and tissue histology as well as proliferation, hormonal and inflammation biomarkers were assessed. Mammary and ovarian morphologies (measured as descriptive histology and dysplasia scores) were normal in vehicle controls. Mammary hyperplasia was observed in DMBA/DMBA (mammary carcinogen/ ovarian carcinogen) and MNU/DMBA -treated rats; however, ovarian preneoplastic changes were seldom observed after these treatments. All E_2/DMBA-treated rats had mammary hyperplasia, atypia, ductal carcinoma in situ and/or invasive adenocarcinoma, while 50% also developed preneoplastic changes in the ovary (ovarian epithelial and stromal hyperplasia and inclusion cyst formation). In both the mammary gland and ovary, decreased estrogen receptor alpha expression

was detected and in the mammary gland elevated Ki-67 and cyclooxygenase-2 expressions were observed. This combined breast and ovarian cancer rat model (systemic E_2 treatment and local ovarian DMBA) may be useful for future dual target breast and ovarian cancer prevention studies.

Introduction

Breast cancer chemoprevention trials are facilitated by minimally invasive techniques to sample breast tissue and the availability of a number of surrogate biomarkers to evaluate prevention drugs in phase II trials as well as the high incidence of this disease in phase III trials [28,29,139]. In contrast, sampling of ovarian tissue is invasive and appropriate biomarkers for ovarian cancer prevention trials are controversial [2]. Additionally, ovarian cancer is a relatively uncommon disease making testing of drugs for primary prevention difficult to justify. One possible solution is to develop breast cancer chemoprevention drugs that simultaneously prevent ovarian cancer. Indeed, successful human ovarian cancer chemoprevention has only been demonstrated incidentally during the course of breast cancer prevention trials (i.e. fenretinide) [47]. An initial obstacle to the development of dual target breast and ovarian cancer prevention drugs is the absence of an appropriate animal model.

Rats are the most frequently used animal model for breast cancer chemoprevention, particularly chemical [7, 12-dimethylbenzanthracene (DMBA) and N-methyl-N-nitrosourea (MNU)] or hormonal [17β-estradiol (E_2)] carcinogen models

[60,62,140]. In intact females, these carcinogens induce a high incidence of mammary adenocarcinoma that express similar histology and biomarker expressions to the human disease within 2-5 months [60,62,140]. No commonly used preclinical model of ovarian cancer has been used to test prevention drugs but one promising method of inducing epithelial ovarian cancer is the direct application of DMBA to the ovarian surface epithelium. This local ovarian DMBA treatment results in an approximate 30-40% occurrence of epithelial ovarian cancer (EOC) and a greater incidence of preneoplastic changes in the ovary [64-66]. In this study, these breast and ovarian cancer models were tested in combination in an effort to develop the first preclinical model of simultaneous breast and ovarian cancer progression.

Materials and methods

Animals and treatments

Female Fischer 344 rats (Harlan Breeding Laboratories, Indianapolis, IN, n=6-8 per treatment*time group) weighing 50-55 g were housed three per cage in a climate- and light- (12L:12D) controlled environment, and received food and water ad libitum. All experimental protocols were approved by the University of Kansas Medical Center Animal Care and Use Committee. Within a week of arrival, rats were anesthetized using ketamine hydrochloride (80 mg/kg), atropine sulfate (0.2 mg/kg) and xylazine (8 mg/kg). Hemiovariectomy was performed aseptically in order to concentrate ovulation upon the treated ovary and hasten a senescent hormonal milieu [141,142]. In addition to increasing ovulation rate on the remaining ovary, Anzalone and

colleagues have shown that hemiovariectomy mimics age-related alterations such as a lower incidence of regular cyclicity altered magnitude of the proestrus LH surge as well as reduced ovarian follicular reserve when compared to age-matched intact rats. The remaining ovary was treated by passing a DMBA-impregnated (2.5mm region dipped in melted DMBA) or vehicle 5-0 silk suture through the ovary twice such that the DMBA or vehicle region was apposed directly and gently secured to the ovarian surface epithelium. Local ovarian DMBA application ultimately results in a 30-50% incidence of ovarian cancer arising from the surface epithelium within 12 months using this model [64-66]. Rats receiving ovarian DMBA were subsequently treated with mammary carcinogens: DMBA (10 mg/kg, p.o.), MNU (50 mg/kg, i.p.), or 17β-estradiol (E_2, 3.0 mg, pellet implant; Hormone Pellet Press, Leawood, KS). Rats (n=12) receiving vehicle-coated sutures were further treated with vehicle (corn oil; 4ml/kg, p.o.; blank implants s.c.).

Tissue Preparation

Rats were sacrificed at 3 or 6 months post-treatment, serum collected and stored at -80ºC. Right thoracic mammary glands were excised, fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Right abdominal-inguinal mammary glands were spread out onto a glass slide, fixed in 4% PFA and infused with alum carmine following a whole mount preparation protocol [143]. The ovary was bisected through the site of DMBA application. One half was fixed in 4% PFA and embedded in paraffin while the remainder was snap frozen for future study.

Immunohistochemistry

Six-micron sections of mammary glands and ovaries were deparaffinized, rehydrated, and stained with hematoxylin & eosin (H&E). H&E sections were evaluated for premalignant morphological changes associated with mammary adenocarcinoma (MAC) and epithelial ovarian cancer (EOC) progression [65,144] by an observer blinded to treatment groups. Additional sections were prepared for immunostaining by antigen retrieval (99.9^{o}C, 10mM citrate buffer, 20 minutes) and incubation with 0.3% hydrogen peroxide (Lab Vision, Fremont, CA). Non-immune serum or primary antibodies against estrogen receptor alpha (ER; 1;100; Clone SP1; rabbit monoclonal antibody; Lab Vision, Fremont, CA), cyclooxygenase-2 (COX-2; 1:50; RB-9072; rabbit polyclonal antibody; Lab Vision, Fremont, CA) and Ki-67 (1:25; Clone Ki-S5; mouse monoclonal antibody; Dako, Carpinteria, CA) were applied and visualized with DAB chromogen and biotinylated secondary antibodies. All incubations were carried using a Dako LV-1 autostainer (Carpinteria, CA).

Hormone assays

Serum concentrations of E_2 were determined by ELISA kit according to manufacturer's protocol (DSL-10-4300, Diagnostic Systems Laboratories, Webster, TX). All samples were run within the same assay and the intra-assay CV was <10%.

Quantitative Analysis of Preneoplastic Lesions

H&E sections of the mammary gland and ovary were evaluated for pre-neoplastic and neoplastic morphological changes associated with breast and epithelial ovarian cancer progression [65,144]. Mammary tissue was evaluated for preneoplastic changes including mild or severe ductal hyperplasia and hyperplasia with atypia as well as neoplastic changes such as ductal carcinoma in situ (DCIS) and invasive DC, corresponding to scores of 1-5, respectively. A score of 0 was given to animals with normal mammary histology. Pre-neoplastic changes of the ovary were defined as surface (bursal flat) hyperplasia, inclusion cysts, stromal hyperplasia and papilloma. Scores of 0, 1 and 2 were given to each section according to the severity or the prevalence of each pre-neoplastic category (a score of 0 represent an absence of preneoplastic changes and a score of 2 indicates a high degree of abnormality). Sections from 3 different levels of the ovary from each animal were evaluated. These preneoplastic criteria are the same as those used by Stewart and colleagues with this rat model of ovarian carcinogenesis [65].

Ki-67 and ER expression in the mammary ductal epithelia cells and ovarian surface epithelia and COX-2 expression in ovarian epithelia were quantified by counting immunoreactive epithelial cells and total epithelial cells (at least 1000 cells were evaluated per section). The intensity of COX-2 immunostaining in the mammary gland was quantified with an automated cellular imaging system (ChromaVision, San Juan Capistrano, CA) [77]. Three random fields of each tissue section were selected for quantification (combined area evaluated = $1.56mm^2$), and

the staining intensity was expressed as percent area occupied by COX-2-immunoreactive cells.

All continuous values are presented as the mean ± SEM and their statistical comparisons were made using two-way analysis of variance. Differences were considered significant when $P \leq 0.05$.

Results

Histopathology

Mammary gland whole mounts: In the mammary gland, DMBA, MNU and E_2 treatment increased ductal branching and area occupied by alveoli by 3 months and the impact of E_2 was the most extensive among these three carcinogens. These effects were further increased after 6 months of treatment (Figure 1B-D). Vehicle-treated rats (3 and 6 month) showed normal mammary morphology (Figure 1A).

Mammary histology: Vehicle-treated (3 and 6 month) and systemic DMBA-treated (3 month) rats displayed normal histology showing scattered acini throughout the mammary gland each bearing a single layer of ductal epithelial cells surrounded by myoepithelial cells (Figure 1E). Increased dysplasia scores were observed in carcinogen-treated animals (Table I). Ductal hyperplasia was observed in some DMBA/DMBA- and MNU/DMBA -treated rats (3 and 6 month) and in all 3-month E_2/DMBA-treated rats (Figure 1F and G). Six months of systemic E_2 induced ductal hyperplasia (2/6), ductal carcinoma in situ (DCIS, 3/6), and invasive adenocarcinoma (1/6) (Figure 1H).

Ovarian histology: Local ovarian DMBA application caused increased ovarian dysplasia and both benign abnormalities such as local inflammation around suture materials, mild stromal hyperplasia and decreased follicle numbers (Figure 1J and K and Table I). Combined systemic E_2 and ovarian DMBA treatment further induced ovarian preneoplastic changes of epithelial origin in 50% rats (i.e. epithelial hyperplasia and inclusion cyst) following 6 month treatment (Figure 1L and Table I). Vehicle-treated rats showed normal ovarian histology with mild inflammation induced by suture materials (Figure 1I).

Epithelial proliferation was stimulated by E_2 treatment

Ki-67 expression was localized in the nucleus of ductal epithelial cells in the mammary gland and surface epithelial cells in the ovary. Following 6 month treatment, Ki-67-immunoreactive cells were increased in E_2/DMBA mammary glands when compared to controls and other carcinogen-treated rats (Figure 2A-C; $P \leq 0.05$). Ovarian epithelial staining for Ki-67 increased significantly from 3 to 6 months in rats treated with E_2/DMBA ($P \leq 0.05$) but did not differ significantly from controls and other carcinogen-treated rats.

Mammary COX-2 expression increased in E_2/DMBA -treated rats

COX-2 expression was elevated in the mammary gland of E_2/DMBA-treated rats when compared to controls and other carcinogen-treated rats (3 month and 6 month) (Figure 2D-E; $P \leq 0.05$). Although COX-2 expression in the ovarian surface epithelia

did not increase after carcinogen treatments, preneoplastic changes of epithelial origin in the ovary of E_2/DMBA-treated rats showed strong immunoreactivity to COX-2 (Figure 3).

ER expression decreased with carcinogen treatment

ER immunoreactivity in the mammary gland was decreased after 6 month systemic DMBA, MNU and E_2 treatment (Figure 4A-C; $P \leq 0.05$). Ovarian DMBA similarly decreased the ER expression in the ovarian surface epithelium by 6 months of treatment when compared to controls (Figure 4D-F; $P \leq 0.05$).

Serum estradiol concentrations

The sustained release E_2 treatment provided a consistent and sustained increase in serum E_2 when compared to DMBA-, MNU-, or vehicle- treatment (Table I; $P \leq 0.05$). E_2-related side effects including pituitary and uterine hyperplasia were observed in two animals. Although ovarian granulosal proliferation was observed (in addition to epithelial and stromal ovarian hyperplasia) in all E_2/DMBA treated rats, it was not considered a preneoplastic lesion for epithelial ovarian cancer when determining dysplasia scores.

Discussion

Breast and ovarian cancer have interdependent risk factors and women at increased risk for one of these cancers are often at risk for the other [145,146]. While active

clinical trials are common for the evaluation of breast cancer prevention drugs, ovarian cancer prevention trials are seldom attempted due to low incidence of the disease, relatively invasive procedures for tissue sampling and the lack of well-established serum or imaging-based biomarkers [2]. A logical approach to ovarian cancer chemoprevention may be the development of breast cancer prevention drugs that simultaneously decrease the risk of ovarian cancer. Towards this end, the present study is intended to produce a preclinical model for the evaluation of simultaneous chemoprevention of breast and ovarian cancer.

Systemic E_2 and local ovarian DMBA induced preneoplastic changes in breast and ovary of the rat as demonstrated by elevated Ki-67 and COX-2 expression in addition to histological analysis. Unlike systemic DMBA and MNU, systemic E_2 appeared to contribute not only to mammary carcinogenesis but also to the progression towards ovarian neoplasia. This additive or synergistic effect of E_2 merits further exploration. One possible explanation is that the proliferative effect of E_2 may increase the mutation rate of ovarian surface epithelial cells [147] and therefore accelerate the incidence of ovarian preneoplastic changes. Similarly, Stewart and colleagues reported that when combined with gonadotropin hormones, local ovarian DMBA induced more ovarian preneoplastic lesions compared to DMBA treatment alone [65].

In the present study, putative ovarian preneoplastic changes such as inclusion cysts, epithelial hyperplasia, papilloma, and stromal hyperplasia were used to evaluate progression towards ovarian cancer instead of actual cancer incidences.

These criteria are the same as those of Stewart et al. in DMBA-induced ovarian adenocarcinoma of the rat [65]. While the presence of inclusion cysts in older women is common [148] and controversy concerning whether inclusion cysts are a preneoplastic lesion remains, many groups agree that ovarian inclusion cysts are a precursor for ovarian adenocarcinoma [149,150]. Studies have shown that the number of inclusion cysts is increased in ovaries from patients with ovarian carcinoma, contralateral epithelial ovarian tumors, or a family history of ovarian cancer compared to healthy subjects [151-154].

Elevated COX-2 expression has been observed in several tumors including ovarian neoplastic lesion [49,50,73,81,155]; however, we found that overall ovarian epithelial COX-2 expression was not altered by E_2/DMBA treatment while COX-2 was highly expressed by ovarian inclusion cysts. Recent studies have also revealed the relevance of COX-1 expression in ovarian tumors development [51,52,66] suggesting another target for ovarian chemoprevention and its role should be investigated using this combined breast and ovarian cancer model.

Although this study was intended primarily to develop a practical model for the evaluation of dual target chemoprevention drugs against breast and ovarian cancer, our results also emphasize the interaction of the etiologies of ovarian and mammary cancer. Previous studies showed that latencies of breast tumor formation induced by MNU, DMBA and E_2 in the rat are approximately 3, 4 and 6 months, respectively [156-159]. However, systemic MNU and DMBA only caused precancerous mammary changes in the current experiment after 6 months of

treatment. This is likely due to hemiovariectomy since the removal of both ovaries completely abolishes the ability of mammary carcinogens to induce mammary tumors [160]. The intention of hemiovariectomy was to concentrate ovulation which is a risk factor for human ovarian cancer on the remaining treated ovary [161,162]. Approximately 50% of E_2/DMBA-treated rats developed preneoplastic changes in the ovary. One way to further increase ovarian cancer progression might be to prolong the treatment (rats develop epithelial ovarian cancer from ovarian DMBA at 10-12 months [66]), but this is difficult due to advanced mammary tumor formation by 6 months of treatment. Additionally, our intention was to parallel the approach of human cancer prevention trials which focus on reversible or preventable preneoplasia and associated biomarkers rather than actual cancer incidence [29,68].

In the present study, we have demonstrated that rats treated with systemic E_2 and local ovarian DMBA develop preneoplastic and neoplastic changes in the breast and ovary simultaneously. This model benefits from apparent additive or synergistic effects of E_2 and DMBA in early ovarian carcinogenesis unlike models addressing breast or ovarian cancer separately. This approach is intended to facilitate the identification of promising cancer prevention drugs that simultaneously decrease progression to breast and ovarian cancer (e.g. postmenopausal SERMs or retinoids [47]) or reveal drugs that might incidentally decrease the incidence of one cancer while predisposing to the other (e.g. progesterone [45,46,163,164]). While all women would potentially benefit from a well tolerated chemoprevention drug against breast and ovarian cancer, a more conservative estimate of benefit in the United

States might be the approximately 10% of the female population considered at elevated risk for these diseases.

Table I. Mammary and ovarian dysplasia scores and serum E_2 concentrations

Treatment	Mammary Gland		Ovaries		E_2 Serum Levels (pg/ml)	
	3.0 Months	6.0 Months	3.0 Months	6.0 Months	3.0 Months	6.0 Months
Vehicle Control	0	0	0.8±0.5	0.8±0.1	60.98±5.98	50.2±8.1
DMBA/ DMBA	0	1.0±0.8*	3.6±0.7*	4.4±1.2*	42.66±1.53	45.0±5.2
NMU/ DMBA	0. 7±0.2*	0.8±0.2*	3.4±0.5*	4.0±0.8*	43.5±3.9	46.5±4.0
E_2/ DMBA	2.4±0.7**	3.5±0.5**	7.4±0.3**	9.5±0.9***	133.6±21.8*	104.0±43.5*

Statistical comparisons were made using two-way analysis of variance. Dysplasia scores are present as mean ± SEM for each treatment group. Histology sections of the mammary gland from each animal were given a score from 0-5, representing normal histology, mild/severe ductal hyperplasia, hyperplasia with atypia, ductal carcinoma in situ, and invasive carcinoma, respectively. Ovarian pre-neoplastic changes include ovarian surface hyperplasia, inclusion cysts, stromal hyperplasia and papilloma. Scores of 0, 1 and 2 was given to each section (3 sections per animal) according to the severity or the prevalence of each change. Three scores from different levels of the ovary were summed to give a dysplasia score for each animal.

* differs significantly to controls ($P < 0.05$).

** differs significantly to controls and * ($P < 0.05$).

*** differs significantly to controls, * and ** ($P < 0.05$).

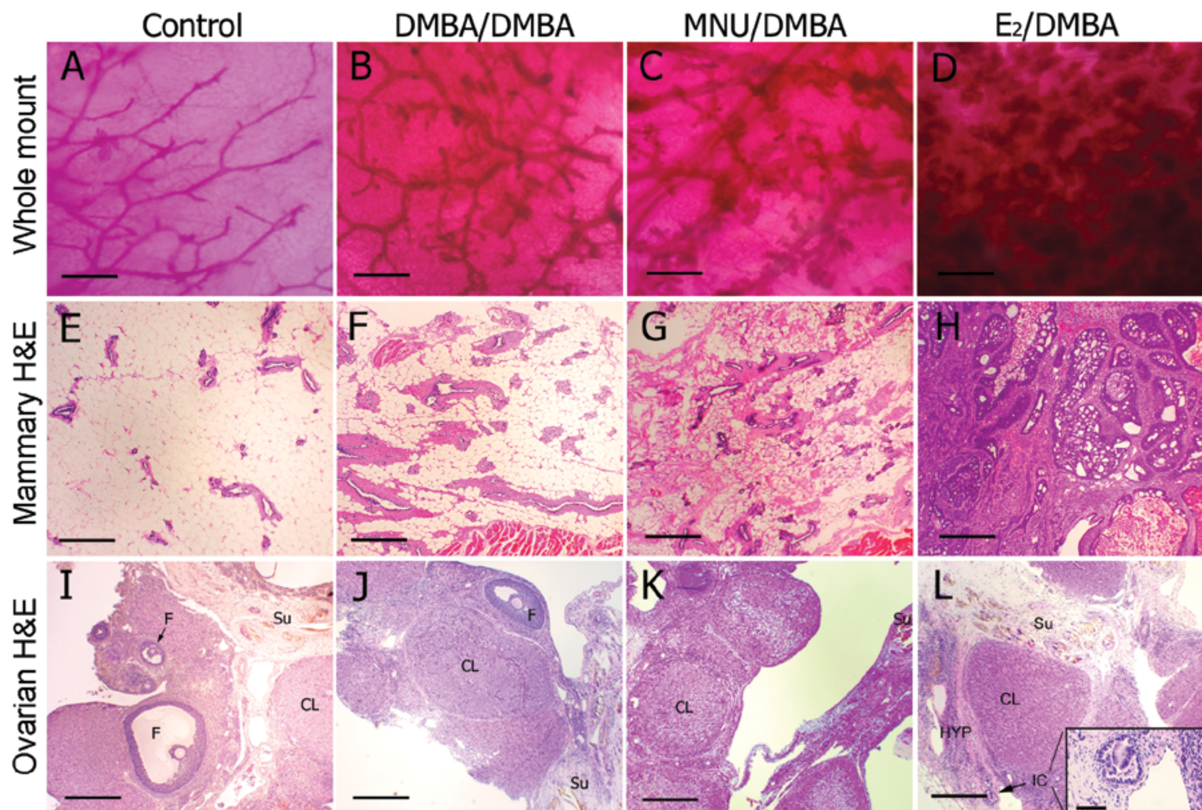


Fig. 1. Whole mounts (A-D) and mammary (E-H) and ovarian histology (I-L). DMBA/DMBA (ovarian/systemic)-treated rats (B, F and J) and MNU/DMBA-treated rats (C, G and K) showed increased ductal branching in whole mounts (B and C), increased alveoli in H&E sections (F and G), and absence of preneoplastic changes in the ovary (J and K). All E_2/DMBA-treated rats developed mammary preneoplastic or neoplastic changes (D and H) and 50% of them also showed ovarian preneoplastic changes (L; insert: magnified image of an inclusion cyst). Scale bars = 1 mm (A-D), 200 µm (E-L) and 50 µm (L insert). Abbreviation: F = ovarian follicle, CL = corpus luteum, Su = suture material, HYP = epithelial hyperplasia, IC = inclusion cyst.

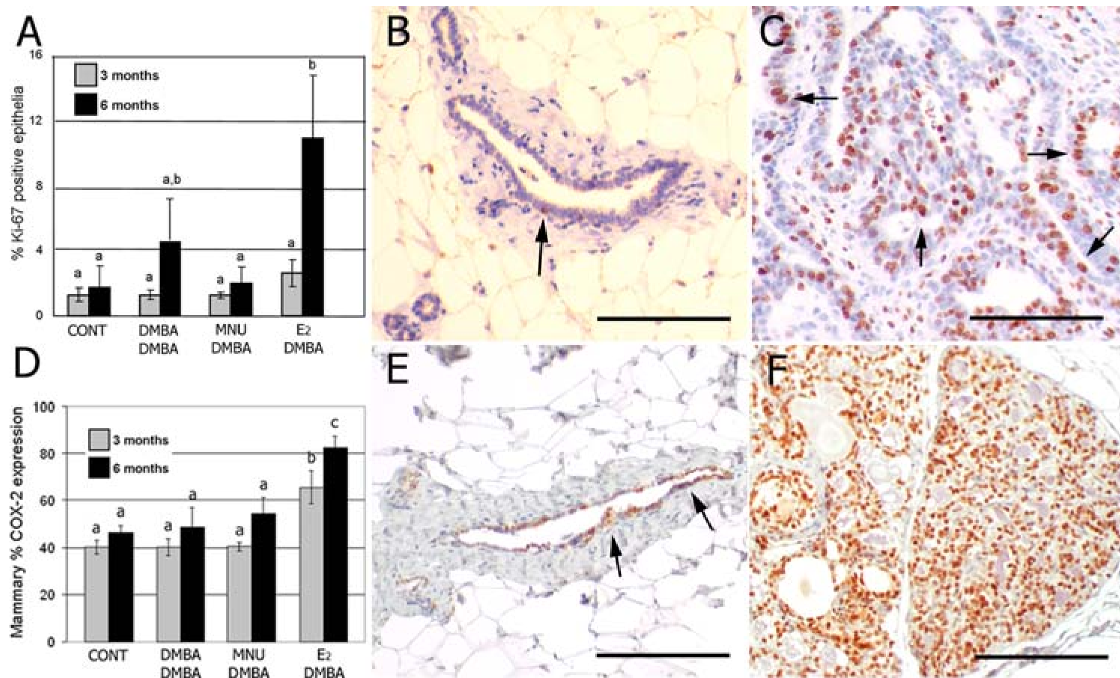


Fig. 2. Mammary epithelial proliferation (Ki-67) was increased above controls at 6 months in E_2/DMBA-treated group (A and C) when compared to controls (B; $P \leq 0.05$). Percentage COX-2 expression in mammary gland also increased in E_2/DMBA-treated groups (F) as compared to controls (E) and other carcinogen-treated groups following 3 month and 6 month treatment (D; $P \leq 0.05$). Arrows indicate positively stained mammary epithelial cells. Scale bars = 100 µm. For A and D, different letters indicates significant difference among different treatment groups.

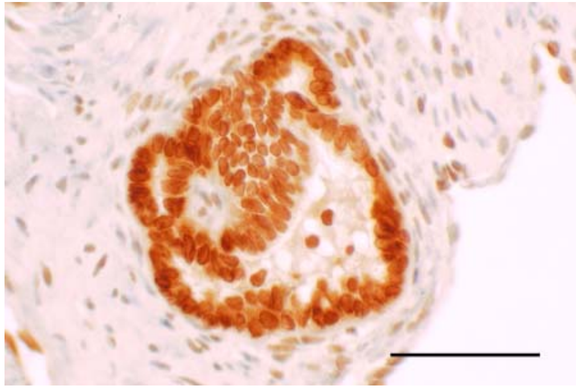


Fig. 3. Inclusion cysts found in the ovary of E_2/DMBA-treatd ovary (adjacent section of Fig. 1L insert) showed positive COX-2 expression. Scale bar = 50 μm. Non-immune control not shown.

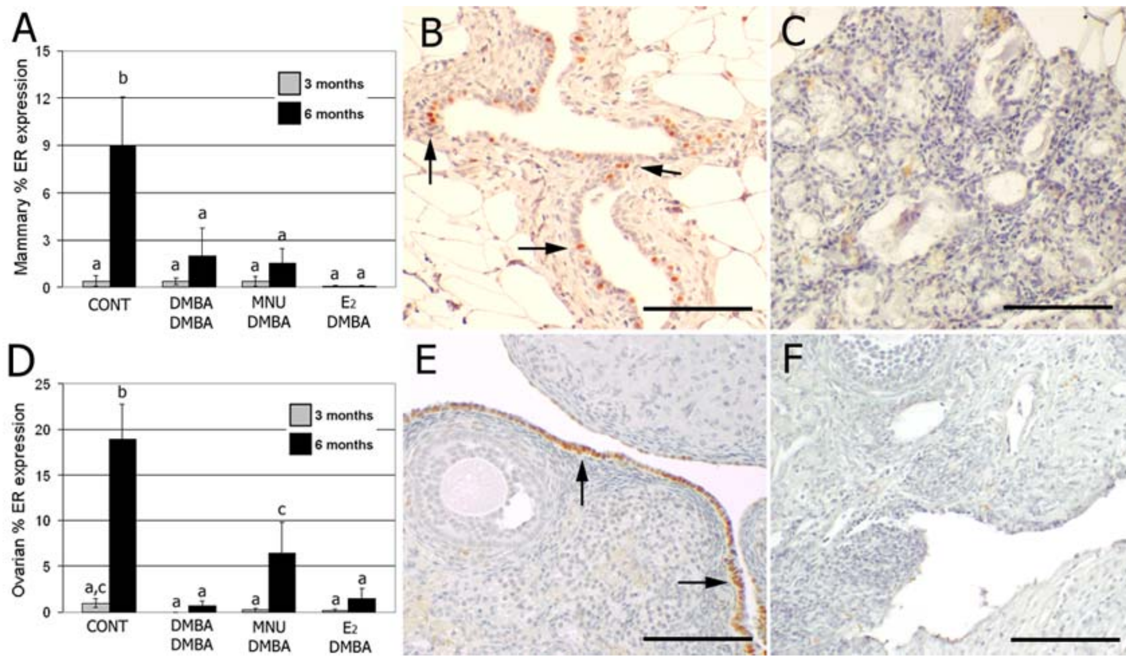


Fig. 4. ER expression was downregulated in the mammary gland (A-C) and ovary (D-F) of carcinogen-treated rats when compared to controls after 6 month treatment ($P \leq 0.05$). Arrows indicate positively stained mammary epithelial cells. Scale bars = 100 μm. For A and D, different letters indicates significant difference among different treatment groups.

Chapter III

Tamoxifen prevents premalignant changes of breast but not ovarian cancer in rats at high risk for both diseases

This work has been published: Alison Y. Ting, Bruce F. Kimler, Carol J. Fabian and Brian K. Petroff. Cancer Prevention Research. 2008; 1(7): p. 546-553

Abstract

Women at increased risk for breast cancer are at increased risk for ovarian cancer as well, reflecting common risk factors and intertwined etiology of the two diseases. We previously developed a rat model of elevated breast and ovarian cancer risk, allowing evaluation of dual target cancer prevention strategies. Tamoxifen, a FDA-approved breast cancer chemoprevention drug, has been shown to promote ovarian cysts in premenopausal women; however, the effect of tamoxifen on ovarian cancer risk is still controversial. In the current experiment, Fischer 344 rats (n=8 per treatment group) received tamoxifen (TAM) or vehicle (CONT) in factorial combination with combined breast and ovarian carcinogen (17β-estradiol and 7, 12 dimethylbenza[α]anthracene, respectively). Mammary and ovarian morphologies were normal in CONT and TAM groups. Carcinogen (CARC) treatment induced mammary dysplasia with elevated cell proliferation and reduced estrogen receptor alpha expression and promoted preneoplastic changes in the ovary. In CARC+TAM-treated group, tamoxifen reduced preneoplastic changes and proliferation rate in the mammary gland but not in the ovary compared to rats treated with carcinogen alone. Putative stem cell markers [Oct-4 and aldehyde dehydrogenase-1 (ALDH-1)] were also elevated in the mammary tissue by carcinogen and this expansion of the stem cell population was not reversed by tamoxifen. Our study suggests that tamoxifen prevents early progression to mammary cancer but has no effect on ovarian cancer progression in this rat model.

Introduction

The development of promising breast cancer chemoprevention agents (i.e. selective estrogen receptor modulators (SERMs), aromatase inhibitors and retinoids [31,48,165]) has been permitted by minimally invasive techniques to access tissue, availability of surrogate biomarkers and relatively high incidence of the disease [25,166]. In contrast, ovarian cancer prevention trials are seldom attempted due to low disease incidence, the absence of accepted disease-specific biomarkers and the invasiveness of sampling for ovarian tissue. Consequently, although most ovarian cancers are diagnosed at advanced stages resulting in high mortality rates, prevention of ovarian cancer remains elusive [2]. One practical approach for successful prevention of ovarian cancer may be the development of chemoprevention agents acting simultaneously against both ovarian and breast cancer.

Breast and ovarian adenocarcinoma share numerous risk factors (e.g. estrogen exposure, ovulation, nulliparity, obesity, family history, BRCA1/2 mutations) and women at increased risk for one of these cancers are often also at risk for the other suggesting intertwined disease pathways [4,59]. Recent studies have shown that women receiving hormone replacement therapy are at increased risk for both cancers [167-169]. Alternatively, drugs that decrease ovarian cancer risk may actually increase the incidence of breast cancer (e.g. progesterone [38,170]). To date, no human chemoprevention trials have been designed simultaneously targeting both breast and ovarian cancers despite the promise of such an approach. Indeed, successful human ovarian cancer chemoprevention has only been demonstrated

incidentally during the course of breast cancer prevention trials (i.e. fenretinide) [47]. To investigate common chemoprevention strategies, our laboratory has developed a preclinical model that exhibits early changes of mammary and ovarian carcinogenesis in the rat [171]. This model allows observation of synergistic and antagonistic drug actions against breast and ovarian cancers that are ignored when each cancer is examined in isolation.

Tamoxifen, the most commonly used breast cancer chemoprevention drug, blocks cell proliferation in the breast and has been shown to cause tumor regression and inhibit tumor formation, especially in ER+ breast tumors [104]. In the ovary, especially in premenopausal women, tamoxifen has been suggested to promote abnormal ovarian function and cyst formation, a putative ovarian preneoplastic change [32,105]. Tamoxifen and other SERMs have also been used to stimulate ovarian function in subfertile women with some question as to impact on ovarian cancer risk [172,173].

Tamoxifen prevents 70% of ER+ breast cancers in high risk women, but fails to prevent ER- and some ER+ tumors [165]. One possibility for the lack of tamoxifen efficacy on 30% of ER+ cancers may be the presence of an E_2-independent breast stem cell population [174]. The existence of self-renewing, pluripotent stem cells have been demonstrated both in human breast and rodent mammary glands [88,89]. Following recurrent carcinogen exposure, these long-lived breast stem cells are thought to accumulate mutations leading to tumor formation. The size of the breast stem cell pool has therefore been hypothesized to serve as a determinant of the

likelihood for breast cancer incidence. Indeed, several studies have suggested a strong correlation between increased number of breast stem cells and elevated breast cancer risk as well as a possible intervention that targets stem cell for cancer treatment and prevention [175-178]. In the current study, we use a combined breast and ovarian cancer model to examine the effect of tamoxifen on markers of cancer risk, stemness and progression in the ovary and mammary gland during carcinogenesis.

Materials and methods

Animals and treatments

Female Fischer 344 rats (Harlan, Indianapolis, IN, n = 8 per treatment group) weighing 50-55 g were housed in a climate and light (12L:12D) controlled environment and received food and water *ad libitum*. All experimental protocols were approved by the University of Kansas Medical Center Animal Care and Use Committee. Animals were randomly assigned into 4 different treatment groups as shown in Table 1. Rats were anesthetized using ketamine hydrochloride and xylazine (80 and 8 mg/kg, respectively). Hemiovariectomy was performed aseptically to concentrate ovulation upon the remaining ovary and hasten a senescent hormonal milieu [141,142] as these are risk factors of human ovarian cancer [171,179,180]. The remaining ovary was treated by passing a 7, 12 dimethylbenza[α]anthracene (DMBA)-impregnated (2.5 mm region dipped in melted DMBA) or vehicle 5-0 silk suture through the ovary twice such that the DMBA or vehicle region was apposed

directly and gently secured to the ovarian surface epithelium. Rats receiving ovarian DMBA were subsequently treated with 17β-estradiol (E_2, 1.5mg, pellet implant, Hormone Pellet Press, Leawood, KS) [62]. Our laboratory has previously shown that this treatment combination promotes progression to simultaneous mammary and ovarian cancer in the rat following 6 months of treatment [171]. Rats were further treated with tamoxifen (5 mg, pellet implant, Hormone Pellet Press) or vehicle to test the effect of tamoxifen in early mammary and ovarian cancer [157].

Tissue preparation

Rats were killed by decapitation at 6 months post-treatment and the right thoracic mammary glands were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Right abdominal mammary glands were spread onto a glass slide, fixed in 4% PFA overnight, hydrated, infused with alum carmine (4 days), dehydrated, cleared in xylene, and stored in methyl salicylate. Left thoracic mammary glands were snap frozen and stored at -80°C. The ovary was bisected through the site of DMBA application. One half was fixed in 4% PFA and embedded in paraffin while the remainder was snap frozen and stored at -80°C.

Immunohistochemistry

Six-micron sections of mammary glands and ovaries were deparaffinized, rehydrated, and stained with hematoxylin and eosin. Mammary and ovarian sections (midsaggital, at 3 different equidistant levels per tissue) were evaluated for

morphological changes associated with early progression to mammary adenocarcinoma (MAC) and epithelial ovarian cancer (EOC) by an observer blinded to treatment group identity [65,144]. Adjacent sections were prepared for immunostaining by antigen retrieval (93°C, 10mM citrate buffer, 25 minutes) and incubation with 0.3% hydrogen peroxide (Lab Vision, Fremont, CA). Non-immune serum or primary antibodies against Ki-67 (1:100; Clone Ki-S5; rabbit monoclonal antibody, Lab Vision), estrogen receptor alpha (ERα; 1:200; MC-20; mouse monoclonal antibody, Santa Cruz), ALDH1A1 (1:150; rabbit polyclonal; Abcam, Cambridge, MA) or Oct-3/4 (1:50, mouse monoclonal, Santa Cruz) were applied and visualized with biotinylated secondary antibodies (Lab Vision) and diaminobenzidine (DAB) chromogen. All incubations were carried out using a Dako LV-1 autostainer (Carpinteria, CA).

Protein isolation and immunoblotting

Samples of mammary gland (n = 4) and ovary (n = 4) from all treatment groups were homogenized in lysis buffer (Cell Signaling Technology, Danvers, MA) with 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 10,000g for 15 minutes at 4°C and supernatant collected. Protein concentrations were measured using a BCA protein assay kit (Pierce, Rockford, IL). Following boiling for 5 minutes in Laemmli sample buffer (Bio-Rad, Hercules, CA), samples (25µg protein) and ladders (Kaleidoscope prestained standards, Bio-Rad) were run on 10% Tris-HCl Criterion Precast gels (Bio-Rad) under reducing conditions and transferred onto

nitrocellulose membrane. Membranes were blocked with 10% milk in Tris-Buffer Saline with Tween-20 (TBST) for 1 hour at room temperature and incubated with antibodies against ALDH1A1 (1μg/ml), cyclooxygenase-2 (COX-2; 2μg/ml, rabbit polyclonal, LabVision), or Oct-3/4 (1:200) at 4°C overnight. Following washing in TBST, blots were incubated in peroxidase-conjugated donkey anti-mouse, anti-rabbit or anti-goat antibodies (Jackson ImmunoResearch, West Grove, PA) for 2 hours at room temperature and washed. Heart tissue lysates were used as positive control [181] and primary antibody omission was used as negative control. Protein signals were visualized using chemiluminescent substrate (Pierce) and protein bands were quantified using GelPro. Equal protein loading was confirmed by stripping (BlotFresh, SignaGen, Gaithersburg, MD) and reprobing the membranes with β-actin antibody (1:20,000, goat polyclonal, sc-1616, Santa Cruz). Data are presented as integrated optical densities.

Quantitative Analysis of Preneoplastic Lesions

Mammary sections stained with H&E were evaluated and each section was assigned a dysplasia score according to the presence of pre-neoplastic and neoplastic lesions associated with breast cancer progression [144,171]. A score of 0 represented normal mammary histology. Preneoplastic changes included mild (score = 1) or severe (2) ductal hyperplasia and/or hyperplasia with atypia (3). Neoplastic changes included ductal carcinoma *in situ* (DCIS, score = 4) and invasive cancer (5). The sum of

scores from all 3 sections from each animal was used as the total dysplasia score; a value ranging from 0 to 15.

Pre-neoplastic changes of the ovary were defined as surface hyperplasia, inclusion cysts, stromal hyperplasia and papilloma, each being a separate histologic parameter [65,66,171]. For each ovarian section, each parameter was given a score of 0, 1, or 2, based on the severity or prevalence of each pre-neoplastic category (i.e. a score of 0 represented normal histology, a score of 1 corresponded to a moderate prevalence or degree of change and a score of 2 indicated a high incidence or degree of abnormality). Scores for all 4 histologic parameters were added up to give a dysplasia score for each ovarian section. The sum of all 3 dysplasia scores for each animal gave rise to the total dysplasia score, a value ranging from 0 to 24. These preneoplastic criteria are the same as those used by Stewart *et al.* with this rat model of ovarian adenocarcinoma [65].

Ki-67, and ERα expression in the mammary ductal epithelia cells and ovarian surface epithelium was quantified by counting immunoreactive cells and total cells (at least 1000 cells were evaluated per section) and presented as % immunoreactive epithelial cells. Location and distribution of ALDH-1 and Oct-3/4 expression were documented. All data are presented as the mean ± SEM. Protein levels of Ki-67, ERα, COX-2, ALDH-1 and Oct-3/4 expression determined by immunohistochemistry and western blot were analyzed using one-way analysis of variance with treatment type as main effect. Dysplasia scores were analyzed using a nonparametric test (Mann Whitney test). Differences were considered significant when $p \leq 0.05$.

Results

Tamoxifen blocks mammary carcinogenesis

Mammary gland whole mounts. Control and TAM rats had normal mammary morphology (Figure 1a, b). Carcinogen (E_2+DMBA) treatment increased area occupied by alveoli (Figure 1c). This effect was markedly reduced by tamoxifen in CARC+TAM group (Figure 1d).

Mammary tissue histology. Controls showed a normal appearance of lobular/acinar units surrounded by abundant adipose tissue (Figure 1e). These units constituted a single layer of myoepithelium and inner mammary epithelial cells. TAM animals also showed normal mammary histology (Table 2; Figure 1f). All CARC animals exhibited pathologic mammary histology ranging from hyperplasia to disseminated DCIS and had higher dysplasia scores when compared to controls (Figure 1g; Table 2, $p < 0.0001$). In CARC+TAM rats, the number and morphology of lobular units were restored to near normality (Figure 1h). These rats also showed mildly increased ductal branching and enlarged intraductal lumen as compared to controls but no dysplastic foci were present in any of these animals (Table 2).

Tamoxifen neither blocks nor accelerates the progression to ovarian cancer in a rat model

Controls showed normal ovarian morphology with mild inflammatory reaction to suture materials and rare inclusion cysts (Figure 2a, e; Table 2). Compared to controls, TAM rats showed a slight increase in dysplasia score mostly due to the

occasional presence of inclusion cysts; however, this difference was not significant (p= 0.20, Figure 2b, f; Table 2). Consistent with our previous findings, CARC rats received higher dysplasia score when compared to controls (p< 0.0001) and showed markedly abnormal ovarian morphology with disorganized granulosal clusters, stromal hyperplasia, epithelial hyperplasia, papilloma and glandular cystic changes resembling inclusion cysts (Figure 2c, g; Table 2). In CARC+TAM group, tamoxifen did not reduce the degree of ovarian preneoplasia following carcinogen treatment (p= 0.09; Figure 2d, h; Table 2). Interestingly, tamoxifen treatment seemed to increase the number of ovarian follicles when compared to those of the CARC group (data not shown).

Expression of Ki-67, ER*α *and COX-2 in mammary gland and ovary under normal or dysplastic conditions

Ki-67 expression was localized in the nucleus of ductal epithelial cells in the mammary gland. Average numbers of Ki-67 positive cells per 100 ductal epithelia were 5.17 ± 2.11, 4.58 ± 1.93, 22.94 ± 3.57, and 8.75 ± 0.79 in CONT, TAM, CARC, and CARC+TAM animals, respectively. These data showed that cellular proliferation was elevated in the mammary gland of CARC-treated animals when compared to CONT (p < 0.0001) and TAM (p < 0.0001) animals. Tamoxifen inhibited carcinogen-induced Ki-67 elevation in CARC+TAM rats when compared to CARC rats (p = 0.0003; Figure 3). Very few ovarian surface epithelial cells expressed Ki-67

(less then 0.2% immunoreactivity) and there was no difference in expression among different treatment groups ($p > 0.05$).

ERα expression. Carcinogen treatment depleted ERα expression (immunoreactivity = 0.25 ± 0.06%; Figure 3) in the mammary gland when compared to control (10.30 ± 1.86%, $p < 0.0001$) and TAM-treated animals (11.87 ± 0.88%, $p < 0.0001$). While no significant differences were detected, there was a trend for ERα expression to increase in response to TAM treatment in CARC+TAM animals (3.61 ± 0.25%, $p = 0.063$) when compared to CARC group. In the ovary, percentages of ERα immunoreactivity in ovarian surface epithelium were 40.97 ± 5.00, 47.45 ± 1.57, 41.69 ± 5.98 and 40.68 ± 6.66 for CONT, TAM, CARC, and CARC+TAM animals, respectively. No change in ERα expression was found in the ovary among different treatment groups.

Inflammation biomarker. COX-2 protein level was elevated in the mammary gland of CARC rats (IOD = 517.49 ± 197.27) when compared to CONT (11.47 ± 0.56, $p = 0.0067$) and TAM (37.61 ± 8.28, $p = 0.0089$) animals. Tamoxifen treatment reduced COX expression in CARC+TAM rats when compared to CARC group (7.60 ± 1.00, $p = 0.0065$; Figure 4A and 4B). In the ovary, COX-2 expression was not altered by CARC treatment ($p > 0.05$).

Levels of putative stem cell markers in the mammary gland and ovary

In the mammary gland, immunoblot analysis showed that Oct-3/4 and ALDH-1 expression were increased in CARC rats compared to controls (Figure 4A and B, p =

0.014 and 0.012, respectively). Surprisingly, while TAM drastically reduced histological progression to breast cancer, TAM had no effect on the induction of stem cell markers by sustained exposure to estrogen. Our results showed that ALDH-1 and Oct-3/4 levels between CARC and CARC+TAM animals and between CONT and TAM animals do not differ ($p > 0.05$, Figure 4). Immunohistochemistry revealed that while no immunoreactivity was observed in ductal epithelial cells of CONT and TAM animals, ALDH-1-positive cells were present in the cytoplasm of a few lobules in CARC and CARC+TAM animals (Figure 4C). However, immunoreactivity of Oct-3/4 was not observed in the selected mammary gland sections. While the stem cell hypothesis has been explored in breast carcinogenesis, no putative stem cell markers have been suggested to be associated with ovarian carcinogenesis. In the current study, immunohistochemistry data suggest that ALDH-1 and Oct-3/4 are not expressed in ovarian surface epithelia.

Discussion

Mammary gland

Tamoxifen inhibited mammary cancer progression in our preclinical model of breast and ovarian carcinogenesis, consistent with previous data from clinical trials and animal studies [165,182]. Ki-67, a proliferation marker, and COX-2, an inflammation marker, are potential markers of breast cancer risk and have been used as surrogate markers of response in human phase II chemoprevention trials [68]. In our rat model, Ki-67 and COX-2 also correlated with progression of mammary

carcinoma. Mammary ERα expression is down-regulated in CARC animals consistent with previous studies showing the loss of ERα following E_2-initiated cell proliferation [171,183]; however, it is also possible that the loss of ERα is temporary and is caused by ligand-induced receptor degradation [184].

Ovary

The current study is the most detailed experiment investigating the effect of tamoxifen on ovarian physiology and cancer progression. While our study showed that tamoxifen does not retard ovarian cancer, this negative finding is very important and in agreement with the human literature while providing more intensive biomarker and histopathology data than in human study. Although there seemed to be a slight increase in dysplasia in the ovary of animals treated with tamoxifen alone when compared to controls, this elevation was not significant. While there is a possibility of cancer incidence with longer tamoxifen administration, six months treatment (one quarter of life-span in rats) in the current experiment far exceeds the recommended treatment time for women taking tamoxifen (less or equal to 5 years).

Women taking tamoxifen have an increased risk for developing follicular cysts in the ovary [32,105], and it has been suggested that tamoxifen-induced ovarian cysts may contribute to increased risk of ovarian cancer [185]. However, in the current experiment, tamoxifen neither augments nor diminishes preneoplastic lesions induced by carcinogen treatment in the ovary in our high risk model. Our results therefore suggest that tamoxifen, as a common prevention therapy for breast cancer,

does not affect ovarian cancer risk in animals at high risk for both mammary and ovarian cancer. In, addition COX-2 levels remained unchanged among difference treatment groups in the current experiment. Recent studies revealed the relevance of COX-1 but not COX-2 expression in ovarian tumors development [51]. The role of COX-1 in mammary and ovarian carcinogenesis should be further investigated using this model.

Stem cell biomarkers

Oct-4 is a transcriptional factor expressed by early embryonic and germ cells and has been used to identify pluripotent cell populations [101]. ALDH-1, an enzyme that is required for the conversion of retinol to retinoic acids, is highly enriched in hematopoietic stem cells and researchers have suggested its presence in breast stem cells as well [102]. Our data showed increased expression of both markers in the mammary gland of rats treated with carcinogens. This finding suggests that stem cell populations are expanded during mammary carcinogenesis in our model.

Estrogen is used to induce mammary carcinogenesis in the current experiment. The mechanism by which estrogen acts on stem cell number is still unclear since most studies agree that breast stem cells are ER- [177]. However, studies have also shown that dysregulation of breast stem cells, or an increased stem cell pool size, can be induced by exposure to elevated breast epithelial mitogens such as insulin-related growth factor-1 and steroid hormones including estrogens [186,187]. One rationale for this effect of estrogen is an indirect mechanism or stem

cell niche; thus, estrogen acts on ER+ cells surrounding the stem cell and promotes paracrine signaling [177,188]. Interestingly, rats treated with carcinogen + tamoxifen were rescued from progression towards mammary cancer but still exhibited elevated mammary stem cell markers. This observation may suggest that tamoxifen, while retarding breast cancer progression, does not act upon the stem cell population but rather has its effects on the differentiated epithelia. This in turn is consistent with the absence of ER in the breast stem cell [177]; however, our understanding of mammary stem cell markers and biology will need to improve to fully answer this question.

Combined model of breast and ovarian cancer prevention

The rat model of breast and ovarian carcinogenesis used here, while allowing us to observe synergistic and antagonistic drug action in our search for a dual target prevention strategy, has some inherent limitations. The human population best modeled by these experiments is probably menopausal women on hormone replacement therapy and the results may be less relevant to other populations. This model is also focused on early changes of breast and ovarian cancer, since these are the intended targets for cancer chemoprevention, rather than following animals to tumor incidence. While this shortens the trials and parallels our human chemoprevention studies [68,189], it does entail the use of surrogate endpoint biomarkers for cancer with their inherent uncertainties.

Breast and ovarian cancer share similar etiology (endocrine background, risk factors, epithelial origin, etc) reflecting common disease pathways; however, these

cancers show discrepancy in terms of development and cancer cell type. This difference in pathology may be due to differences in the cells of origin or the hormonal milieu surrounding them. Despite these differences in the later stages of disease, the initiation factors for breast and ovarian cancer are similar and therefore, it is plausible to target both cancers simultaneously for prevention.

In the present study, we have demonstrated that, while tamoxifen is an effective breast cancer prevention drug for ER+ disease, it does not retard the development of ovarian preneoplasia and therefore is not ideal for simultaneous prevention of breast and ovarian cancer. Our results also suggest that while tamoxifen has been shown to induce ovarian cyst formation, it does not increase ovarian cancer risk in this model. Mechanistically, hormonal mammary carcinogenesis in this model is accompanied with elevated expression of ALDH-1 and Oct-4 and this putative expansion of the ALDH-1- or Oct-4-positive stem cell population is not reversed by tamoxifen cancer chemoprevention. These data also confirm that our combined breast and ovarian cancer model allows the observation of synergistic and antagonistic drug action on the breast and ovary. Simultaneous breast and ovarian cancer prevention is biologically feasible and may offer the best possibility for ovarian cancer prevention. Future studies will include investigation of common disease pathways and evaluation of other candidate drugs for simultaneous chemoprevention of both breast and ovarian cancers using this model.

Table 1: Experimental groups to examine the effect of tamoxifen on the progression towards concurrent mammary and ovarian cancer. CONT = vehicle-treated animals, TAM = tamoxifen-treated animals, and CARC = carcinogen-treated animals.

Treatment Group	**CONT**	**TAM**	**CARC**	**CARC+TAM**
Ovarian treatment	Vehicle	Vehicle	DMBA	DMBA
Systemic treatment	Vehicle	Vehicle	E_2	E_2
Tamoxifen treatment	Vehicle	Tamoxifen	Vehicle	Tamoxifen

Table 2. Mammary and ovarian dysplasia scores.

Animal group	**Mammary Gland**	**Ovaries**
CONT	0	0.44±0.24
TAM	0	1.38±0.32
CARC	7.88±0.79*	7.29±1.09*
CARC+TAM	0	5.83±0.54*

Dysplasia scores are present as mean ± SEM for each treatment group. CONT = control, TAM = tamoxifen-treated animals, and CARC = carcinogen-treated animals. * differs significantly from control ($p < 0.05$). In the ovary, the differences between CONT and TAM as well as CARC and CARC+TAM were not significant ($p = 0.20$ and 0.085, respectively).

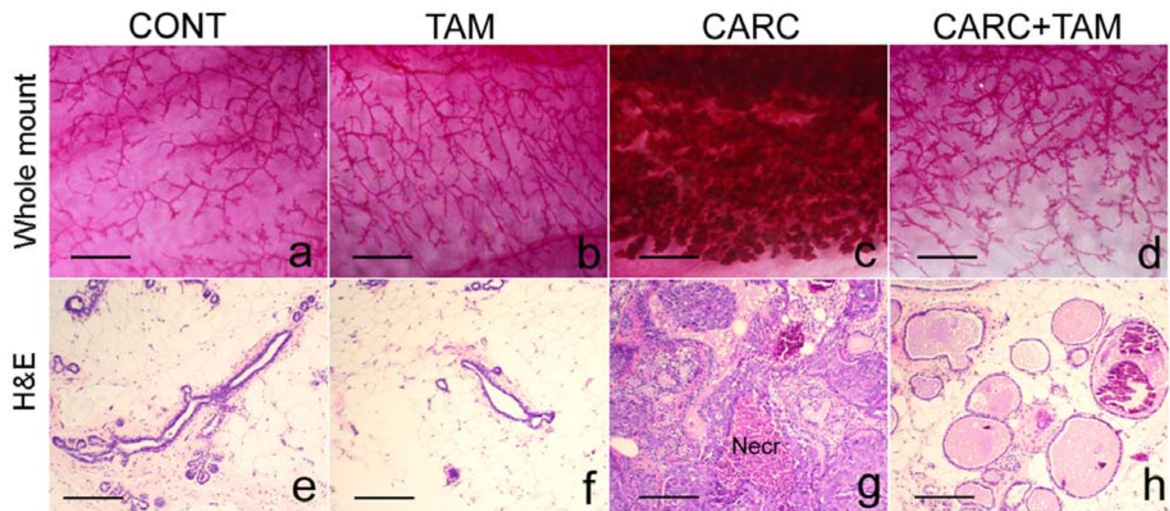


Figure 1: Whole mounts (a-d, 20x), and H&E sections (e-h, 100x) of the mammary gland. Controls (CONT) and tamoxifen-treated (TAM) animals showed normal mammary histology (a-b, e-f). Carcinogen treatment (CARC) caused preneoplasia and neoplasia (c and g) and this effect was blocked by CARC+TAM (d and h). Secreting mammary glands were observed in CARC+TAM rats. Scale bars = 5 mm (a-d), 200 μm (e-h).

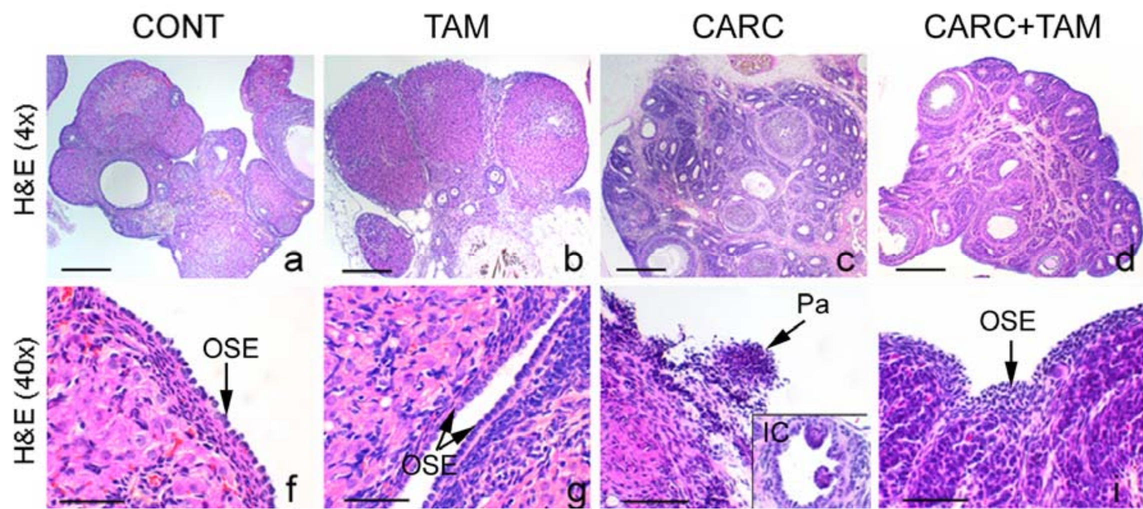


Figure 2: H&E sections of the ovary. Scale bars = 600 µm (a-d), 50 µm (e-h and insert). CONT = controls, TAM = tamoxifen-treated rats, CARC = E_2/DMBA-treated rats, OSE = ovarian surface epithelia, Pa = papilloma, IC = inclusion cyst.

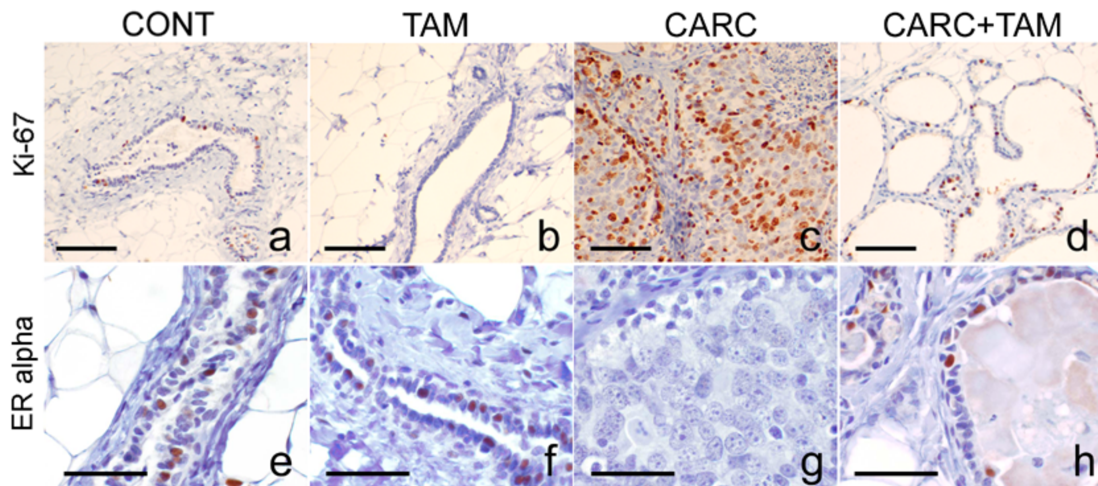
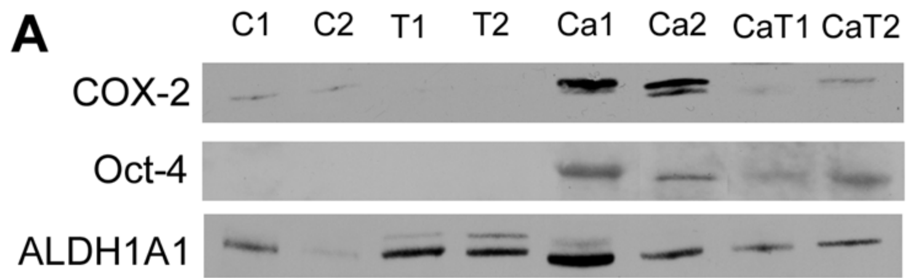


Figure 3: Ki-67 and ER alpha immunostaining in the mammary gland. Mammary epithelial proliferation (% Ki-67) was increased above controls in CARC animals (a and c, $p<0.05$). CARC+TAM treatment reduced this elevation (c and d, $p<0.05$). TAM alone did not effect % Ki-67 compared to controls (a and b). Carcinogen treatment depleted ER expression in the mammary gland when compared to control and TAM-treated animals (e-g, $p<0.05$). There was a trend for ERα expression to increase in response to TAM treatment in CARC+TAM animals when compared to CARC group; however, this difference was not significant (g and h, $p= 0.063$). Scale bars = 100 µm (a-d), 50 µm (e-h).



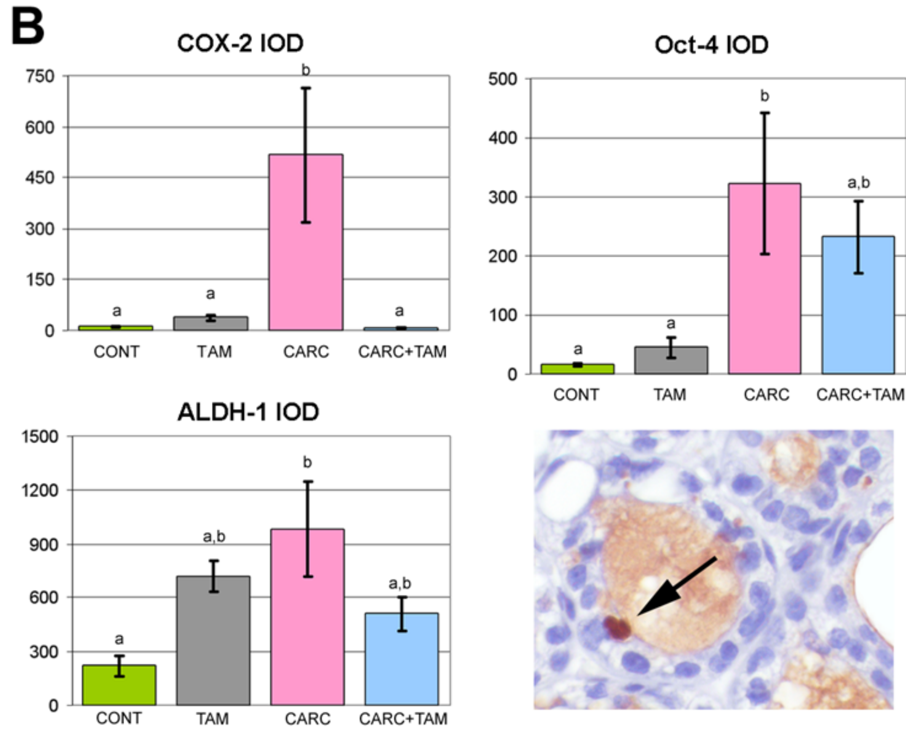


Figure 4: Cyclooxygenase 2 (COX-2), Oct-4, and aldehyde dehydrogenase 1 (ALDH-1) expression in the mammary gland. **A.** Representative western blots of mammary glands from rats treated with vehicle (C1, C2), tamoxifen (T1, T2), carcinogen (CA1, CA2), and carcinogen + tamoxifen (CA+T1, CA+T2). β-actin was used as a loading control. **B.** Quantitative analysis of western blots showing that COX-2, Oct-4 and ALDH-1 levels (y axis represents IOD, Integrated Optical Density) were elevated following carcinogen treatment (CARC) compared to controls (CONT). COX-2 expression was reduced by tamoxifen (TAM) in CARC+TAM

group. Letters indicate significant differences among different treatment groups. **C.** Cytoplasmic ALDH-1 immunostaining was observed in a few luminal epithelial cells in the mammary gland of CARC animals (Arrow). No immunoreactivity was observed in CONT and TAM animals. Scale bars = 50 μm.

Chapter IV

Tamoxifen as a molecular shield to protect the ovary against DMBA and cyclophosphamide-induced follicle loss in the rat

Abstract

Infertility and premature ovarian failure are common side effects of cytotoxic chemotherapy. Embryo freezing is the only established option to preserve fertility and it has significant limitations in efficacy. Our laboratory has serendipitously discovered a protective effect of tamoxifen, a selective estrogen receptor modulator, against the destruction of oocytes by toxic chemicals. The current study is designed to explore this novel ovoprotective property of tamoxifen. *In vivo*, 5-week-old female rats received placebo or tamoxifen pellets (5mg) followed by weekly injections of vehicle, 7,12-dimethylbenzanthracene (DMBA) or cyclophosphamide (Cy, the most ovotoxic chemotherapy), and total numbers of follicles in the ovary were determined. *In vitro*, ovaries from 4 day old pups and mature oocytes were exposed to DMBA and doxorubicin, respectively, with or without 4-hydroxytamoxifen (4HT) to examine local effects of tamoxifen on DMBA-induced follicle loss and doxorubicin-induced oocyte fragmentation. Follicle populations were classified and counted in every 10th sections of the ovary. Results confirmed that numbers of primordial and total follicles were reduced by DMBA and Cy ($p<0.05$) and this reduction was prevented by tamoxifen *in vivo* and 4HT *in vitro*. In oocyte culture, doxorubicin elevated the percentage of fragmented oocytes, an effect that was antagonized by 4HT. In summary, this study demonstrated a protective effect of tamoxifen against DMBA, a well characterized ovotoxicant, Cy, the most ovotoxic cancer drug and another chemotherapy agent, doxorubicin. Tamoxifen may therefore provide a novel protection to the ovary against chemotherapy-induced infertility in premenopausal

cancer patients. Tamoxifen has already been tested for safe first-line use for several cancers allowing these observations to be readily translated from bench to bedside.

Introduction

With over 2 million oocytes at birth, a woman ovulates only 300-500 eggs during her reproductive life span while the remaining majority becomes atretic [190]. Standard cytotoxic cancer therapy can destroy ovarian follicles and often leads to infertility or premature ovarian failure, permanently affecting quality of life for young women with cancer [111,191]. Previously this infertility and iatrogenic menopause was viewed by physicians and patients as an acceptable cost of curative chemotherapy and radiation regimens. However, as more women survive their cancers and strive to resume normal family life, sterilization and premature menopause from cancer therapy has become a major survivorship issue [192]. With earlier diagnoses and higher survival rates, the problem of infertility in cancer patients will only increase. To date, there is no established treatment capable of protecting the ovary from the toxic effects of chemotherapy.

The only approved fertility preservation method for female cancer patients is assisted reproduction followed by embryo cryopreservation for future transfer; however, this procedure causes a delay in chemotherapy by several weeks to months, is expensive and invasive, requires current partners or sperms and has low success rate [193]. Experimental methods such as oocyte freezing also delay cancer treatment and has little success to date due to fragility of the oocytic meiotic spindle and formation of ice crystals during freezing [117]. Ovarian tissue cryopreservation is also available experimentally but it is surgically invasive, has a risk of possible cancer cell transmission, and causes ischemic damage to the ovary leading to poor oocyte

viability [120]. One experimental therapy that appears to protect gonadal function is hypothalamic suppression with GnRH agonists [122,194]. Blumenfeld and colleagues have documented chemoprotective effects of GnRH suppression against infertility in women following chemotherapy for lupus and lymphoma [121,124,195]. However, treatment with GnRH agonists does not appear to protect from radiation-induced ovotoxicity [126] and other studies have not observed consistent outcome from this protocol [125]. Fertility preservation options that provide protection of the ovary for young female cancer patients are urgently needed.

Our laboratory serendipitously observed a protective effect of the commonly used anticancer drug tamoxifen (TAM) against ovarian follicular loss caused by 7,12-dimethylbenzanthracene (DMBA) in a preclinical cancer prevention study [196]. TAM is a selective estrogen receptor modulator (SERM) and the most commonly used drug for treatment and prevention of hormonal-responsive breast cancer [197]. The estrogenic or anti-estrogenic effects of TAM are determined in large part by the complement of ER isoforms, estrogen response element polymorphisms, corepressor and coactivator plentitudes, and existing concentrations of endogenous estrogens [198]. While chronic (5 years) use of tamoxifen is associated with an increased risk of endometrial cancer [165], short term use of tamoxifen has been tested extensively and proven to be safe as first line combination therapy for lymphoma due to its potential alleviation of multidrug resistance [199-201].

Two of the most extensively studied ovotoxic agents are DMBA and cyclophosphamide (Cy). DMBA, a polycyclic aromatic hydrocarbon, is commonly

used in rodent models to induce a variety of cancers [64,127]. In addition to being a carcinogen, DMBA also destroys follicular reserve and causes ovarian failure [128,129]. While the mechanism by which DMBA targets and destroys follicles in the ovary is not entirely understood, many investigators have suggested that apoptosis plays a critical role [129,130]. Cy, also known as Cytoxan, is commonly used as a part of standard regimen therapy of many malignancies including lymphomas, leukemia, and solid tumors. Cy is a nitrogen mustard alkylating agent which forms adducts with DNA and is considered to be the most ovotoxic cancer drug resulting in a high rate of ovarian failure in cancer patients [110,134]. Doxorubicin (DOX), also known as Adriamycin, intercalates with DNA and inhibits helicases and topoisomerases and is another frequently studied chemotherapeutic agent known for its ovotoxicity. Similar to DMBA-induced ovarian toxicity, Cy and DOX has been shown to target the ovary directly and cause atresia [138]. However, while some studies have shown that DMBA and DOX deplete ovarian follicles by elevating Bax protein and apoptosis in oocytes [129]; others have suggested Cy-induced follicle depletion is driven by apoptosis in oocyte supporting cells or granulosa cells [138].

In the current study, we test a novel use of tamoxifen to protect the ovary and rescue ovarian reserve from DMBA, Cy and DOX-induced gonadal toxicities in female Sprague Dawley rats. Results from this study may lead to improved fertility preservation and quality of life, especially following cancers with high incidence for young females.

Materials and methods

Animals and treatments

Pregnant and 4-6 week old virgin female Sprague Dawley rats (Harlan Breeding Laboratories, Indianapolis, IN) were housed in a climate and light (12L:12D) controlled environment and received food and water *ad libitum*. All experimental protocols are approved by the University of Kansas Medical Center Animal Care and Use Committee.

DMBA experiment: One week after arrival, rats (n = 6 per treatment group) were randomly assigned into the following groups:1) controls, 2) 5mg TAM [196], 3) 5mg DMBA, 4) 10mg DMBA [202], 5) 5mg DMBA+TAM, 6) 10mg DMBA+TAM. Rats were anesthetized with ketamine hydrochloride and xylazine (80 and 8 mg/kg, respectively) and pellets containing 5mg TAM or matching placebos (Hormone Pellet Press, Leawood, KS) were implanted subcutaneously between the scapulae on day 0. Each animal also received 2 injections of DMBA (5 or 10mg dissolved in 0.5ml corn oil per dose, Sigma St. Louis, MO) or vehicle on day 0 and 7 [202].

Cy experiment: Rats (n = 6 per treatment group) were randomly assigned into 4 treatment groups: 1) controls, 2) 5mg TAM, 3) 35mg/kg Cy [203], 4) 50mg/kg Cy, 5) 35mg/kg Cy + TAM, 6) 50mg/kg Cy + TAM. Rats were anesthetized as described above and implanted with pellets containing 5mg TAM or matching placebos (60 day release; Innovative Research of America, Sarasota, Florida) on day 0. On day 2 and

weekly thereafter, rats received Cy (35 or 50mg/kg in 0.9% NaCl, i.p.; Sigma) or vehicle injections for 4 weeks [136].

Tissue preparation

One week following the last DMBA treatment or three days following the last Cy injection, rats were sacrificed by decapitation and serum was collected and stored at -80°C. The left ovary was excised, weighed, fixed in 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA) and embedded in paraffin and right ovary was snap-frozen and stored at -80°C.

Ovarian organ culture

Postnatal day 4 female pups were killed by decapitation [204]. Ovaries (n= 6 per treatment) were removed, oviduct and excess tissue trimmed and placed on a piece of Millicell-CM membrane which was pre-incubated and floating on 250µl of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/ F12, 1:1) containing 0.1% bovine serum albumin (BSA), 50 µg/ml ascorbic acid, 2.75 µg/ml transferrin, 1mg/ml Albumax, and 50 I.U./ml penicillin/ 50 µg/ml streptomycin in a 24-well plate [205] with the following treatments; 1) Vehicle control: 0.1% DMSO and 0.1% ethanol, 2) DMBA: 1µM DMBA [129,206] in DMSO and 0.1% ethanol, 3) 1µM 4-hydroxytamoxifen (4HT): 0.1% DMSO and 1µM 4HT in ethanol, 4) 10µM 4HT: 0.1% DMSO and 10µM 4HT, 5) DMBA+1µM 4HT and 6) DMBA+10µM 4HT. A drop of culture medium (approximately 10 µl) was placed to cover the ovary

to prevent drying. Ovaries were incubated at 37ºC with 5% CO_2 and media were removed and fresh media and treatment were replaced every 2 days. Following 1 week of treatment, ovaries were collected, fixed in 4% PFA, and embedded in paraffin. BSA, ascorbic acid, transferrin and penicillin-streptomycin were purchased from Sigma. D-MEM/ F12 without phenol red and Albumax II were purchased from Invitrogen (Carlsbad, CA). Millicell cell culture plate inserts (12mm, 0.4µm) were purchased from Millipore (Bedford, MA).

Oocyte culture

Sprague Dawley rats (4 weeks old) were superovulated with 20 IU of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) 48 hours later. Sixteen hours after hCG injection, cumulus-oocyte-complexes were collected from the oviducts and oocytes were denuded of cumulus cells in 1000 IU/ml hyaluronidase followed by 3 washes of Modified Rat Embryo Culture Medium (MRECM1) HEPES media [207,208]. Groups of 15-25 oocytes per treatment per replication (n=9 replication per treatment group) were cultured in 0.1 ml drops of culture medium containing 25mM $NaHCO_3$, 0.5mM sodium pyruvate, 22mM HEPES, 0.5x Minimal Essential Medium Non-essential Amino acids (MEM NEAA), 0.5x Minimal Essential Medium Essential Amino Acids (MEM EAA), 0.1mM GlutaMAX1, 110mM NaCl, 3.2mM KCl, 7.5mM D-glucose, 100µg/ml penicillin G K salt, 50µg/ml streptomycin sulfate, 13.53mM sodium lactate (60% syrup), 2mM $CaCl_2$-$2H_2O$ and 0.5mM $MgCl_2$-$6H_2O$ under sterile mineral oil. Oocytes were

incubated with the following treatment: 1) vehicle, 0.1% ethanol, 2) 40μM 4HT in ethanol, 3) 200nM doxorubicin (DOX) [208] + 0.1% ethanol, or 4) 200nM DOX+ 40μM 4HT at 37ºC in a humidified atmosphere with 5% CO_2 for 26 hours.

Histological and morphological analysis

Six-micron serial sections of ovaries from *in vivo* and organ culture experiments were deparaffinized, rehydrated, and stained with hematoxylin and eosin for histological analysis. Follicles were classified as primordial (with one layer of flattened pregranulosa cells), primary (one expanded layer of cuboidal granulosa cells), pre-antral (2-5 layers of granulosa cells), early antral, and late antral follicles and counted in every 10th section [209]. Only follicles with visible nuclei were used for enumeration. The number of follicles in each category per ovary is expressed by the sum of counted follicle in every 10th section multiplied by 10. Numbers of total follicles per ovary were estimated by summing up numbers of follicles of all categories. Over 90% of all follicles in cultured postnatal day 4 ovaries were primordial follicles, thus, only this population was quantified [204]. At the end of the treatment period for oocyte cultures, morphological changes indicative of apoptosis or oocyte fragmentation were examined by a light microscope.

Data analysis

Data for follicular numeration are presented as the mean ± SEM and analyzed for main effects of DMBA or Cy and TAM, as well as their interaction on numbers of

follicles using a one or two way ANOVA. Results from oocyte cultures are presented as percent apoptosis, and analyzed using nonparametric Kruskal-Wallis. Bonferroni analysis was used for multiple comparisons. Differences are considered significant when $p \leq 0.05$.

Results

Tamoxifen rescued ovarian follicular loss caused by DMBA

The mean number of total ovarian follicles per ovary in vehicle-treated controls was 5.1×10^3. Compared to controls, the higher dose of DMBA (10mg/wk) induced loss of total and primordial follicles ($p < 0.05$) while the lower dose of DMBA (5mg/wk) only induced significant total ($p < 0.05$) but not primordial follicle loss ($p = 0.07$, Fig. 1 and 2). Rats treated with TAM plus DMBA (both doses) inhibited DMBA-induced follicle loss and showed numbers of total and primordial follicles comparable to those of vehicle controls (Fig. 2). Tamoxifen treatment alone caused an elevation in numbers of total and primordial follicles compared with vehicle controls (Fig. 2). Numbers of primary, pre-antral and early antral follicles were reduced by both doses of DMBA, while late antral follicle population was not affected by DMBA (Fig. 3). Tamoxifen caused a reduction in numbers of pre-antral and early antral follicles, and had no effect in primary and late antral population (Fig. 3).

Tamoxifen inhibited Cy-induced follicular loss in the ovary

The average number of total ovarian follicles in control animals was 8.1×10^3 per ovary. Both doses of Cy (35 and 50mg/kg/wk) induced a depletion in numbers of total and primordial follicles in comparison to controls ($p < 0.05$, Fig. 4 and 5) and had no effect on other follicle populations (Fig. 6). The addition of tamoxifen inhibited Cy-induced follicular loss resulting in numbers of total and primordial follicles similar to vehicle controls ($p < 0.05$, Fig. 4 and 5). Tamoxifen alone decreased the number of pre-antral follicles but had no effect in other follicle populations (Fig. 5 and 6).

Protection against DMBA and DOX -induced ovotoxicity by tamxoifen is in part via local mechanisms

One μM 4HT treatment alone induced moderate follicle loss in comparison with vehicle-treated ovaries ($p < 0.05$, Fig. 7). DMBA induced even greater follicle loss in cultured ovaries ($p < 0.05$) compared to vehicle- and 4HT -treated ovaries (Fig. 7). Both 1 and 10 μM 4HT partially inhibited DMBA-induced toxicity (Fig. 7). Results from oocyte culture showed that 12 and18% of oocytes exposed to vehicle and 4HT, respectively, underwent spontaneous fragmentation (Fig. 8 and 9). Doxorubicin doubled ($p < 0.05$) the amount of fragmented oocytes compared with vehicle-treated controls, an effect that is alleviated by 4HT ($p < 0.05$, Fig. 8 and 9).

Discussion

Tamoxifen rescues ovarian follicular reserves from DMBA and Cy in vivo

In the current study, we have shown that tamoxifen protects the ovary from the three most studied ovotoxicants, DMBA, Cy, and DOX. *In vivo*, both 5 and 10 mg/kg DMBA depleted total ovarian follicles by 39% and 55%, respectively when compared to those of vehicle-treated animals. Since over 80% of total ovarian follicles are primordial follicles, changes observed in numbers of total follicles often correlate with changes observed in those of primordial population. TAM inhibited the loss of primordial follicles caused by both dosages of DMBA. For pre-antral and early antral follicles, tamoxifen alone, DMBA alone and DMBA plus tamoxifen all decreased the number of these follicles. Tamoxifen and other SERMs have been shown to induce anovulatory cysts and arrest follicle growth by triggering hypogonadoism [108] while DMBA has been shown to inactivate the ovary by inhibiting preovulatory gonadotropin secretion as well as promoting apoptosis in all follicles populations [128,210].

Like DMBA, a single treatment cycle of Cy reduced numbers of total follicles, and the addition of TAM inhibited the follicle loss. Similar changes were observed in primordial follicle population. Unlike DMBA, Cy does not affect the size of pre-antral and early antral follicle populations when compared to controls. This suggests a possible mechanism of action caused by Cy that directly targets primordial/primary follicles and promote cell death.

Tamoxifen exerts its protection against DOX-induced oocyte fragmentation and DMBA-induced follicle loss in vitro

In ovulated oocytes, doxorubicin increased the rate of fragmentation when compared to vehicle-treated controls and 4HT was able to antagonize this effect. Studies have suggested that oocyte fragmentation in vitro occurs through apoptosis [208]; therefore, a possible protective role of tamoxifen on DOX-treated oocytes may occur through inhibition of apoptosis. When we investigated the effect of 4HT on ovarian histology in cultured ovaries exposed to DMBA, a moderate protective effect of tamoxifen was observed in endangered ovarian follicles. 4HT alone in culture moderately reduced the number of follicles when compared to vehicle controls. This observation is not consistent with results from the *in vivo* study in which TAM did not have any ovotoxic effect, and this may be explained by the serum-free and hormone-free culture medium that the ovary was exposed to in culture, differing from the physiological environment. DMBA further depleted the number of follicles by 66% in comparison to that of control ovaries and the combination of DMBA and 4HT partially inhibited this loss resulting in similar follicle numbers in 4HT and DMBA+4HT-treated ovaries. Together, our results suggest that the optimal effects of tamoxifen on protecting the ovary from ovotoxic insults are likely to be through local plus systemic mechanisms.

Effects of tamoxifen on reproductive endocrinology of cancer patients undergoing chemotherapy

Tamoxifen is a commonly used drug in cancer patients and the subject of many clinical and preclinical trials [157,165,211], and adjuvant tamoxifen therapy has already been used in premenopausal female cancer patients without adverse side effects [199,201]. However, past studies have overlooked changes in the ovary and have been inappropriate in design, treatment duration, patient age and endpoints to address fertility. Interestingly, a study by Rose and Davis conducted in 1980 to examine reproductive endocrinology during treatment in premenopausal breast cancer patients receiving cyclophosphamide + methotrexate + 5-fluorouracil (CMF) chemotherapy with or without simultaneous prednisone or prednisone + tamoxifen showed similar results to that in our study [109]. While the clinical study did not directly measure markers of follicular reserve or reproductive function post-treatment, Rose and Davis revealed that the addition of tamoxifen to the regimen was associated with elevated estradiol, decreased FSH concentrations and delayed iatrogenic amenorrhea during 6 and 10 months of treatment [109]. This observation suggests protection of ovarian function provided by tamoxifen in patients undergoing chemotherapy.

Possible mechanisms by which tamoxifen protects ovarian follicular reserves

The mechanism by which tamoxifen acts to rescue ovarian follicles is unclear and needs further investigation. Systemically, tamoxifen may cause effects similar to

GnRH agonist by disrupting the hypothalamic-pituitary-ovarian axis [195], therefore, protecting the ovary by keeping it a quiescent anovulatory state. Similar observations have also been reported in rats treated with another SERM, raloxifene [108]. The advantage of tamoxifen over GnRH agonist for the protection of the ovary may be the addition of local beneficial effect exerted by tamoxifen. Locally, the role of tamoxifen, whether estrogenic or anti-estrogenic, in the ovary has not been clarified due to the cellular complexity of the ovary. Interestingly, E_2 has been shown by our group (unpublished data) and others to provide protection against chemical-induced ovarian follicle loss in the rat [212]; however, clinically, E_2 treatment in premenopausal women is dangerous and tumorigenic in several tissues and is therefore unpractical for therapeutic usage [213]. Premenopausal women taking SERMs including tamoxifen have been shown to have slightly increased E_2 production in the ovary due to a compensatory mechanism suggesting an estrogenic role of tamoxifen in the ovary [57]. In addition, tamoxifen can act as an anti-oxidant which is associated with cell survival and affects the retinoic acid pathway that is also important for cell survival [214], although the potentiation of cancer cytotoxicity by tamoxifen in past studies argues against the primacy of such general mechanisms during ovarian protection.

In conclusion, we have shown in the current study that tamoxifen rescues ovarian follicular reserves from the ovotoxic chemical DMBA and the chemotherapy drugs Cy and DOX. The only currently approved option for fertility preservation following chemotherapy is cryopreservation of the embryo. This method does not

prevent ovarian toxicity from cancer therapy and premature menopause remains a problem even in women with cancer actively seeking fertility preservation.

Tamoxifen may serve as a better candidate drug for translation to fertility preservation in several ways: 1) tamoxifen-related side effects including menopausal-like symptoms and impairment of the hypothalamus-gonadal axis are reversible following the discontinuation of tamoxifen, 2) tamoxifen has a long and extensive safety record in the cancer patient population, 3) the use of tamoxifen for fertility preservation offers clear advantages in cost, convenience and efficacy over current options, 4) tamoxifen administered concurrently with chemotherapy appears to have no negative effect on cancer progression and has been suggested to alleviate chemotherapy resistance in preclinical studies.

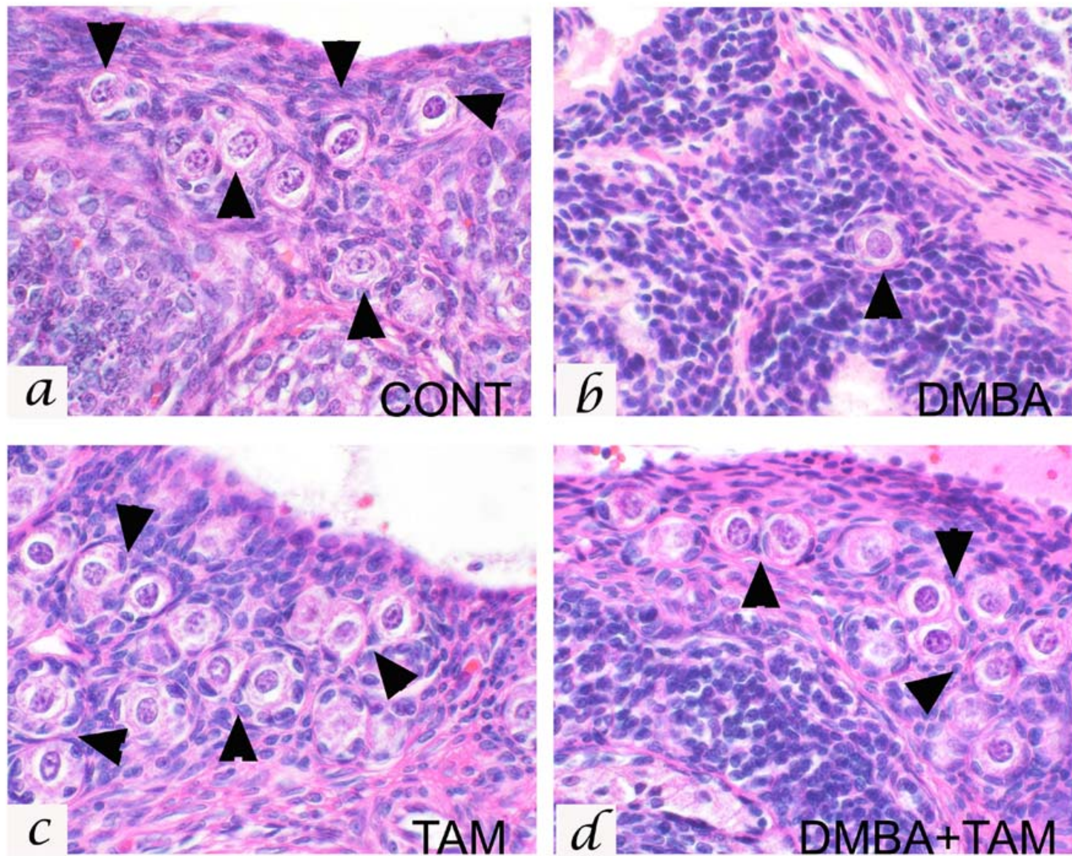
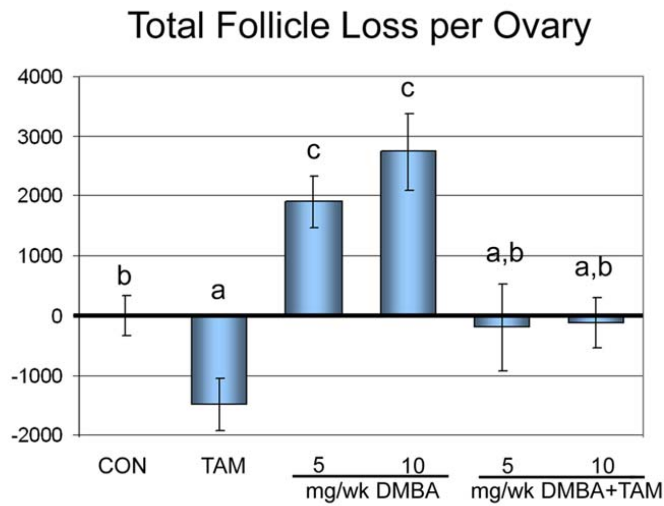


Figure 1: H&E sections of the ovary from rats exposed to vehicle (CONT, a), DMBA (10mg/wk, b), tamoxifen (TAM, c), or DMBA + TAM (d) for 2 weeks. Arrows = primordial follicles. DMBA decreased follicular reserves in the ovary compared with vehicle-treated controls and the addition of tamoxifen inhibited this depletion. Tamoxifen alone elevated the number of follicles in the ovary compared with vehicle-treated controls.



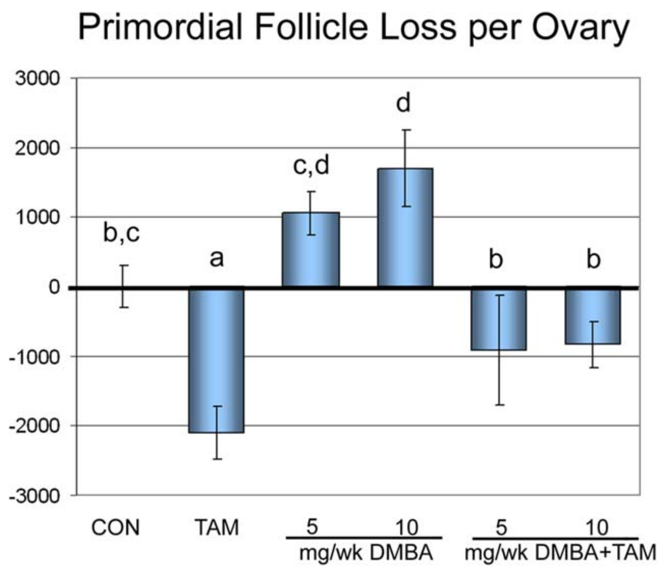


Figure 2: Numbers of follicle loss relative to vehicle-treated controls for total (follicles at all developmental stages, upper panel) and primordial (bottom panel) follicle populations. Average number of follicles per ovary in vehicle-treated controls was 5.1×10^3. DMBA treatment at 5 and 10 mg/wk induced follicles loss compared with controls and the addition of tamoxifen (TAM) to both doses of DMBA inhibited

this depletion. TAM alone elevated total follicle numbers in comparison with vehicle controls. Different letters indicate significant differences among different treatment groups.

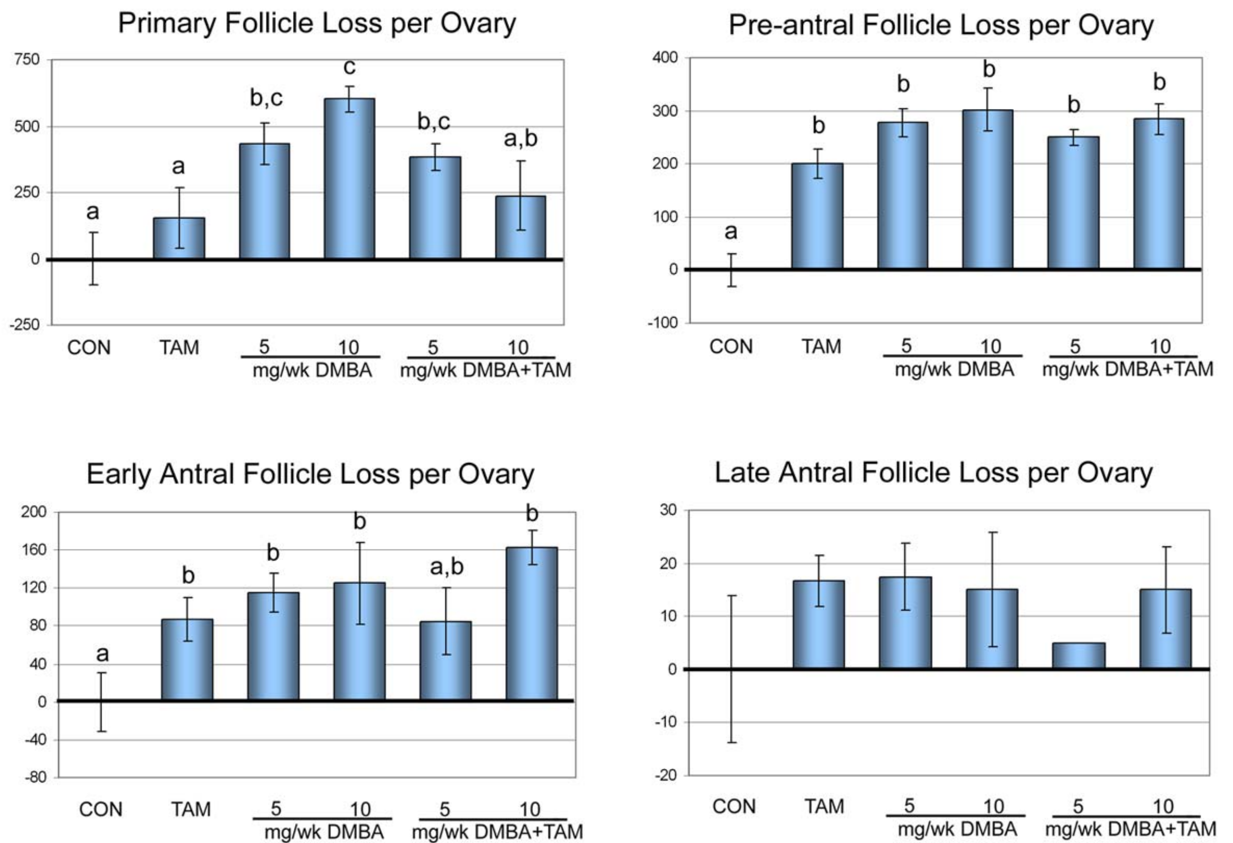


Figure 3: Numbers of follicle loss relative to vehicle-treated controls for primary, pre-antral, early antral and late antral follicles remaining in the ovary of rats treated with vehicle, tamoxifen (TAM), DMBA (5 or 10 mg/wk) or DMBA+TAM. Average numbers of follicles per ovary in vehicle-treated controls were 0.87×10^3, 0.47×10^3, 0.19×10^3 and 0.035×10^3 for primary, pre-antral, early antral and late antral follicle populations, respectively. In primary follicles, DMBA treatment at 10 mg/wk induced follicles loss compared with vehicle controls and the addition of TAM to 10 mg/wk DMBA inhibited this depletion. TAM alone did not affect primary follicle

numbers. For pre-antral and early antral follicle populations, DMBA at both doses, TAM, and DMBA+TAM all induced a loss of follicles. Different letters indicate significant differences among different treatment groups.

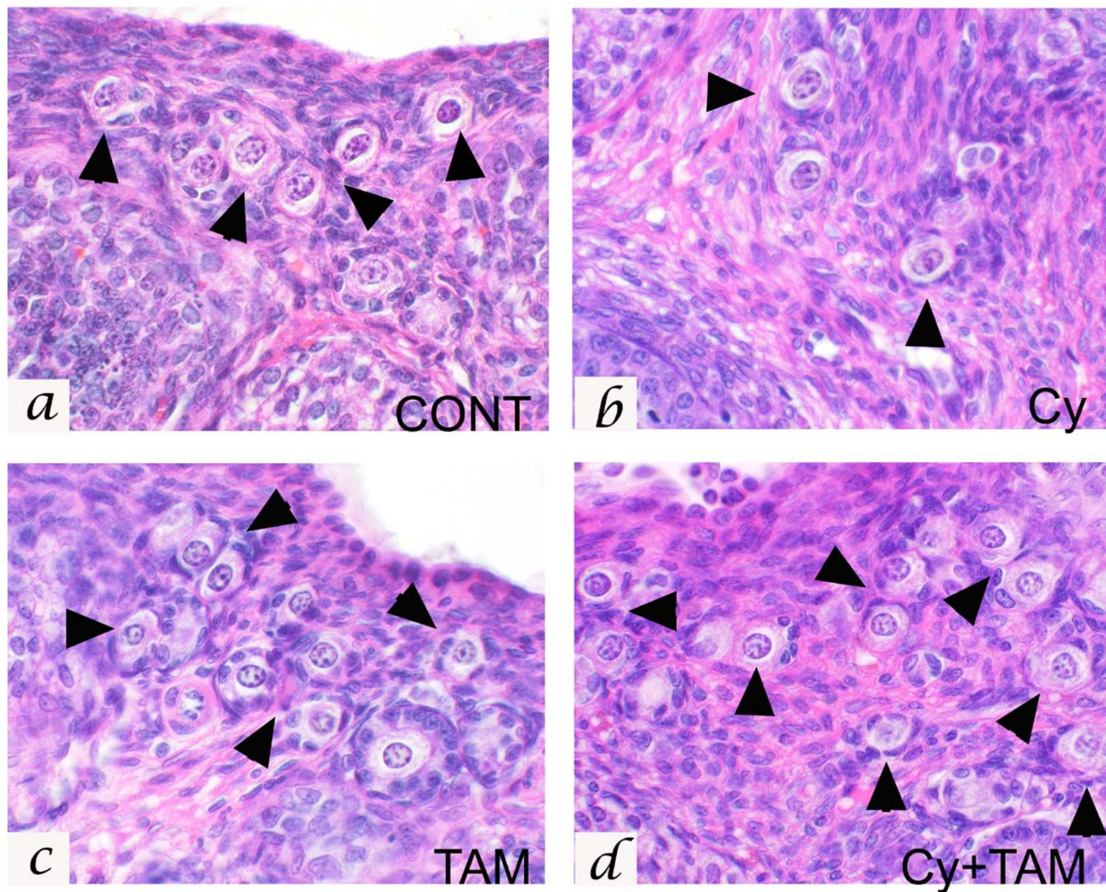


Figure 4: H&E sections of the ovary from rats exposed to vehicle (CONT, a), cyclophosphamide (Cy, 50mg/kg, b), tamoxifen (TAM, c), or Cy + TAM (d) for 4 weeks. Arrows = primordial and primary follicles. Cy decreased total numbers of follicles in the ovary compared with vehicle-treated controls and the addition of tamoxifen inhibited this depletion. TAM did not affect the number of follicles in the ovary compared with vehicle-treated controls.

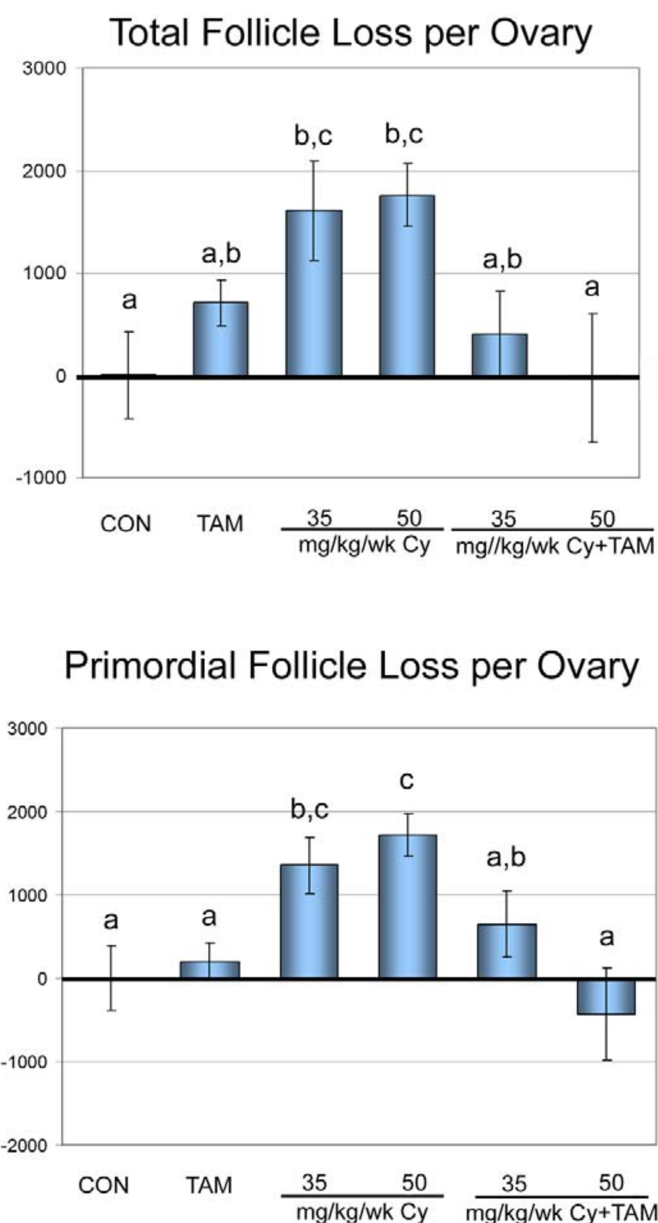


Figure 5: Numbers of follicle loss relative to vehicle-treated controls for total (follicles at all developmental stages, upper panel) and primordial (bottom panel) follicle populations. Average number of follicles per ovary in vehicle-treated controls was 6.1×10^3. Cyclophosphamide (Cy) treatment at 35 and 50 mg/kg/wk induced follicles loss compared with controls and the addition of TAM to both doses of Cy

inhibited this depletion. TAM alone did not affect follicle numbers. Different letters indicate significant differences among different treatment groups.

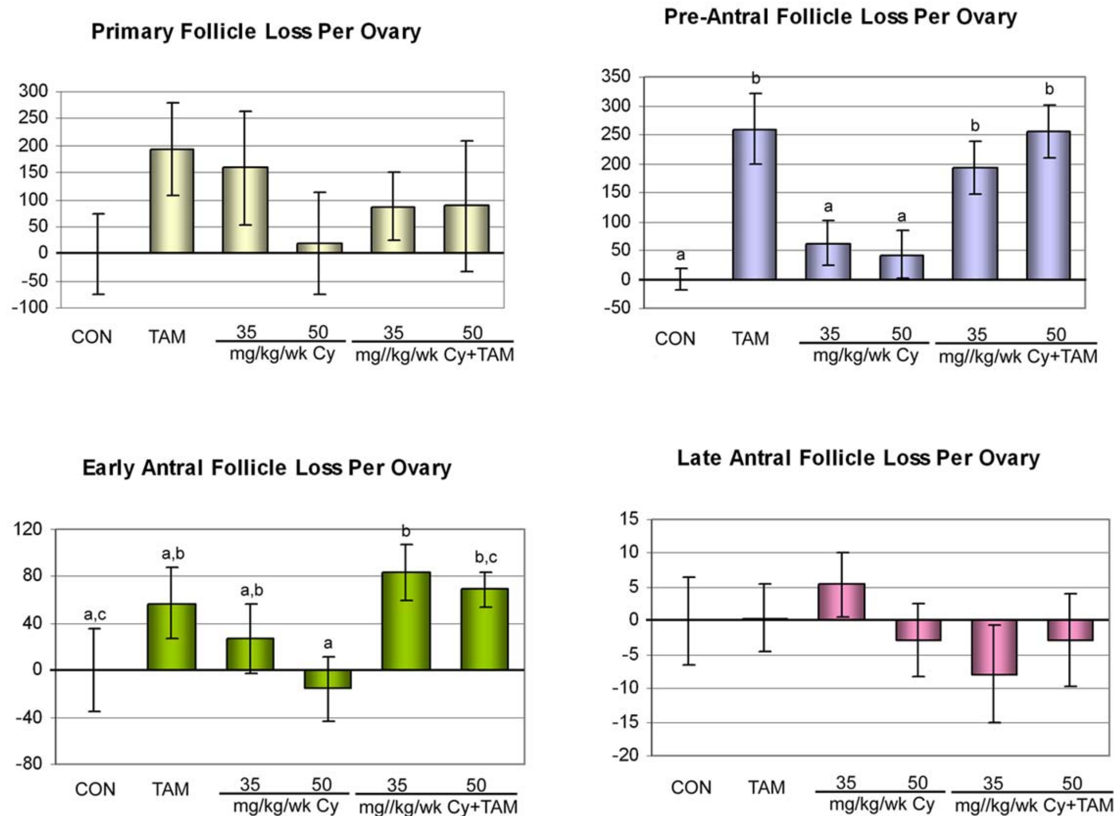


Figure 6: Numbers of follicle loss relative to vehicle-treated controls for primary, pre-antral, early antral and late antral follicles remaining in the ovary of rats treated with vehicle, tamoxifen (TAM), Cy (35 or 50 mg/kg/wk) or Cy+TAM. Average numbers of follicles per ovary in vehicle-treated controls were $1.1x10^3$, $0.70x10^3$, $0.22x10^3$ and $0.017x10^3$ for primary, pre-antral, early antral and late antral follicle populations, respectively. In pre-antral follicles, TAM and Cy+TAM induced follicle loss compared with vehicle controls while Cy alone did not affect follicle numbers. Follicles of primary, early antral and late antral did not show a treatment-dependent

effect in their numbers. Different letters indicate significant differences among different treatment groups.

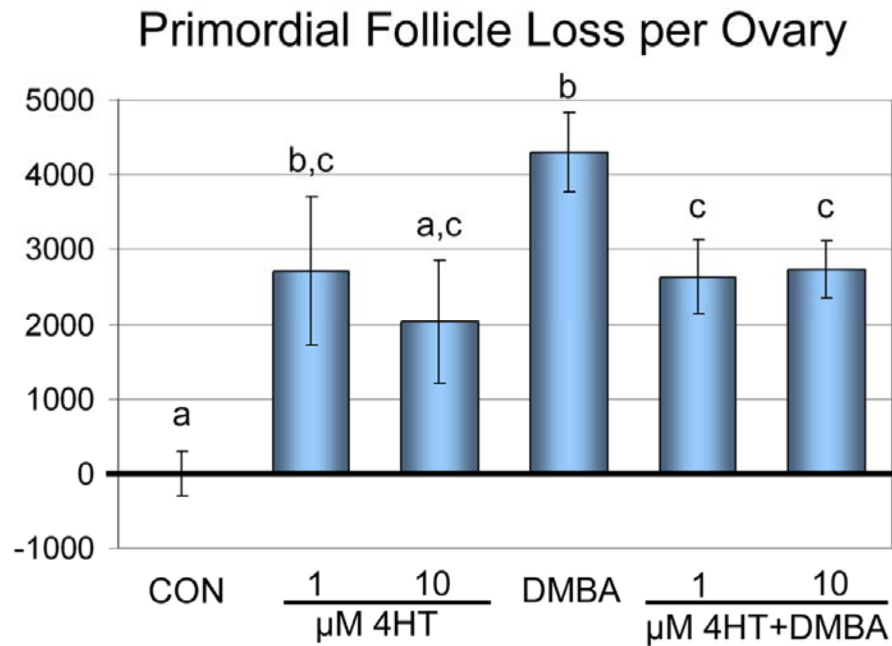


Figure 7: Numbers of primordial follicle loss relative to vehicle-treated controls in ovaries exposed to vehicle (CON), 4-hydroxytamoxifen (1 or 10 µM 4HT), DMBA or DMBA+TAM. DMBA induced follicle loss compared with controls and the addition of tamoxifen at both doses inhibited this depletion. Tamoxifen at 1 µM elevated the number of lost follicles while 10 µM did not affect follicle numbers. Different letters indicate significant differences among different treatment groups.

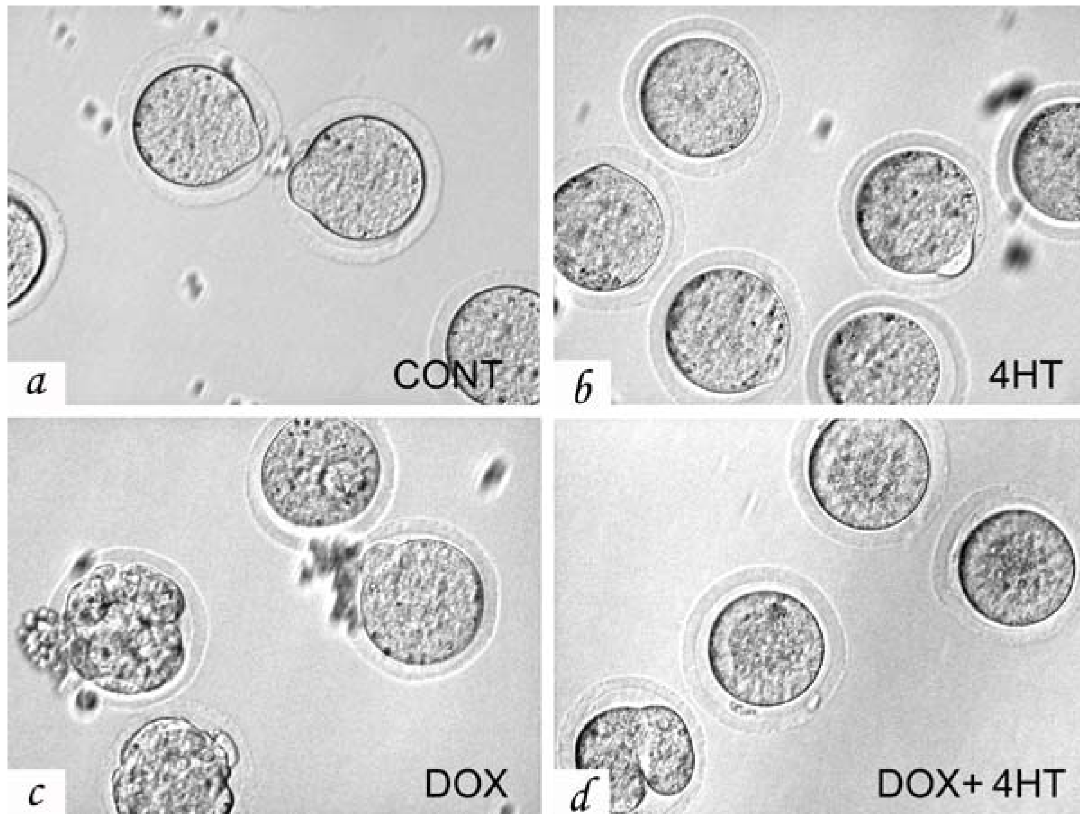


Figure 8: Mature oocytes exposed to vehicle (a), 4-hydroxytamoxifen (4HT, b), doxorubicin (DOX, c), or DOX+4HT (d) for 26 hours. Healthy oocytes surround by zona pellucida were observed after vehicle and 4HT treatment (a, b). Doxorubicin induces fragmentation in cultured oocytes (c), an effect that is antagonized by the addition of 4HT (d).

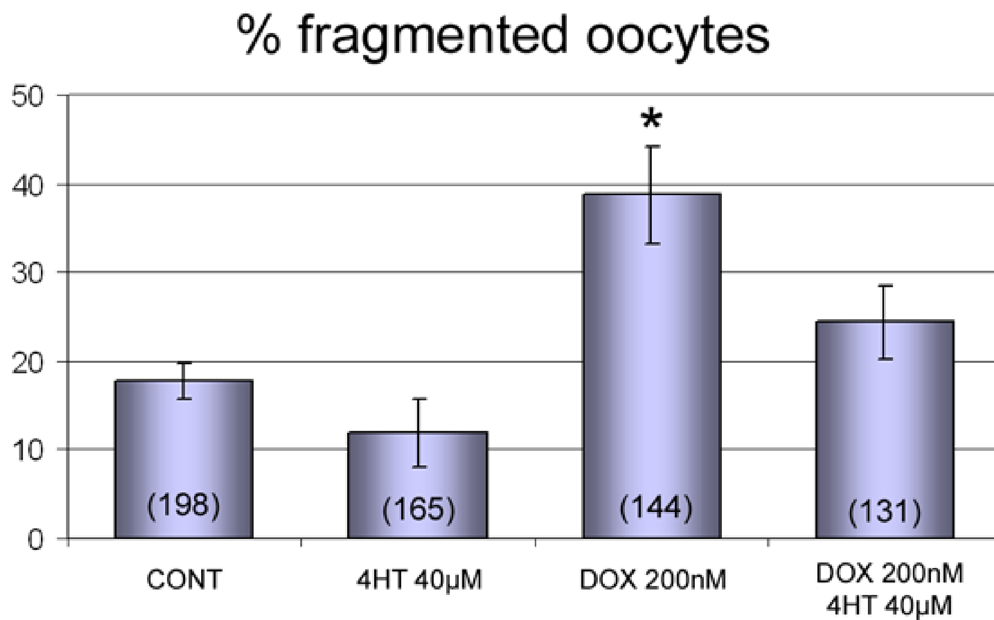


Figure 9: Quantitative analysis of the percentage of oocytes undergoing fragmentation. Oocytes incubated with doxorubicin (DOX) showed increased fragmentation rate compared with vehicle-treated controls. This elevated fragmentation rate was inhibited by the addition of 4-hydroxytamoxifen. The total number of oocytes cultured in each treatment group is indicated in parentheses inside the respective bar. * indicates a significant difference in comparison to vehicle-treated controls.

CHAPTER V

OVERALL CONCLUSION AND DISCUSSION

Feasibility and rationale for dual cancer prevention:

To overcome the difficulties of ovarian cancer prevention, our laboratory proposed a strategy that prevents breast and ovarian cancer simultaneously. This novel idea takes advantage of the fact that women at elevated risk for breast cancer are often also at elevated risk for ovarian cancer suggesting intertwined etiologies and possible common prevention targets. We developed a rat model that exhibits concurrent mammary and ovarian cancer and used this to screen drug candidates that may decrease risks for both cancers. Although the effects of chemoprevention drugs can be tested separately in preclinical models, this approach ignores the intertwined etiologies of breast and ovarian cancer as well as potential synergism between effects in the breast and ovary of prevention drugs. Our data showed for the first time that the combination of systemic E_2 and local ovarian application of DMBA induced mammary and ovarian cancer simultaneously and effectively in the rat [171]. This model produced 100% mammary preneoplasia/ neoplasia and 50% ovarian preneoplasia following 6 months of treatment.

Tamoxifen is not suitable as a dual cancer prevention therapy for breast and ovarian cancer, however, it does not accelerate ovarian cancer progression:

Clinically, tamoxifen was first approved by the FDA as a contraceptive agent in the 1970s, but this application was later abandoned. Currently, tamoxifen is used to treat hormonal-responsive breast cancer, to reduce breast cancer risk and is currently the most prescribed cancer drug worldwide [165]. However, the effect of tamoxifen on

ovarian cancer risk is still unclear. In premenopausal women, tamoxifen and other SERMs have been shown to promote abnormal ovarian function and cystic changes (follicular) in the ovary; therefore, many researchers have suggested that the use of tamoxifen may be associated with increased risk for ovarian cancer [32,33,57,58]. Using the dual cancer model, we showed that tamoxifen, while preventing mammary carcinogenesis, did not retard ovarian cancer progression indicating that tamoxifen is not suitable for use as a simultaneous prevention agent to reduce risks of both breast and ovarian cancer [196]. Interestingly, tamoxifen did not accelerate the progression towards ovarian cancer in this model supporting the safety of the use of tamoxifen to reduce breast cancer risk in a population that are very likely to predispose to elevated ovarian cancer risk [196].

Other drug candidates that may simultaneously prevent breast and ovarian cancer:
In the future, our dual cancer model will continue to be used for the screening of drug candidates that may simultaneously decrease breast and ovarian cancer risks. One potential candidate that may soon be tested for its dual prevention efficacy is letrozole, an aromatase inhibitor. Aromatase is responsible for the majority of estrogen synthesis and estrogen plays a critical role in both breast and ovarian carcinogenesis. In addition, prolonged use of hormone replacement therapy is associated with increased breast as well as ovarian cancer risk [215-217]. While tamoxifen partially antagonizes estrogen's action by working at the level of receptors,

aromatase inhibitors, on the other hand, block estrogen production and may offer the best efficacy of hormonal responsive breast and ovarian cancer prevention [218].

Results from the IBIS-II (International Breast Cancer Intervention Study) trial have confirmed the efficacy of aromatase inhibitors in preventing breast cancer in postmenopausal women, with fewer undesirable side effects such as endometrial cancer or thromboembolic events caused by the use of tamoxifen [219,220]. Aromatase inhibitors have been associated with bone density loss and fracture from profound estrogen depletion. However, these symptoms are usually manageable with supplemental hormones and biophosphonates [221]. The potential of using aromatase inhibitors to prevent ovarian cancer while not clear, is supported by studies showing their ability to sensitize ovarian tumor cells to chemotherapy and prevent recurrent ovarian cancer [222].

In our studies, tamoxifen did not prevent ovarian cancer progression and is therefore not suitable for dual cancer prevention. Letrozole, based on its mechanism of action, may serve as a plausible candidate to lower risks for both breast and ovarian cancer. The potential efficacy of letrozole to prevent both cancers should be tested using our dual cancer model.

The use of tamoxifen to preserve fertility in premenopausal women undergoing cytotoxic chemotherapy treatment regimen:

While examining the ovary of our dual cancer model treated with tamoxifen, we serendipitously discovered a novel protective effect of tamoxifen against DMBA-

induced follicle loss in the ovary. From several follow-up studies, we were able to show for the first time that tamoxifen inhibits DMBA, cyclophosphamide and doxorubicin-induced ovotoxicity in the rat. These results suggest a potential therapeutic intervention with tamoxifen to preserve fertility in premenopausal women undergoing chemotherapy. Currently, the only established fertility preservation method for women undergoing chemotherapy is embryo cryopreservation. This method is expensive, invasive, and requires a partner and, more importantly, it requires a delay in cancer treatment by several weeks to months.

One experimental protocol is the use of GnRH agonists to protect the ovary against chemotherapy-induced toxicity by disrupting hypothalamic-pituitary-ovarian axis and suppressing ovarian function [223]. GnRH agonists have been shown to be effective in rodents and non-human primates [122], but its efficacy to protect the ovary in women undergoing chemotherapy is still controversial [125,224]. In contrast to GnRH agonist-induced systemic hypogonadism which was ineffective in human trials to preserve fertility, our results demonstrated that tamoxifen seems to protect the ovary in part through local mechanisms suggesting an alternative action by tamoxifen to protect ovarian follicles.

Possible mechanisms of action by which tamoxifen inhibit environmental toxicant- and chemotherapy-induced follicle loss:

The mechanism responsible for the protective effect of tamoxifen when rescuing ovarian follicles from DMBA and cyclophosphamide-induced toxicity is still unclear

and needs further investigation. We speculate that the observed results may be a combined effect of local and systemic actions induced by tamoxifen. Systemically, tamoxifen may act similarly to GnRH agonists, inactivating normal ovarian function and therefore preserving a quiescent follicular reserve. Locally, tamoxifen may prevent toxicant-induced follicular loss by inhibiting follicular apoptosis and disrupting drug transfer into the ovary.

Apoptosis-driven ovarian damage has been thought to play a critical role in oocyte destruction and premature ovarian failure caused by environmental toxicants and chemotherapy [129,225-227]. Chemotherapy induces elevated levels of pro-apoptotic molecules such as caspase-2/3 and Bax, and decreased levels of the anti-apoptotic marker Bcl-2 in ovarian follicles [228,229]. Interestingly, E_2 has been shown to protect against 4-vinylcyclohexene diepoxide-induced follicle loss in the ovary due to anti-oxidant and anti-apoptosis properties including decreasing levels of caspase-3 [212]. Of course, E_2 is tumorigenic in several tissues and the use of E_2 to preserve fertility in cancer patients is unfeasible. Tamoxifen, when it promotes selective estrogenic actions, also has anti-oxidant properties that contribute to the protective effect of tamoxifen in the cardiovascular system [214,230]. Therefore, tamoxifen may promote estrogen-like actions in ovarian follicles and offer similar protection against cyclophosphamide-induced ovotoxicity without the carcinogenic effect of E_2 in women. In addition, tamoxifen has been shown to alter levels of aldehyde dehydrogenase, an enzyme that is responsible for the conversion of retinol to retinoic acid [231]. Retinoic acid has been identified as a potent survival factor for

female germ cells in rodents [232]. Tamoxifen may therefore promote survival of oocytes and prevent chemotherapy-induced follicle loss by affecting the retinoic acid pathway coupled with estrogenic anti-oxidant effects, in a tissue specific manner.

Tamoxifen may also protect the ovary by inhibiting drug transfer to the ovary. Cyclophosphamide is a prodrug that is metabolized to active 4-hydroxycyclophosphamide (4HC) by hepatic CYP enzymes; therefore, it can not be metabolized in the ovary and the only route for its active metabolite's delivery to the ovary is via blood supply [233]. Tamoxifen may suppress normal ovarian function and the lack of folliculogenesis may lead to diminished blood flow to the ovary resulting in local hypoxia and decreased 4HC accumulation in the ovary [234]. Tamoxifen may also inhibit toxicant accumulation in the ovary by altering levels of p-glycoprotein. P-glycoprotein is a membrane pump located in the wall of ovarian capillaries and is responsible for detoxification and steroid transport [235]. In cancer cells, p-glycoprotein is responsible for pumping chemotherapy drugs out of the cell and causes multi-drug resistance [236]. Tamoxifen has been shown in preclinical and *in vitro* studies to down regulate p-glycoprotein and reverse this phenotype assuming that down regulation of p-glycoprotein is associated with decreased exposure to chemotherapy [201,237,238]. In addition, tamoxifen may inhibit drug transfer to the oocyte by moderating the expression of gap junctions such as connexin 43 and 37. Connexin 43 and 37 form granulosa-to-granulosa and granulosa-to-oocyte communications, respectively, and are essential for follicular survival and development [239,240]. A reduction in the expression of these gap junctions may

interrupt drug transport into the oocyte resulting in decreased drug accumulation in these cells. Elucidation of the mechanism responsible for tamoxifen protection of ovarian follicles will be crucial to development of novel strategies for fertility preservation following chemotherapy in females.

Radiation-induced ovotoxicity:

Both chemotherapy and radiation therapy destroy ovarian follicles and cause premature reproductive aging. Whether tamoxifen can also protect ovarian follicles from radiation-induced damage is likely to be dependent upon tamoxifen's mechanism of action. If tamoxifen protects ovarian follicles only by suppressing ovarian function and keeping primordial follicles quiescent, radiation therapy while less effectively will still damage DNA in oocytes and granulosa cells directly and cause follicular death. If tamoxifen rescues ovarian follicles by altering cell-to-cell communication within a follicle, or expression of membrane pumps responsible for cyclophosphamide transport, it may still fail to protect follicles from radiation-induced injury.

However, if tamoxifen reduces blood flow to the ovary, therefore reducing local oxygen concentrations, it may be able to weaken radiation-induced ovarian damage in comparison to ovaries under normal oxygen conditions. Oxygen is a potent radiosensitizer that increases the effectiveness of a given dose of radiation by forming DNA-damaging free radicals [241,242]. Studies have shown that tumor cells in a hypoxic environment are more resistant to radiation damage than those in a

normal oxygen environment [243,244]. Alternatively, if tamoxifen protects ovarian follicles by inhibiting apoptosis, while not blocking radiation-induced DNA damage, it may allow additional DNA repair mechanisms to take place and fix the damaged DNA [245-247]. However, if this happens, the integrity of the germline DNA following repair will need to be examined carefully.

Safety issues of using tamoxifen as fertility preservation treatment:

Tamoxifen has been prescribed to patients with hormonal responsive breast cancer for the past 30 years and has a very extensive safety record [211,248]. The recommended continuous use of tamoxifen (20mg/day) has been limited to 5 years due to the adverse side effects (i.e. blood clots, stroke, and uterine cancer) observed with longer usage [165]. Typical chemotherapy regimens, such as CMF (cyclophosphamide + methotrexate + 5-fluorouracil) and CHOP (cyclophosphamide + doxorubicin + prednisone + vincristine) are usually given in cycles over a period of 3-6 months. Results from our studies suggest that a concurrent administration of tamoxifen with cytotoxic chemotherapy for the duration of the chemotherapy regimen will protect ovarian follicles from destruction and therefore preserve fertility. In premenopausal women, short term (<2 years) tamoxifen has been shown to induce menopausal symptoms and irregular menstruation, and these symptoms are reversible upon drug withdrawal.

Furthermore, a major concern of the use of any adjuvant treatment is its potential to counteract or interfere with the efficacy of standard chemotherapy.

Tamoxifen has actually been suggested to increase the efficacy of chemotherapy in animal models due to the ability of tamoxifen to downregulate p-glycoprotein, a membrane transporter that is responsible for multi-drug resistant phenotype in cancer cells. This potential effect of tamoxifen was tested in phase III trials without establishing such benefit [201], nonetheless, results from these trials showed that even high dose tamoxifen has little effect on the toxicities and pharmacokinetics of standard cancer chemotherapy.

Translational use of tamoxifen for fertility preservation; from bench side to bedside:

Results from our studies suggest a novel use of tamoxifen for fertility preservation in premenopausal women undergoing cancer chemotherapy. While tamoxifen has been used for cancer treatment for more than 3 decades and has been safely tested as concurrent cancer treatment, past human studies focused on older women and ignored any reproductive endpoints. The translation of this preclinical research into clinical trials should be facilitated by the established use and safety of tamoxifen with and without standard chemotherapy. This novel approach for the use of tamoxifen in preserving fertility can also be easily tested by adding reproductive endpoints (serum hormone concentrations, regularity of menstrual cycle, ability to conceive) to existing clinical trials testing the effect of tamoxifen as adjuvant chemotherapy in young female patients undergoing cytotoxic cancer therapy.

More than 100,000 women under the age of 45 are diagnosed with cancer each year in the United States and 50-80% of these patients that receive cytotoxic chemotherapy suffer from premature ovarian failure and infertility. Previously, this infertility and iatrogenic menopause was viewed by physicians and patients as an acceptable cost of curative chemotherapy and radiation regimens. However, with early diagnosis and as more women survive their cancers and strive to resume normal family life, cancer therapy-induced infertility has become a major survivorship issue. Our result, for the first time, showed that tamoxifen prevents chemotherapy-induced follicle destruction and may offer a dramatic clinical benefit to preserve fertility in young female patients undergoing chemotherapy or even radiation therapy.

REFERENCES

1. AmericanCancerSociety (2009) Cancer Facts and Figures 2009.

2. Gershenson, D.M., Tortolero-Luna, G., Malpica, A., Baker, V.V., Whittaker, L., Johnson, E. and Follen Mitchell, M. (1996) Ovarian intraepithelial neoplasia and ovarian cancer. *Obstet Gynecol Clin North Am*, **23**, 475-543.

3. Metcalfe, K.A., Lynch, H.T., Ghadirian, P., Tung, N., Olivotto, I.A., Foulkes, W.D., Warner, E., Olopade, O., Eisen, A., Weber, B., McLennan, J., Sun, P. and Narod, S.A. (2005) The risk of ovarian cancer after breast cancer in BRCA1 and BRCA2 carriers. *Gynecol Oncol*, **96**, 222-6.

4. Brekelmans, C.T. (2003) Risk factors and risk reduction of breast and ovarian cancer. *Curr Opin Obstet Gynecol*, **15**, 63-8.

5. Dahl, E., Sadr-Nabavi, A., Klopocki, E., Betz, B., Grube, S., Kreutzfeld, R., Himmelfarb, M., An, H.X., Gelling, S., Klaman, I., Hinzmann, B., Kristiansen, G., Grutzmann, R., Kuner, R., Petschke, B., Rhiem, K., Wiechen, K., Sers, C., Wiestler, O., Schneider, A., Hofler, H., Nahrig, J., Dietel, M., Schafer, R., Rosenthal, A., Schmutzler, R., Durst, M., Meindl, A. and Niederacher, D. (2005) Systematic identification and molecular characterization of genes differentially expressed in breast and ovarian cancer. *J Pathol*, **205**, 21-8.

6. Narod, S., Ford, D., Devilee, P., Barkardottir, R.B., Eyfjord, J., Lenoir, G., Serova, O., Easton, D. and Goldgar, D. (1995) Genetic heterogeneity of

breast-ovarian cancer revisited. Breast Cancer Linkage Consortium. *Am J Hum Genet*, **57**, 957-8.

7. Ford, D., Easton, D.F., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D.T., Weber, B., Lenoir, G., Chang-Claude, J., Sobol, H., Teare, M.D., Struewing, J., Arason, A., Scherneck, S., Peto, J., Rebbeck, T.R., Tonin, P., Neuhausen, S., Barkardottir, R., Eyfjord, J., Lynch, H., Ponder, B.A., Gayther, S.A., Zelada-Hedman, M. and et al. (1998) Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *Am J Hum Genet*, **62**, 676-89.

8. Risch, H.A., McLaughlin, J.R., Cole, D.E., Rosen, B., Bradley, L., Kwan, E., Jack, E., Vesprini, D.J., Kuperstein, G., Abrahamson, J.L., Fan, I., Wong, B. and Narod, S.A. (2001) Prevalence and penetrance of germline BRCA1 and BRCA2 mutations in a population series of 649 women with ovarian cancer. *Am J Hum Genet*, **68**, 700-10.

9. Easton, D.F., Ford, D. and Bishop, D.T. (1995) Breast and ovarian cancer incidence in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. *Am J Hum Genet*, **56**, 265-71.

10. Eeles, R.A. (2000) Future possibilities in the prevention of breast cancer: intervention strategies in BRCA1 and BRCA2 mutation carriers. *Breast Cancer Res*, **2**, 283-90.

11. Trichopoulos, D., MacMahon, B. and Cole, P. (1972) Menopause and breast cancer risk. *J Natl Cancer Inst*, **48**, 605-13.

12. Mogren, I., Stenlund, H. and Hogberg, U. (2001) Long-term impact of reproductive factors on the risk of cervical, endometrial, ovarian and breast cancer. *Acta Oncol*, **40**, 849-54.

13. Neale, R.E., Darlington, S., Murphy, M.F., Silcocks, P.B., Purdie, D.M. and Talback, M. (2005) The effects of twins, parity and age at first birth on cancer risk in Swedish women. *Twin Res Hum Genet*, **8**, 156-62.

14. Marchbanks, P.A., McDonald, J.A., Wilson, H.G., Folger, S.G., Mandel, M.G., Daling, J.R., Bernstein, L., Malone, K.E., Ursin, G., Strom, B.L., Norman, S.A., Wingo, P.A., Burkman, R.T., Berlin, J.A., Simon, M.S., Spirtas, R. and Weiss, L.K. (2002) Oral contraceptives and the risk of breast cancer. *N Engl J Med*, **346**, 2025-32.

15. Walker, G.R., Schlesselman, J.J. and Ness, R.B. (2002) Family history of cancer, oral contraceptive use, and ovarian cancer risk. *Am J Obstet Gynecol*, **186**, 8-14.

16. (1987) The reduction in risk of ovarian cancer associated with oral-contraceptive use. The Cancer and Steroid Hormone Study of the Centers for Disease Control and the National Institute of Child Health and Human Development. *N Engl J Med*, **316**, 650-5.

17. Li, C.I., Moe, R.E. and Daling, J.R. (2003) Risk of mortality by histologic type of breast cancer among women aged 50 to 79 years. *Arch Intern Med*, **163**, 2149-53.

18. Li, C.I., Anderson, B.O., Daling, J.R. and Moe, R.E. (2003) Trends in incidence rates of invasive lobular and ductal breast carcinoma. *Jama*, **289**, 1421-4.

19. Li, C.I., Uribe, D.J. and Daling, J.R. (2005) Clinical characteristics of different histologic types of breast cancer. *Br J Cancer*, **93**, 1046-52.

20. London, S.J., Connolly, J.L., Schnitt, S.J. and Colditz, G.A. (1992) A prospective study of benign breast disease and the risk of breast cancer. *Jama*, **267**, 941-4.

21. Carter, C.L., Corle, D.K., Micozzi, M.S., Schatzkin, A. and Taylor, P.R. (1988) A prospective study of the development of breast cancer in 16,692 women with benign breast disease. *Am J Epidemiol*, **128**, 467-77.

22. Wang, J., Costantino, J.P., Tan-Chiu, E., Wickerham, D.L., Paik, S. and Wolmark, N. (2004) Lower-category benign breast disease and the risk of invasive breast cancer. *J Natl Cancer Inst*, **96**, 616-20.

23. Dupont, W.D. and Page, D.L. (1985) Risk factors for breast cancer in women with proliferative breast disease. *N Engl J Med*, **312**, 146-51.

24. Dupont, W.D., Parl, F.F., Hartmann, W.H., Brinton, L.A., Winfield, A.C., Worrell, J.A., Schuyler, P.A. and Plummer, W.D. (1993) Breast cancer risk

associated with proliferative breast disease and atypical hyperplasia. *Cancer*, **71**, 1258-65.

25. Fabian, C.J., Kimler, B.F., Zalles, C.M., Klemp, J.R., Kamel, S., Zeiger, S. and Mayo, M.S. (2000) Short-term breast cancer prediction by random periareolar fine-needle aspiration cytology and the Gail risk model. *J Natl Cancer Inst*, **92**, 1217-27.

26. Society, A.C. (2007) Cancer Facts and Figures 2007. . *Atlanta: American Cancer Society*.

27. Fabian, C.J. and Kimler, B.F. (2005) Selective estrogen-receptor modulators for primary prevention of breast cancer. *J Clin Oncol*, **23**, 1644-55.

28. Higgins, S.A., Matloff, E.T., Rimm, D.L., Dziura, J., Haffty, B.G. and King, B.L. (2005) Patterns of reduced nipple aspirate fluid production and ductal lavage cellularity in women at high risk for breast cancer. *Breast Cancer Res*, **7**, R1017-22.

29. Khan, Q.J., Kimler, B.F., Clark, J., Metheny, T., Zalles, C.M. and Fabian, C.J. (2005) Ki-67 expression in benign breast ductal cells obtained by random periareolar fine needle aspiration. *Cancer Epidemiol Biomarkers Prev*, **14**, 786-9.

30. NationalCancerInstitute (2009) Current breast cancer prevention trials. cancer .gov.

31. Vogel, V.G., Costantino, J.P., Wickerham, D.L., Cronin, W.M., Cecchini, R.S., Atkins, J.N., Bevers, T.B., Fehrenbacher, L., Pajon, E.R., Jr., Wade, J.L.,

3rd, Robidoux, A., Margolese, R.G., James, J., Lippman, S.M., Runowicz, C.D., Ganz, P.A., Reis, S.E., McCaskill-Stevens, W., Ford, L.G., Jordan, V.C. and Wolmark, N. (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *Jama*, **295**, 2727-41.

32. Metindir, J., Aslan, S. and Bilir, G. (2005) Ovarian cyst formation in patients using tamoxifen for breast cancer. *Jpn J Clin Oncol*, **35**, 607-11.

33. Cohen, I., Rosen, D.J., Altaras, M., Beyth, Y., Shapira, J. and Yigael, D. (1994) Tamoxifen treatment in premenopausal breast cancer patients may be associated with ovarian overstimulation, cystic formations and fibroid overgrowth. *Br J Cancer*, **69**, 620-1.

34. Casagrande, J.T., Louie, E.W., Pike, M.C., Roy, S., Ross, R.K. and Henderson, B.E. (1979) "Incessant ovulation" and ovarian cancer. *Lancet*, **2**, 170-3.

35. Murdoch, W.J. (2005) Carcinogenic potential of ovulatory genotoxicity. *Biol Reprod*, **73**, 586-90.

36. Fathalla, M.F. (1971) Incessant ovulation--a factor in ovarian neoplasia? *Lancet*, **2**, 163.

37. Harlow, B.L., Cramer, D.W., Baron, J.A., Titus-Ernstoff, L. and Greenberg, E.R. (1998) Psychotropic medication use and risk of epithelial ovarian cancer. *Cancer Epidemiol Biomarkers Prev*, **7**, 697-702.

38. Risch, H.A. (1998) Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. *J Natl Cancer Inst*, **90**, 1774-86.

39. Cramer, D.W. and Welch, W.R. (1983) Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. *J Natl Cancer Inst*, **71**, 717-21.

40. Hoskins, W.J. (1995) Prospective on ovarian cancer: why prevent? *J Cell Biochem Suppl*, **23**, 189-99.

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

42. Collaborative Group on Epidemiological Studies of Ovarian, C., Beral, V., Doll, R., Hermon, C., Peto, R. and Reeves, G. (2008) Ovarian cancer and oral contraceptives: collaborative reanalysis of data from 45 epidemiological studies including 23,257 women with ovarian cancer and 87,303 controls. *Lancet*, **371**, 303-14.

43. Schildkraut, J.M., Calingaert, B., Marchbanks, P.A., Moorman, P.G. and Rodriguez, G.C. (2002) Impact of progestin and estrogen potency in oral contraceptives on ovarian cancer risk. *J Natl Cancer Inst*, **94**, 32-8.

44. Rosenberg, L., Palmer, J.R., Zauber, A.G., Warshauer, M.E., Lewis, J.L., Jr., Strom, B.L., Harlap, S. and Shapiro, S. (1994) A case-control study of oral contraceptive use and invasive epithelial ovarian cancer. *Am J Epidemiol*, **139**, 654-61.

45. Bu, S.Z., Yin, D.L., Ren, X.H., Jiang, L.Z., Wu, Z.J., Gao, Q.R. and Pei, G. (1997) Progesterone induces apoptosis and up-regulation of p53 expression in human ovarian carcinoma cell lines. *Cancer*, **79**, 1944-50.

46. Ivarsson, K., Sundfeldt, K., Brannstrom, M. and Janson, P.O. (2001) Production of steroids by human ovarian surface epithelial cells in culture: possible role of progesterone as growth inhibitor. *Gynecol Oncol*, **82**, 116-21.

47. De Palo, G., Mariani, L., Camerini, T., Marubini, E., Formelli, F., Pasini, B., Decensi, A. and Veronesi, U. (2002) Effect of fenretinide on ovarian carcinoma occurrence. *Gynecol Oncol*, **86**, 24-7.

48. Veronesi, U., De Palo, G., Marubini, E., Costa, A., Formelli, F., Mariani, L., Decensi, A., Camerini, T., Del Turco, M.R., Di Mauro, M.G., Muraca, M.G., Del Vecchio, M., Pinto, C., D'Aiuto, G., Boni, C., Campa, T., Magni, A., Miceli, R., Perloff, M., Malone, W.F. and Sporn, M.B. (1999) Randomized trial of fenretinide to prevent second breast malignancy in women with early breast cancer. *J Natl Cancer Inst*, **91**, 1847-56.

49. Singhal, P.K., Spiegel, G., Driscoll, D., Odunsi, K., Lele, S. and Rodabaugh, K.J. (2005) Cyclooxygenase 2 expression in serous tumors of the ovary. *Int J Gynecol Pathol*, **24**, 62-6.

50. Denkert, C., Kobel, M., Pest, S., Koch, I., Berger, S., Schwabe, M., Siegert, A., Reles, A., Klosterhalfen, B. and Hauptmann, S. (2002) Expression of cyclooxygenase 2 is an independent prognostic factor in human ovarian carcinoma. *Am J Pathol*, **160**, 893-903.

51. Daikoku, T., Tranguch, S., Trofimova, I.N., Dinulescu, D.M., Jacks, T., Nikitin, A.Y., Connolly, D.C. and Dey, S.K. (2006) Cyclooxygenase-1 is overexpressed in multiple genetically engineered mouse models of epithelial ovarian cancer. *Cancer Res*, **66**, 2527-31.

52. Li, S., Miner, K., Fannin, R., Carl Barrett, J. and Davis, B.J. (2004) Cyclooxygenase-1 and 2 in normal and malignant human ovarian epithelium. *Gynecol Oncol*, **92**, 622-7.

53. Harris, R.E., Beebe-Donk, J., Doss, H. and Burr Doss, D. (2005) Aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs in cancer prevention: a critical review of non-selective COX-2 blockade (review). *Oncol Rep*, **13**, 559-83.

54. Rodriguez-Burford, C., Barnes, M.N., Oelschlager, D.K., Myers, R.B., Talley, L.I., Partridge, E.E. and Grizzle, W.E. (2002) Effects of nonsteroidal anti-inflammatory agents (NSAIDs) on ovarian carcinoma cell lines: preclinical evaluation of NSAIDs as chemopreventive agents. *Clin Cancer Res*, **8**, 202-9.

55. (2006) Cardiovascular and cerebrovascular events in the randomized, controlled Alzheimer's Disease Anti-Inflammatory Prevention Trial (ADAPT). *PLoS Clin Trials*, **1**, e33.

56. Shushan, A., Peretz, T., Uziely, B., Lewin, A. and Mor-Yosef, S. (1996) Ovarian cysts in premenopausal and postmenopausal tamoxifen-treated women with breast cancer. *Am J Obstet Gynecol*, **174**, 141-4.

57. Ravdin, P.M., Fritz, N.F., Tormey, D.C. and Jordan, V.C. (1988) Endocrine status of premenopausal node-positive breast cancer patients following adjuvant chemotherapy and long-term tamoxifen. *Cancer Res*, **48**, 1026-9.

58. Neven, P., Goldstein, S.R., Ciaccia, A.V., Zhou, L., Silfen, S.L. and Muram, D. (2002) The effect of raloxifene on the incidence of ovarian cancer in postmenopausal women. *Gynecol Oncol*, **85**, 388-90.

59. MacMahon, B. (2006) Epidemiology and the causes of breast cancer. *Int J Cancer*, **118**, 2373-8.

60. Thompson, H.J., McGinley, J.N., Rothhammer, K. and Singh, M. (1995) Rapid induction of mammary intraductal proliferations, ductal carcinoma in situ and carcinomas by the injection of sexually immature female rats with 1-methyl-1-nitrosourea. *Carcinogenesis*, **16**, 2407-11.

61. Dao, T.L. (1969) Mammary cancer induction by 7,12-dimethylbenz(a)anthracene: relation to age. *Science*, **165**, 810-1.

62. Li, J.J., Papa, D., Davis, M.F., Weroha, S.J., Aldaz, C.M., El-Bayoumy, K., Ballenger, J., Tawfik, O. and Li, S.A. (2002) Ploidy differences between hormone- and chemical carcinogen-induced rat mammary neoplasms: comparison to invasive human ductal breast cancer. *Mol Carcinog*, **33**, 56-65.

63. Gullino, P.M., Pettigrew, H.M. and Grantham, F.H. (1975) N-nitrosomethylurea as mammary gland carcinogen in rats. *J Natl Cancer Inst*, **54**, 401-14.

64. Nishida, T., Sugiyama, T., Kataoka, A., Ushijima, K. and Yakushiji, M. (1998) Histologic characterization of rat ovarian carcinoma induced by intraovarian insertion of a 7,12-dimethylbenz[a]anthracene-coated suture: common epithelial tumors of the ovary in rats? *Cancer*, **83**, 965-70.

65. Stewart, S.L., Querec, T.D., Ochman, A.R., Gruver, B.N., Bao, R., Babb, J.S., Wong, T.S., Koutroukides, T., Pinnola, A.D., Klein-Szanto, A., Hamilton, T.C. and Patriotis, C. (2004) Characterization of a carcinogenesis rat model of ovarian preneoplasia and neoplasia. *Cancer Res*, **64**, 8177-83.

66. Crist, K.A., Zhang, Z., You, M., Gunning, W.T., Conran, P.B., Steele, V.E. and Lubet, R.A. (2005) Characterization of rat ovarian adenocarcinomas developed in response to direct instillation of 7,12-dimethylbenz[a]anthracene (DMBA) coated suture. *Carcinogenesis*, **26**, 951-7.

67. Kelloff, G.J., Boone, C.W., Crowell, J.A., Nayfield, S.G., Hawk, E., Malone, W.F., Steele, V.E., Lubet, R.A. and Sigman, C.C. (1996) Risk biomarkers and current strategies for cancer chemoprevention. *J Cell Biochem Suppl*, **25**, 1-14.

68. Fabian, C.J., Kimler, B.F., Anderson, J., Tawfik, O.W., Mayo, M.S., Burak, W.E., Jr., O'Shaughnessy, J.A., Albain, K.S., Hyams, D.M., Budd, G.T., Ganz, P.A., Sauter, E.R., Beenken, S.W., Grizzle, W.E., Fruehauf, J.P., Arneson, D.W., Bacus, J.W., Lagios, M.D., Johnson, K.A. and Browne, D. (2004) Breast cancer chemoprevention phase I evaluation of biomarker

modulation by arzoxifene, a third generation selective estrogen receptor modulator. *Clin Cancer Res*, **10**, 5403-17.

69. Esteva, F.J. and Hortobagyi, G.N. (2004) Prognostic molecular markers in early breast cancer. *Breast Cancer Res*, **6**, 109-18.

70. Gerdes, J., Lemke, H., Baisch, H., Wacker, H.H., Schwab, U. and Stein, H. (1984) Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol*, **133**, 1710-5.

71. Perrone, G., Santini, D., Vincenzi, B., Zagami, M., La Cesa, A., Bianchi, A., Altomare, V., Primavera, A., Battista, C., Vetrani, A., Tonini, G. and Rabitti, C. (2005) COX-2 expression in DCIS: correlation with VEGF, HER-2/neu, prognostic molecular markers and clinicopathological features. *Histopathology*, **46**, 561-8.

72. Vital-Reyes, V., Rodriguez-Burford, C., Chhieng, D.C., Oelschlager, D.K., Reyes-Fuentes, A., Barnes, M. and Grizzle, W.E. (2006) Celecoxib inhibits cellular growth, decreases ki-67 expression and modifies apoptosis in ovarian cancer cell lines. *Arch Med Res*, **37**, 689-95.

73. Shigemasa, K., Tian, X., Gu, L., Shiroyama, Y., Nagai, N. and Ohama, K. (2003) Expression of cyclooxygenase-2 and its relationship to p53 accumulation in ovarian adenocarcinomas. *Int J Oncol*, **22**, 99-105.

74. Daikoku, T., Wang, D., Tranguch, S., Morrow, J.D., Orsulic, S., DuBois, R.N. and Dey, S.K. (2005) Cyclooxygenase-1 is a potential target for prevention and treatment of ovarian epithelial cancer. *Cancer Res*, **65**, 3735-44.

75. Santillan, A., Garg, R., Zahurak, M.L., Gardner, G.J., Giuntoli, R.L., 2nd, Armstrong, D.K. and Bristow, R.E. (2005) Risk of epithelial ovarian cancer recurrence in patients with rising serum CA-125 levels within the normal range. *J Clin Oncol*, **23**, 9338-43.

76. Rosenthal, A.N., Menon, U. and Jacobs, I.J. (2006) Screening for ovarian cancer. *Clin Obstet Gynecol*, **49**, 433-47.

77. Tawfik, O.W., Kimler, B.F., Davis, M., Donahue, J.K., Persons, D.L., Fan, F., Hagemeister, S., Thomas, P., Connor, C., Jewell, W. and Fabian, C.J. (2006) Comparison of immunohistochemistry by automated cellular imaging system (ACIS) versus fluorescence in-situ hybridization in the evaluation of HER-2/neu expression in primary breast carcinoma. *Histopathology*, **48**, 258-67.

78. Lindgren, P.R., Cajander, S., Backstrom, T., Gustafsson, J.A., Makela, S. and Olofsson, J.I. (2004) Estrogen and progesterone receptors in ovarian epithelial tumors. *Mol Cell Endocrinol*, **221**, 97-104.

79. Smith, W.L., Garavito, R.M. and DeWitt, D.L. (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J Biol Chem*, **271**, 33157-60.

80. Oliveira, V.M., Piato, S. and Silva, M.A. (2005) Correlation of cyclooxygenase-2 and aromatase immunohistochemical expression in invasive ductal carcinoma, ductal carcinoma in situ, and adjacent normal epithelium. *Breast Cancer Res Treat*, 1-7.

81. Matsumoto, Y., Ishiko, O., Deguchi, M., Nakagawa, E. and Ogita, S. (2001) Cyclooxygenase-2 expression in normal ovaries and epithelial ovarian neoplasms. *Int J Mol Med*, **8**, 31-6.

82. Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A. and et al. (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*, **244**, 707-12.

83. Revillion, F., Bonneterre, J. and Peyrat, J.P. (1998) ERBB2 oncogene in human breast cancer and its clinical significance. *Eur J Cancer*, **34**, 791-808.

84. Karlan, B.Y. and McIntosh, M. (2007) The quest for ovarian cancer's Holy Grail: can CA-125 still be the chalice of early detection? *J Clin Oncol*, **25**, 1303-4.

85. Hermsen, B.B., von Mensdorff-Pouilly, S., Berkhof, J., van Diest, P.J., Gille, J.J., Menko, F.H., Blankenstein, M.A., Kenemans, P. and Verheijen, R.H. (2007) Serum CA-125 in relation to adnexal dysplasia and cancer in women at hereditary high risk of ovarian cancer. *J Clin Oncol*, **25**, 1383-9.

86. Raspollini, M.R., Amunni, G., Villanucci, A., Boddi, V. and Taddei, G.L. (2006) COX-2 and preoperative CA-125 level are strongly correlated with survival and clinical responsiveness to chemotherapy in ovarian cancer. *Acta Obstet Gynecol Scand*, **85**, 493-8.

87. Ginestier, C., Hur, M.H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., Jacquemier, J., Viens, P., Kleer, C.G., Liu, S., Schott, A., Hayes,

D., Birnbaum, D., Wicha, M.S. and Dontu, G. (2007) ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell*, **1**, 555-67.

88. Proia, D.A. and Kuperwasser, C. (2006) Reconstruction of human mammary tissues in a mouse model. *Nat Protoc*, **1**, 206-14.

89. Russo, J., Balogh, G.A., Chen, J., Fernandez, S.V., Fernbaugh, R., Heulings, R., Mailo, D.A., Moral, R., Russo, P.A., Sheriff, F., Vanegas, J.E., Wang, R. and Russo, I.H. (2006) The concept of stem cell in the mammary gland and its implication in morphogenesis, cancer and prevention. *Front Biosci*, **11**, 151-72.

90. Welm, B.E., Tepera, S.B., Venezia, T., Graubert, T.A., Rosen, J.M. and Goodell, M.A. (2002) Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. *Dev Biol*, **245**, 42-56.

91. Kordon, E.C. and Smith, G.H. (1998) An entire functional mammary gland may comprise the progeny from a single cell. *Development*, **125**, 1921-30.

92. Shackleton, M., Vaillant, F., Simpson, K.J., Stingl, J., Smyth, G.K., Asselin-Labat, M.L., Wu, L., Lindeman, G.J. and Visvader, J.E. (2006) Generation of a functional mammary gland from a single stem cell. *Nature*, **439**, 84-8.

93. Dontu, G., Abdallah, W.M., Foley, J.M., Jackson, K.W., Clarke, M.F., Kawamura, M.J. and Wicha, M.S. (2003) In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev*, **17**, 1253-70.

94. Dontu, G., Liu, S. and Wicha, M.S. (2005) Stem cells in mammary development and carcinogenesis: implications for prevention and treatment. *Stem Cell Rev*, **1**, 207-13.

95. Woodward, W.A., Chen, M.S., Behbod, F. and Rosen, J.M. (2005) On mammary stem cells. *J Cell Sci*, **118**, 3585-94.

96. Marx, J. (2003) Cancer research. Mutant stem cells may seed cancer. *Science*, **301**, 1308-10.

97. Fillmore, C.M. and Kuperwasser, C. (2008) Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res*, **10**, R25.

98. Hadnagy, A., Gaboury, L., Beaulieu, R. and Balicki, D. (2006) SP analysis may be used to identify cancer stem cell populations. *Exp Cell Res*, **312**, 3701-10.

99. Clayton, H., Titley, I. and Vivanco, M. (2004) Growth and differentiation of progenitor/stem cells derived from the human mammary gland. *Exp Cell Res*, **297**, 444-60.

100. Trosko, J.E. (2006) From adult stem cells to cancer stem cells: Oct-4 Gene, cell-cell communication, and hormones during tumor promotion. *Ann N Y Acad Sci*, **1089**, 36-58.

101. Tai, M.H., Chang, C.C., Kiupel, M., Webster, J.D., Olson, L.K. and Trosko, J.E. (2005) Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis. *Carcinogenesis*, **26**, 495-502.

102. Ferrell, C.M., Dorsam, S.T., Ohta, H., Humphries, R.K., Derynck, M.K., Haqq, C., Largman, C. and Lawrence, H.J. (2005) Activation of stem-cell specific genes by HOXA9 and HOXA10 homeodomain proteins in CD34+ human cord blood cells. *Stem Cells*, **23**, 644-55.

103. Shang, Y. and Brown, M. (2002) Molecular determinants for the tissue specificity of SERMs. *Science*, **295**, 2465-8.

104. Vogel, V.G. (2001) Reducing the risk of breast cancer with tamoxifen in women at increased risk. *J Clin Oncol*, **19**, 87S-92S.

105. Cohen, I., Figer, A., Tepper, R., Shapira, J., Altaras, M.M., Yigael, D. and Beyth, Y. (1999) Ovarian overstimulation and cystic formation in premenopausal tamoxifen exposure: comparison between tamoxifen-treated and nontreated breast cancer patients. *Gynecol Oncol*, **72**, 202-7.

106. Jordan, V.C. and O'Malley, B.W. (2007) Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. *J Clin Oncol*, **25**, 5815-24.

107. Yding Andersen, C., Rosendahl, M. and Byskov, A.G. (2008) Concentration of anti-Mullerian hormone and inhibin-B in relation to steroids and age in follicular fluid from small antral human follicles. *J Clin Endocrinol Metab*, **93**, 2344-9.

108. Long, G.G., Cohen, I.R., Gries, C.L., Young, J.K., Francis, P.C. and Capen, C.C. (2001) Proliferative lesions of ovarian granulosa cells and reversible

hormonal changes induced in rats by a selective estrogen receptor modulator. *Toxicol Pathol*, **29**, 719-26.

109. Rose, D.P. and Davis, T.E. (1980) Effects of adjuvant chemohormonal therapy on the ovarian and adrenal function of breast cancer patients. *Cancer Res*, **40**, 4043-7.

110. Warne, G.L., Fairley, K.F., Hobbs, J.B. and Martin, F.I. (1973) Cyclophosphamide-induced ovarian failure. *N Engl J Med*, **289**, 1159-62.

111. Simon, B., Lee, S.J., Partridge, A.H. and Runowicz, C.D. (2005) Preserving fertility after cancer. *CA Cancer J Clin*, **55**, 211-28; quiz 263-4.

112. Bines, J., Oleske, D.M. and Cobleigh, M.A. (1996) Ovarian function in premenopausal women treated with adjuvant chemotherapy for breast cancer. *J Clin Oncol*, **14**, 1718-29.

113. Blumenfeld, Z., Dann, E., Avivi, I., Epelbaum, R. and Rowe, J.M. (2002) Fertility after treatment for Hodgkin's disease. *Ann Oncol*, **13 Suppl 1**, 138-47.

114. Byrne, J., Fears, T.R., Gail, M.H., Pee, D., Connelly, R.R., Austin, D.F., Holmes, G.F., Holmes, F.F., Latourette, H.B., Meigs, J.W. and et al. (1992) Early menopause in long-term survivors of cancer during adolescence. *Am J Obstet Gynecol*, **166**, 788-93.

115. CenterforDiseaseControl (2004) Cancer Survivorship --- United States, 1971--2001. In Rowland, J. (ed.).

116. U.S.CensusBureau (2003) Fertility for women.

117. Pacey, A.A. (2007) Fertility issues in survivors from adolescent cancers. *Cancer Treat Rev*, **33**, 646-55.

118. Kim, S.S. (2006) Fertility preservation in female cancer patients: current developments and future directions. *Fertil Steril*, **85**, 1-11.

119. Sonmezer, M. and Oktay, K. (2006) Fertility preservation in young women undergoing breast cancer therapy. *Oncologist*, **11**, 422-34.

120. Lobo, R.A. (2005) Potential options for preservation of fertility in women. *N Engl J Med*, **353**, 64-73.

121. Blumenfeld, Z. (2008) GnRH-agonists in fertility preservation. *Curr Opin Endocrinol Diabetes Obes*, **15**, 523-8.

122. Ataya, K., Rao, L.V., Lawrence, E. and Kimmel, R. (1995) Luteinizing hormone-releasing hormone agonist inhibits cyclophosphamide-induced ovarian follicular depletion in rhesus monkeys. *Biol Reprod*, **52**, 365-72.

123. Blumenfeld, Z. (2002) Treatment with gonadotropin-releasing hormone agonists and prevention of multiple gestations in lupus or antiphospholipid syndrome patients undergoing in vitro fertilization: comment on the article by Guballa et al. *Arthritis Rheum*, **46**, 2542; author reply 2542.

124. Blumenfeld, Z. (2007) How to preserve fertility in young women exposed to chemotherapy? The role of GnRH agonist cotreatment in addition to cryopreservation of embrya, oocytes, or ovaries. *Oncologist*, **12**, 1044-54.

125. Oktay, K., Sonmezer, M., Oktem, O., Fox, K., Emons, G. and Bang, H. (2007) Absence of conclusive evidence for the safety and efficacy of gonadotropin-

releasing hormone analogue treatment in protecting against chemotherapy-induced gonadal injury. *Oncologist*, **12**, 1055-66.

126. Ataya, K., Pydyn, E., Ramahi-Ataya, A. and Orton, C.G. (1995) Is radiation-induced ovarian failure in rhesus monkeys preventable by luteinizing hormone-releasing hormone agonists?: Preliminary observations. *J Clin Endocrinol Metab*, **80**, 790-5.

127. Geyer, R.P., Bleisch, V.R., Bryant, J.E., Robbins, A.N., Saslaw, I.M. and Stare, F.J. (1951) Tumor production in rats injected intravenously with oil emulsions containing 9,10-dimethyl-1,2-benzanthracene. *Cancer Res*, **11**, 474-8.

128. Borman, S.M., Christian, P.J., Sipes, I.G. and Hoyer, P.B. (2000) Ovotoxicity in female Fischer rats and B6 mice induced by low-dose exposure to three polycyclic aromatic hydrocarbons: comparison through calculation of an ovotoxic index. *Toxicol Appl Pharmacol*, **167**, 191-8.

129. Matikainen, T., Perez, G.I., Jurisicova, A., Pru, J.K., Schlezinger, J.J., Ryu, H.Y., Laine, J., Sakai, T., Korsmeyer, S.J., Casper, R.F., Sherr, D.H. and Tilly, J.L. (2001) Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. *Nat Genet*, **28**, 355-60.

130. Tsai-Turton, M., Nakamura, B.N. and Luderer, U. (2007) Induction of apoptosis by 9,10-dimethyl-1,2-benzanthracene in cultured preovulatory rat

follicles is preceded by a rise in reactive oxygen species and is prevented by glutathione. *Biol Reprod*, **77**, 442-51.

131. Evens, A.M., Hutchings, M. and Diehl, V. (2008) Treatment of Hodgkin lymphoma: the past, present, and future. *Nat Clin Pract Oncol*, **5**, 543-56.

132. Hudis, C.A. and Schmitz, N. (2004) Dose-dense chemotherapy in breast cancer and lymphoma. *Semin Oncol*, **31**, 19-26.

133. Plowchalk, D.R. and Mattison, D.R. (1991) Phosphoramide mustard is responsible for the ovarian toxicity of cyclophosphamide. *Toxicol Appl Pharmacol*, **107**, 472-81.

134. Kumar, R., Biggart, J.D., McEvoy, J. and McGeown, M.G. (1972) Cyclophosphamide and reproductive function. *Lancet*, **1**, 1212-4.

135. Shiromizu, K., Thorgeirsson, S.S. and Mattison, D.R. (1984) Effect of cyclophosphamide on oocyte and follicle number in Sprague-Dawley rats, C57BL/6N and DBA/2N mice. *Pediatr Pharmacol (New York)*, **4**, 213-21.

136. Letterie, G.S. (2004) Anovulation in the prevention of cytotoxic-induced follicular attrition and ovarian failure. *Hum Reprod*, **19**, 831-7.

137. Jurisicova, A., Lee, H.J., D'Estaing, S.G., Tilly, J. and Perez, G.I. (2006) Molecular requirements for doxorubicin-mediated death in murine oocytes. *Cell Death Differ*, **13**, 1466-74.

138. Lopez, S.G. and Luderer, U. (2004) Effects of cyclophosphamide and buthionine sulfoximine on ovarian glutathione and apoptosis. *Free Radic Biol Med*, **36**, 1366-77.

139. Fabian, C.J., Kimler, B.F., Mayo, M.S. and Khan, S.A. (2005) Breast-tissue sampling for risk assessment and prevention. *Endocr Relat Cancer*, **12**, 185-213.

140. Swanson, S.M. and Unterman, T.G. (2002) The growth hormone-deficient Spontaneous Dwarf rat is resistant to chemically induced mammary carcinogenesis. *Carcinogenesis*, **23**, 977-82.

141. Anzalone, C.R., Hong, L.S., Lu, J.K. and LaPolt, P.S. (2001) Influences of age and ovarian follicular reserve on estrous cycle patterns, ovulation, and hormone secretion in the Long-Evans rat. *Biol Reprod*, **64**, 1056-62.

142. Chatterjee, A. and Greenwald, G.S. (1972) The long-term effects of unilateral ovariectomy of the cycling hamster and rat. *Biol Reprod*, **7**, 238-46.

143. Cotroneo, M.S., Wang, J., Fritz, W.A., Eltoum, I.E. and Lamartiniere, C.A. (2002) Genistein action in the prepubertal mammary gland in a chemoprevention model. *Carcinogenesis*, **23**, 1467-74.

144. Thompson, H.J. and Singh, M. (2000) Rat models of premalignant breast disease. *J Mammary Gland Biol Neoplasia*, **5**, 409-20.

145. Martin, A.M. and Weber, B.L. (2000) Genetic and hormonal risk factors in breast cancer. *J Natl Cancer Inst*, **92**, 1126-35.

146. Antoniou, A.C., Gayther, S.A., Stratton, J.F., Ponder, B.A. and Easton, D.F. (2000) Risk models for familial ovarian and breast cancer. *Genet Epidemiol*, **18**, 173-90.

147. Syed, V., Ulinski, G., Mok, S.C., Yiu, G.K. and Ho, S.M. (2001) Expression of gonadotropin receptor and growth responses to key reproductive hormones in normal and malignant human ovarian surface epithelial cells. *Cancer Res*, **61**, 6768-76.

148. Scully, R.E. (1995) Pathology of ovarian cancer precursors. *J Cell Biochem Suppl*, **23**, 208-18.

149. Feeley, K.M. and Wells, M. (2001) Precursor lesions of ovarian epithelial malignancy. *Histopathology*, **38**, 87-95.

150. Brewer, M.A., Ranger-Moore, J., Greene, M.H., Alberts, D.S., Liu, Y., Bartels, H.G., Baruch, A.C. and Bartels, P.H. (2004) Preneoplastic changes in ovarian tissues. *Anal Quant Cytol Histol*, **26**, 207-16.

151. Mittal, K.R., Zeleniuch-Jacquotte, A., Cooper, J.L. and Demopoulos, R.I. (1993) Contralateral ovary in unilateral ovarian carcinoma: a search for preneoplastic lesions. *Int J Gynecol Pathol*, **12**, 59-63.

152. Resta, L., Russo, S., Colucci, G.A. and Prat, J. (1993) Morphologic precursors of ovarian epithelial tumors. *Obstet Gynecol*, **82**, 181-6.

153. Werness, B.A., Afify, A.M., Bielat, K.L., Eltabbakh, G.H., Piver, M.S. and Paterson, J.M. (1999) Altered surface and cyst epithelium of ovaries removed prophylactically from women with a family history of ovarian cancer. *Hum Pathol*, **30**, 151-7.

154. Okamura, H. and Katabuchi, H. (2001) Detailed morphology of human ovarian surface epithelium focusing on its metaplastic and neoplastic capability. *Ital J Anat Embryol*, **106**, 263-76.

155. Roland, I.H., Yang, W.L., Yang, D.H., Daly, M.B., Ozols, R.F., Hamilton, T.C., Lynch, H.T., Godwin, A.K. and Xu, X.X. (2003) Loss of surface and cyst epithelial basement membranes and preneoplastic morphologic changes in prophylactic oophorectomies. *Cancer*, **98**, 2607-23.

156. Turan, V.K., Sanchez, R.I., Li, J.J., Li, S.A., Reuhl, K.R., Thomas, P.E., Conney, A.H., Gallo, M.A., Kauffman, F.C. and Mesia-Vela, S. (2004) The effects of steroidal estrogens in ACI rat mammary carcinogenesis: 17beta-estradiol, 2-hydroxyestradiol, 4-hydroxyestradiol, 16alpha-hydroxyestradiol, and 4-hydroxyestrone. *J Endocrinol*, **183**, 91-9.

157. Li, S.A., Weroha, S.J., Tawfik, O. and Li, J.J. (2002) Prevention of solely estrogen-induced mammary tumors in female aci rats by tamoxifen: evidence for estrogen receptor mediation. *J Endocrinol*, **175**, 297-305.

158. Thompson, H.J. and Adlakha, H. (1991) Dose-responsive induction of mammary gland carcinomas by the intraperitoneal injection of 1-methyl-1-nitrosourea. *Cancer Res*, **51**, 3411-5.

159. Jang, T.J., Jung, H.G., Jung, K.H. and O, M.K. (2002) Chemopreventive effect of celecoxib and expression of cyclooxygenase-1 and cyclooxygenase-2 on chemically-induced rat mammary tumours. *Int J Exp Pathol*, **83**, 173-82.

160. Dao, T.L. (1962) The role of ovarian hormones in initiating the induction of mammary cancer in rats by polynuclear hydrocarbons. *Cancer Res*, **22**, 973-81.

161. Murdoch, W.J. and McDonnel, A.C. (2002) Roles of the ovarian surface epithelium in ovulation and carcinogenesis. *Reproduction*, **123**, 743-50.

162. Dietl, J. and Marzusch, K. (1993) Ovarian surface epithelium and human ovarian cancer. *Gynecol Obstet Invest*, **35**, 129-35.

163. Liang, Y. and Hyder, S.M. (2005) Proliferation of endothelial and tumor epithelial cells by progestin-induced vascular endothelial growth factor from human breast cancer cells: paracrine and autocrine effects. *Endocrinology*, **146**, 3632-41.

164. Jabara, A.G. and Harcourt, A.G. (1971) Effects of progesterone, ovariectomy and adrenalectomy on mammary tumours induced by 7,12-dimethylbenz(a)anthracene in Sprague-Dawley rats. *Pathology*, **3**, 209-14.

165. Fisher, B., Costantino, J.P., Wickerham, D.L., Redmond, C.K., Kavanah, M., Cronin, W.M., Vogel, V., Robidoux, A., Dimitrov, N., Atkins, J., Daly, M., Wieand, S., Tan-Chiu, E., Ford, L. and Wolmark, N. (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst*, **90**, 1371-88.

166. Kelloff, G.J., Lippman, S.M., Dannenberg, A.J., Sigman, C.C., Pearce, H.L., Reid, B.J., Szabo, E., Jordan, V.C., Spitz, M.R., Mills, G.B., Papadimitrakopoulou, V.A., Lotan, R., Aggarwal, B.B., Bresalier, R.S., Kim,

J., Arun, B., Lu, K.H., Thomas, M.E., Rhodes, H.E., Brewer, M.A., Follen, M., Shin, D.M., Parnes, H.L., Siegfried, J.M., Evans, A.A., Blot, W.J., Chow, W.H., Blount, P.L., Maley, C.C., Wang, K.K., Lam, S., Lee, J.J., Dubinett, S.M., Engstrom, P.F., Meyskens, F.L., Jr., O'Shaughnessy, J., Hawk, E.T., Levin, B., Nelson, W.G. and Hong, W.K. (2006) Progress in chemoprevention drug development: the promise of molecular biomarkers for prevention of intraepithelial neoplasia and cancer--a plan to move forward. *Clin Cancer Res*, **12**, 3661-97.

167. Katalinic, A. and Rawal, R. (2007) Decline in breast cancer incidence after decrease in utilisation of hormone replacement therapy. *Breast Cancer Res Treat*.

168. Danforth, K.N., Tworoger, S.S., Hecht, J.L., Rosner, B.A., Colditz, G.A. and Hankinson, S.E. (2007) A prospective study of postmenopausal hormone use and ovarian cancer risk. *Br J Cancer*, **96**, 151-6.

169. Lacey, J.V., Jr., Brinton, L.A., Leitzmann, M.F., Mouw, T., Hollenbeck, A., Schatzkin, A. and Hartge, P. (2006) Menopausal hormone therapy and ovarian cancer risk in the National Institutes of Health-AARP Diet and Health Study Cohort. *J Natl Cancer Inst*, **98**, 1397-405.

170. Birrell, S.N., Butler, L.M., Harris, J.M., Buchanan, G. and Tilley, W.D. (2007) Disruption of androgen receptor signaling by synthetic progestins may increase risk of developing breast cancer. *Faseb J*.

171. Ting, A.Y., Kimler, B.F., Fabian, C.J. and Petroff, B.K. (2007) Characterization of a preclinical model of simultaneous breast and ovarian cancer progression. *Carcinogenesis*, **28**, 130-5.

172. Steiner, A.Z., Terplan, M. and Paulson, R.J. (2005) Comparison of tamoxifen and clomiphene citrate for ovulation induction: a meta-analysis. *Hum Reprod*, **20**, 1511-5.

173. Laven, J.S. and Fauser, B.C. (2006) What role of estrogens in ovarian stimulation. *Maturitas*, **54**, 356-62.

174. Song, L.L. and Miele, L. (2007) Cancer stem cells--an old idea that's new again: implications for the diagnosis and treatment of breast cancer. *Expert Opin Biol Ther*, **7**, 431-8.

175. Anderson, E. and Clarke, R.B. (1999) Epithelial stem cells in the mammary gland: casting light into dark corners. *Breast Cancer Res*, **1**, 11-3.

176. Wicha, M.S. (2006) Identification of murine mammary stem cells: implications for studies of mammary development and carcinogenesis. *Breast Cancer Res*, **8**, 109.

177. Sleeman, K.E., Kendrick, H., Robertson, D., Isacke, C.M., Ashworth, A. and Smalley, M.J. (2007) Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. *J Cell Biol*, **176**, 19-26.

178. Ginestier, C., Hur, M.H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., Jacquemier, J., Viens, P., Kleer, C.G., Liu, S., Schott, A., Hayes, D., Birnbaum, D., Wicha, M.S. and Dontu, G. (2007) ALDH1 Is a Marker of

Normal and Malignant Human Mammary Stem Cells and a Predictor of Poor Clinical Outcome. *Cell Stem Cell*, **1**, 555-567.

179. Riman, T., Nilsson, S. and Persson, I.R. (2004) Review of epidemiological evidence for reproductive and hormonal factors in relation to the risk of epithelial ovarian malignancies. *Acta Obstet Gynecol Scand*, **83**, 783-95.

180. Kreiger, N., Sloan, M., Cotterchio, M. and Parsons, P. (1997) Surgical procedures associated with risk of ovarian cancer. *Int J Epidemiol*, **26**, 710-5.

181. Oyama, T., Isse, T., Kagawa, N., Kinaga, T., Kim, Y.D., Morita, M., Sugio, K., Weiner, H., Yasumoto, K. and Kawamoto, T. (2005) Tissue-distribution of aldehyde dehydrogenase 2 and effects of the ALDH2 gene-disruption on the expression of enzymes involved in alcohol metabolism. *Front Biosci*, **10**, 951-60.

182. Decensi, A., Robertson, C., Viale, G., Pigatto, F., Johansson, H., Kisanga, E.R., Veronesi, P., Torrisi, R., Cazzaniga, M., Mora, S., Sandri, M.T., Pelosi, G., Luini, A., Goldhirsch, A., Lien, E.A. and Veronesi, U. (2003) A randomized trial of low-dose tamoxifen on breast cancer proliferation and blood estrogenic biomarkers. *J Natl Cancer Inst*, **95**, 779-90.

183. Cheng, G., Weihua, Z., Warner, M. and Gustafsson, J.A. (2004) Estrogen receptors ER alpha and ER beta in proliferation in the rodent mammary gland. *Proc Natl Acad Sci U S A*, **101**, 3739-46.

184. Nawaz, Z., Lonard, D.M., Dennis, A.P., Smith, C.L. and O'Malley, B.W. (1999) Proteasome-dependent degradation of the human estrogen receptor. *Proc Natl Acad Sci U S A*, **96**, 1858-62.

185. Spicer, D.V., Pike, M.C. and Henderson, B.E. (1991) Ovarian cancer and long-term tamoxifen in premenopausal women. *Lancet*, **337**, 1414.

186. Savarese, T.M., Strohsnitter, W.C., Low, H.P., Liu, Q., Baik, I., Okulicz, W., Chelmow, D.P., Lagiou, P., Quesenberry, P.J., Noller, K.L. and Hsieh, C.C. (2007) Correlation of umbilical cord blood hormones and growth factors with stem cell potential: implications for the prenatal origin of breast cancer hypothesis. *Breast Cancer Res*, **9**, R29.

187. De Assis, S. and Hilakivi-Clarke, L. (2006) Timing of dietary estrogenic exposures and breast cancer risk. *Ann N Y Acad Sci*, **1089**, 14-35.

188. Mallepell, S., Krust, A., Chambon, P. and Brisken, C. (2006) Paracrine signaling through the epithelial estrogen receptor alpha is required for proliferation and morphogenesis in the mammary gland. *Proc Natl Acad Sci U S A*, **103**, 2196-201.

189. Fabian, C.J., Kimler, B.F., Zalles, C.M., Khan, Q.J., Mayo, M.S., Phillips, T.A., Simonsen, M., Metheny, T. and Petroff, B.K. (2007) Reduction in proliferation with six months of letrozole in women on hormone replacement therapy. *Breast Cancer Res Treat*.

190. Wallace, W.H. and Kelsey, T.W. (2004) Ovarian reserve and reproductive age may be determined from measurement of ovarian volume by transvaginal sonography. *Hum Reprod*, **19**, 1612-7.

191. Absolom, K., Eiser, C., Turner, L., Ledger, W., Ross, R., Davies, H., Coleman, R., Hancock, B., Snowden, J. and Greenfield, D. (2008) Ovarian failure following cancer treatment: current management and quality of life. *Hum Reprod*, **23**, 2506-12.

192. Wallace, W.H. and Anderson, R.A. (2006) Cancer survivors and infertility: where do we go from here? *J Support Oncol*, **4**, 183-4.

193. Meirow, D. (2000) Reproduction post-chemotherapy in young cancer patients. *Mol Cell Endocrinol*, **169**, 123-31.

194. Bokser, L., Szende, B. and Schally, A.V. (1990) Protective effects of D-Trp6-luteinising hormone-releasing hormone microcapsules against cyclophosphamide-induced gonadotoxicity in female rats. *Br J Cancer*, **61**, 861-5.

195. Blumenfeld, Z., Avivi, I., Ritter, M. and Rowe, J.M. (1999) Preservation of fertility and ovarian function and minimizing chemotherapy-induced gonadotoxicity in young women. *J Soc Gynecol Investig*, **6**, 229-39.

196. Ting, A.Y., Kimler, B.F., Fabian, C.J. and Petroff, B.K. (2008) Tamoxifen prevents premalignant changes of breast but not ovarian cancer in rats at high risk for both disease. *Cancer Prev Res*, **in press**.

197. Wickerham, D.L., Fisher, B., Wolmark, N., Bryant, J., Costantino, J., Bernstein, L. and Runowicz, C.D. (2002) Association of tamoxifen and uterine sarcoma. *J Clin Oncol*, **20**, 2758-60.

198. Jordan, V.C. (2006) The science of selective estrogen receptor modulators: concept to clinical practice. *Clin Cancer Res*, **12**, 5010-3.

199. Berman, E., McBride, M., Lin, S., Menedez-Botet, C. and Tong, W. (1995) Phase I trial of high-dose tamoxifen as a modulator of drug resistance in combination with daunorubicin in patients with relapsed or refractory acute leukemia. *Leukemia*, **9**, 1631-7.

200. Smith, D.C. and Trump, D.L. (1995) A phase I trial of high-dose oral tamoxifen and CHOPE. *Cancer Chemother Pharmacol*, **36**, 65-8.

201. Ezzat, A.A., Ibrahim, E.M., Stuart, R.K., Ajarim, D., Bazarbashi, S., El-Foudeh, M.O., Rahal, M., Al-Sayed, A. and Berry, J. (2000) Adding high-dose tamoxifen to CHOP does not influence response or survival in aggressive non-Hodgkin's lymphoma: an interim analysis of a randomized phase III trial. *Med Oncol*, **17**, 39-46.

202. Kossoy, G., Ben-Hur, H., Elhayany, A., Schneider, D.F., Kossoy, N. and Zusman, I. (2005) Effects of tamoxifen and soluble tumor-associated antigens on ovarian structure in mammary tumor-bearing rats. *Oncol Rep*, **14**, 1317-21.

203. Ataya, K.M., McKanna, J.A., Weintraub, A.M., Clark, M.R. and LeMaire, W.J. (1985) A luteinizing hormone-releasing hormone agonist for the

prevention of chemotherapy-induced ovarian follicular loss in rats. *Cancer Res*, **45**, 3651-6.

204. Devine, P.J., Sipes, I.G., Skinner, M.K. and Hoyer, P.B. (2002) Characterization of a rat in vitro ovarian culture system to study the ovarian toxicant 4-vinylcyclohexene diepoxide. *Toxicol Appl Pharmacol*, **184**, 107-15.

205. Kezele, P., Nilsson, E.E. and Skinner, M.K. (2005) Keratinocyte growth factor acts as a mesenchymal factor that promotes ovarian primordial to primary follicle transition. *Biol Reprod*, **73**, 967-73.

206. Rajapaksa, K.S., Sipes, I.G. and Hoyer, P.B. (2007) involvement of microsomal epoxide hydrolase enzyme in ovotoxicity caused by 7,12-dimethylbenz[a]anthracene. *Toxicol Sci*, **96**, 327-34.

207. Oh, S.H., Miyoshi, K. and Funahashi, H. (1998) Rat oocytes fertilized in modified rat 1-cell embryo culture medium containing a high sodium chloride concentration and bovine serum albumin maintain developmental ability to the blastocyst stage. *Biol Reprod*, **59**, 884-9.

208. Perez, G.I., Tao, X.J. and Tilly, J.L. (1999) Fragmentation and death (a.k.a. apoptosis) of ovulated oocytes. *Mol Hum Reprod*, **5**, 414-20.

209. Tilly, J.L. (2003) Ovarian follicle counts--not as simple as 1, 2, 3. *Reprod Biol Endocrinol*, **1**, 11.

210. Kerdelhue, B. and El Abed, A. (1979) Inhibition of preovulatory gonadotropin secretion and stimulation of prolactin secretion by 7,12-dimethylbenz(a)anthracene in Sprague-Dawley rats. *Cancer Res*, **39**, 4700-5.

211. Jordan, V.C. (2006) Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. *Br J Pharmacol*, **147 Suppl 1**, S269-76.

212. Thompson, K.E., Sipes, I.G., Greenstein, B.D. and Hoyer, P.B. (2002) 17beta-estradiol affords protection against 4-vinylcyclohexene diepoxide-induced ovarian follicle loss in Fischer-344 rats. *Endocrinology*, **143**, 1058-65.

213. Persson, I. (2000) Estrogens in the causation of breast, endometrial and ovarian cancers - evidence and hypotheses from epidemiological findings. *J Steroid Biochem Mol Biol*, **74**, 357-64.

214. Dubey, R.K., Tyurina, Y.Y., Tyurin, V.A., Gillespie, D.G., Branch, R.A., Jackson, E.K. and Kagan, V.E. (1999) Estrogen and tamoxifen metabolites protect smooth muscle cell membrane phospholipids against peroxidation and inhibit cell growth. *Circ Res*, **84**, 229-39.

215. Lacey, J.V., Jr., Mink, P.J., Lubin, J.H., Sherman, M.E., Troisi, R., Hartge, P., Schatzkin, A. and Schairer, C. (2002) Menopausal hormone replacement therapy and risk of ovarian cancer. *Jama*, **288**, 334-41.

216. Russo, I.H. and Russo, J. (1998) Role of hormones in mammary cancer initiation and progression. *J Mammary Gland Biol Neoplasia*, **3**, 49-61.

217. Chlebowski, R.T., Kuller, L.H., Prentice, R.L., Stefanick, M.L., Manson, J.E., Gass, M., Aragaki, A.K., Ockene, J.K., Lane, D.S., Sarto, G.E., Rajkovic, A., Schenken, R., Hendrix, S.L., Ravdin, P.M., Rohan, T.E., Yasmeen, S. and Anderson, G. (2009) Breast cancer after use of estrogen plus progestin in postmenopausal women. *N Engl J Med*, **360**, 573-87.

218. Perez, E.A. (2006) Appraising adjuvant aromatase inhibitor therapy. *Oncologist*, **11**, 1058-69.

219. Cuzick, J. (2008) IBIS II: a breast cancer prevention trial in postmenopausal women using the aromatase inhibitor anastrozole. *Expert Rev Anticancer Ther*, **8**, 1377-85.

220. Thurlimann, B., Keshaviah, A., Coates, A.S., Mouridsen, H., Mauriac, L., Forbes, J.F., Paridaens, R., Castiglione-Gertsch, M., Gelber, R.D., Rabaglio, M., Smith, I., Wardley, A., Price, K.N. and Goldhirsch, A. (2005) A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med*, **353**, 2747-57.

221. Gasser, J.A., Green, J.R., Shen, V., Ingold, P., Rebmann, A., Bhatnagar, A.S. and Evans, D.B. (2006) A single intravenous administration of zoledronic acid prevents the bone loss and mechanical compromise induced by aromatase inhibition in rats. *Bone*, **39**, 787-95.

222. Langdon, S.P. and Smyth, J.F. (2008) Hormone therapy for epithelial ovarian cancer. *Curr Opin Oncol*, **20**, 548-53.

223. Ataya, K. and Moghissi, K. (1989) Chemotherapy-induced premature ovarian failure: mechanisms and prevention. *Steroids*, **54**, 607-26.

224. Ataya, K. and Ramahi-Ataya, A. (1993) Reproductive performance of female rats treated with cyclophosphamide and/or LHRH agonist. *Reprod Toxicol*, **7**, 229-35.

225. Livera, G., Petre-Lazar, B., Guerquin, M.J., Trautmann, E., Coffigny, H. and Habert, R. (2008) p63 null mutation protects mouse oocytes from radio-induced apoptosis. *Reproduction*, **135**, 3-12.

226. Hu, X., Christian, P., Sipes, I.G. and Hoyer, P.B. (2001) Expression and redistribution of cellular Bad, Bax, and Bcl-X(L) protein is associated with VCD-induced ovotoxicity in rats. *Biol Reprod*, **65**, 1489-95.

227. Tsai-Turton, M., Luong, B.T., Tan, Y. and Luderer, U. (2007) Cyclophosphamide-induced apoptosis in COV434 human granulosa cells involves oxidative stress and glutathione depletion. *Toxicol Sci*, **98**, 216-30.

228. Morita, Y., Perez, G.I., Maravei, D.V., Tilly, K.I. and Tilly, J.L. (1999) Targeted expression of Bcl-2 in mouse oocytes inhibits ovarian follicle atresia and prevents spontaneous and chemotherapy-induced oocyte apoptosis in vitro. *Mol Endocrinol*, **13**, 841-50.

229. Takai, Y., Canning, J., Perez, G.I., Pru, J.K., Schlezinger, J.J., Sherr, D.H., Kolesnick, R.N., Yuan, J., Flavell, R.A., Korsmeyer, S.J. and Tilly, J.L. (2003) Bax, caspase-2, and caspase-3 are required for ovarian follicle loss caused by 4-vinylcyclohexene diepoxide exposure of female mice in vivo. *Endocrinology*, **144**, 69-74.

230. Nathan, L. and Chaudhuri, G. (1998) Antioxidant and prooxidant actions of estrogens: potential physiological and clinical implications. *Semin Reprod Endocrinol*, **16**, 309-14.

231. Pappas, P., Stephanou, P., Sotiropoulou, M., Murphy, C., Salminen, L. and Marselos, M. (1999) Effects of tamoxifen and toremifene on ALDH1 and ALDH3 in human retinal pigment epithelial cells and rat liver. *Adv Exp Med Biol*, **463**, 151-8.

232. Morita, Y. and Tilly, J.L. (1999) Segregation of retinoic acid effects on fetal ovarian germ cell mitosis versus apoptosis by requirement for new macromolecular synthesis. *Endocrinology*, **140**, 2696-703.

233. Ataya, K., Pydyn, E., Young, J. and Struck, R. (1990) The uptake and metabolism of cyclophosphamide by the ovary. *Sel Cancer Ther*, **6**, 83-92.

234. Balakier, H. and Stronell, R.D. (1994) Color Doppler assessment of folliculogenesis in in vitro fertilization patients. *Fertil Steril*, **62**, 1211-6.

235. Stewart, P.A., Beliveau, R. and Rogers, K.A. (1996) Cellular localization of P-glycoprotein in brain versus gonadal capillaries. *J Histochem Cytochem*, **44**, 679-85.

236. Pastan, I. and Gottesman, M.M. (1991) Multidrug resistance. *Annu Rev Med*, **42**, 277-86.

237. Shen, L.Z., Hua, Y.B., Yu, X.M., Xu, Q., Chen, T., Wang, J.H. and Wu, W.X. (2005) Tamoxifen can reverse multidrug resistance of colorectal carcinoma in vivo. *World J Gastroenterol*, **11**, 1060-4.

238. Darvari, R. and Boroujerdi, M. (2005) Investigation of the influence of modulation of P-glycoprotein by a multiple dosing regimen of tamoxifen on

the pharmacokinetics and toxicodynamics of doxorubicin. *Cancer Chemother Pharmacol*, **56**, 497-509.

239. Gershon, E., Plaks, V. and Dekel, N. (2008) Gap junctions in the ovary: expression, localization and function. *Mol Cell Endocrinol*, **282**, 18-25.

240. Kidder, G.M. and Mhawi, A.A. (2002) Gap junctions and ovarian folliculogenesis. *Reproduction*, **123**, 613-20.

241. Teicher, B.A. (1995) Physiologic mechanisms of therapeutic resistance. Blood flow and hypoxia. *Hematol Oncol Clin North Am*, **9**, 475-506.

242. Harrison, L. and Blackwell, K. (2004) Hypoxia and anemia: factors in decreased sensitivity to radiation therapy and chemotherapy? *Oncologist*, **9 Suppl 5**, 31-40.

243. Jordan, B.F., Gregoire, V., Demeure, R.J., Sonveaux, P., Feron, O., O'Hara, J., Vanhulle, V.P., Delzenne, N. and Gallez, B. (2002) Insulin increases the sensitivity of tumors to irradiation: involvement of an increase in tumor oxygenation mediated by a nitric oxide-dependent decrease of the tumor cells oxygen consumption. *Cancer Res*, **62**, 3555-61.

244. Harrison, L.B., Chadha, M., Hill, R.J., Hu, K. and Shasha, D. (2002) Impact of tumor hypoxia and anemia on radiation therapy outcomes. *Oncologist*, **7**, 492-508.

245. Wang, J.Y. (2001) DNA damage and apoptosis. *Cell Death Differ*, **8**, 1047-8.

246. Lobascio, A.M., Klinger, F.G., Scaldaferri, M.L., Farini, D. and De Felici, M. (2007) Analysis of programmed cell death in mouse fetal oocytes. *Reproduction*, **134**, 241-52.

247. Ghafari, F., Gutierrez, C.G. and Hartshorne, G.M. (2007) Apoptosis in mouse fetal and neonatal oocytes during meiotic prophase one. *BMC Dev Biol*, **7**, 87.

248. White, I.N. (1999) The tamoxifen dilemma. *Carcinogenesis*, **20**, 1153-60.