2011

p,p' DDE Regulated Gene Expression and Possible Mechanisms of Action in Breast Tumor

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p,p′ DDE REGULATED GENE EXPRESSION AND POSSIBLE MECHANISMS OF ACTION IN BREAST TUMOR FORMATION

A Dissertation

Submitted to the Graduate School of Pharmaceutical Sciences

Duquesne University

In partial fulfillment of the requirements for

the degree of Doctor of Philosophy

By

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December 2011

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ABSTRACT

p,p′ DDE REGULATED GENE EXPRESSION AND POSSIBLE MECHANISMS OF ACTION IN BREAST TUMOR FORMATION

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Dissertation supervised by Paula A. Witt-Enderby, Ph.D.

Background: The insecticide DDT (dichlorodiphenyltrichloroethane) has been speculated to increase breast cancer risk due to its environmental persistence, levels of bioaccumulation in breast adipose tissue, and endocrine disrupting actions. Epidemiological studies have had inconsistent findings, however a study in MMTV-*neu* mice determined that localized, developmental exposure to the reported anti-androgen p,p′ DDE accelerated mammary tumor development. This study tested the potential cancer-promoting actions of p,p′ DDE, the most prevalent and persistent DDT metabolite.

Objectives: To identify and characterize the expression of p,p′ DDE -regulated genes to determine how developmental exposure may influence mammary tumorigenesis.

Methods: For localized delivery, ELVAX 40P pellets containing various doses of p,p′ DDE, hydroxyflutamide (another anti-androgen), and mixtures of p,p′ DDE with other

congeners like o,p′ DDE and p,p′ DDT were implanted into the mammary fatpads of prepubertal female mice. p,p′ DDE-regulated genes were identified by microarray analysis and analyzed by real time RT-PCR.

Results: Lipid-adjusted levels of p,p′ DDE in mammary adipose tissue and serum in young mice were within the ranges of human exposure. p,p′ DDE significantly upregulated casein gamma (*csn1s2a*), keratin 18 (*krt18*) and interferon-induced protein 44 (*ifi44*) genes in mammary tissue. These genes were similarly, but not significantly regulated by hydroxyflutamide. The dose of p,p′ DDE that caused early tumor onset in a previous study resulted in unique expression for all three genes and concentrations of p,p′ DDE also influenced gene responses for the mixtures. However, no qualitative changes were observed in gland morphology. Significant upregulation of transforming growth factor beta (*tgfb1*) and downregulation of interleukin 10 (*il10*) in splenic leukocytes indicated that localized delivery of p,p′ DDE to the mammary gland also influences systemic immune responses. Significant upregulation of *il10* by p,p′ DDE and hydroxyflutamide suggest that some of p,p′ DDE actions may be through its anti-androgenic activity.

Conclusions: Relevant human exposure levels of p,p′ DDE induce significant increases in expression of *csn1s2a, krt18* and *ifi44*. This activity as well as those induced by other doses, ratios and hydroxyflutamide suggest p,p′DDE actions may involve anti-androgenic activity and influence local and systemic effects in a $HER2^{+}$ breast cancer mouse model.

DEDICATION

This dissertation is dedicated to my beautiful, loving, amazing, strong, inspirational, wise and god-spirited mom. I am who I am because of you and could not have accomplished any of this without your constant LOVE, PRAYERS, SUPPORT and ENCOURAGEMENT. I am so truly blessed to have you as my mom. I love you.

This is also in loving memory of my nana, Elizabeth Johnson, my childhood caregiver, Lydia Kennebrew, my uncle, William Littlejohn and my cousin Steven Ruffin.

ACKNOWLEDGEMENT

First, giving an honor to God, my Lord and Savior Jesus Christ; the author of my life. Thank you for all the many blessings you have bestowed upon me. It is my heart's desire that this education be used to glorify you and bless your people, so that when I stand before you, you will say, "job well done, my good and faithful servant."

To my Doctoral Advisor, Dr. Vicki Davis, thank you for your constant guidance, support and patience over the past six years. Thank you for making my educational dream a reality.

To my Dissertation Chair; Dr. Paula Witt-Enderby, thank you for always believing in me and providing not only guidance but encouragement every step of the way. Also, thank you for accepting me into your lab and making what could have been a difficult time, virtually seamless.

To my Dissertation Committee; Drs. Wilson Meng, Warren Foster, Jane Cavanaugh and Rehana Leak, thank you for your guidance, support and input on my project. I am forever grateful.

To the Davis Lab; Drs. Rachel Kirker and Mary Kotlarczyk; thank you for taking me under your wing and showing me the ropes when I got to DU. You have both taught me so many things over the years! Thank you for your never-ending guidance and support.

Also, to Sheri Rhea and Katie Gallagher, thank you for all of the work you did to support me on my projects. Your help has been invaluable.

To the Witt-Enderby lab; Corry Bondi and Bala Reddy Doda, thank you for your support. A special thank you to Dr. Bill Clafshenkel and Dr. Mary Kotlarczyk, we did it!! Having the both of you go through this process with me was absolutely amazing. Thank you. Words can't explain what your encouragement and support has meant to me.

To Jackie Farrer; thank you. You are amazing. God's love shines through you every day.

To all the graduate students in the Mylan School of Pharmacy Graduate School of Pharmaceutical Sciences; thank you for your support

To all of the PharmD students that gave of their free time to work in the lab and do research with me, thank you; and to the high school students that worked in the lab during the summers, thank you for your help.

To my Bethel Baptist Church family; thank you for your love, support and prayers. No matter where I go, I know where home is.

To Pastor Curtis; you have touched my life in so many ways. Your sermons have blessed me; filling my spiritual tank every Saturday night. I thank God for you.

To the Mt. Ararat Baptist Church family; thank you for your love and support.

To Assata; I love you.

To Antonette Sotillio, Dr.Tiffany Evans, Sonja Littlejohn, Jason Lewis, Rory Ivey, Natasha Graham, James Kelly and Rahim Johnson, thank you and I love you. Having you in my life is a blessing. Our daily conversations and/or interactions speak for themselves. Your love, support, encouragement and belief in me has been incredible.

To all those who are not personally mentioned, I have not forgotten you or the impact you've had on my life during this time. Each and every phone call, text, facebook message, prayer and/or thought was felt. Thank you.

To my entire extended family, I love you and I thank you!

To my Daddy, my sister Joi, my niece Sanai (my Pinkibear), my stepdad Robert and my brother-in-law Steve, I LOVE YOU! Thank you for always being there for me.

And last but certainly not least; to my sweet, sweet, girl PRISSA and my heart, PRETTY BOY, I LOVE YOU SO MUCH! The unconditional love that you gave and continue to give has gotten me through. Thank you for making me smile and laugh EVERYDAY. My heart is so full because of you.

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LIST OF ABBREVIATIONS

akr1c18	Aldo-keto reductase fam1 memC18
1l12	Interleukin 12
acly	ATP citrate lyase
aacs	acetoacetyl-CoA synthetase
ahr	Arylhydrocarbon receptor
ar	Androgen Receptor
bcl2l11	Bcl2-like 11
bex1	Brain expressed gene 1
brca1	Breast Cancer 1
BW	Body Weight
ccd1	Cyclin D1
ccl19	Chemokine (C-C motif) ligand 19
ccl21b	Chemokine (C-C motif) ligand 21b
ccl5	Chemokine (C-C motif) ligand 5
ccr7	Chemokine (C-C motif) receptor 7
cd3d	CD3 antigen
cd52	CD52 antigen
cd81	CD81 antigen
csn1s2a	Casein gamma
csn2	Casein beta
Ct	Cycle threshold
DDE	Dichlordiphenyldichloroethylene
DDT	Dichlorodiphenlydichloroethane
E_2	17-β Estradiol
egr2	Early growth response 2
esr1	Estrogen receptor 1 (alpha)
F	Forward
flt4	FMS-like tyrosine kinase 4
glycam1	Glycosylation adhesion molecule
gpr30	G protein-coupled receptor 30
hdac3	Histone deacetylase 3
HRE	Hormone Response Element
id1	Inhibitor of DNA binding 1
id4	Inhibitor of DNA binding 4
ifi44	Interferon-induced protein 44
il10	Interleukin 10
il18	Interleukin 18
il18	Interleukin 18

il2rg	Interleukin 2 receptor, gamma
il7r	Interleukin 7 receptor
insig1	Insulin induced gene 1
itk	IL2-inducible T-cell kinase
krt18	Keratin 18
lck	Lymphocyte protein tyrosine kinase
LM	Lower Mammary
LN(+)	Lymph Node positive
LTR	Long Terminal Repeat
lyve1	Lymphatic vessel endothelial hyaluronan receptor 1
Methyl-T	Methyltestosterone
MMTV	Mouse Mammary Tumor Virus
ms4a1	Membrane-spanning 4-domains, subfamily 1, member 1
neu	Mouse neu
neu	Rat neu
nrp2	Neuropilin 2
o,p' DDE	ortho, para Dichlorodiphenyldichloroethylene
OC	Organochlorine pesticide
OH-flut	Hydroxyflutamide
p,p' DDE	para, para Dichlorodiphenyldichloroethylene
p,p' DDT	para, para Dichlorodiphenyldichloroethane
pdpn	Podoplanin
pou2af1	Pou domain, class 2, associating factor 1
ppia	Cyclophilin
pgr	Progesterone Receptor
PR-A	Progesterone Receptor A
PR-B	Progesterone Receptor B
prox1	Prospero homeobox-1
prune2	Prune homolog 2
R	Reverse
rankl	Tumor necrosis factor sf 11
rps12	Ribosomal protein S12
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
saa1	Serum Amyloid A1
saa2	Serum Amyloid A2
sell	Selectin
sfrp5	Secreted frizzled-related sequence protein 5

LIST OF ABBREVIATIONS

SL	Splenic Leukocytes
slc2a5	Solute carrier member2 fam5
slco3a1	Solute carrier organic anion transporter family, member 3a1
slfn2	Schlafen 2
sprr1a	Small proline-rich protein 1A
tcra	T-cell receptor alpha chain
tert	telomerase reverse transcriptase
tgfb1	Transforming Growth Factor β1
thbs1	Thrombospondin 1
thrsp	Thyroid hormone responsive SPOT14 homologue
tnfrsf13c	Tumor necrosis factor receptor superfamily member 13c
tpm2	Tropomyosin-2 F1
txn2	Thioredoxin 2
UT	Uterus

Chapter 1

Introduction

INTRODUCTION

There have been many human epidemiology studies on the insecticide and environmental contaminant DDT (dicholorodiphenyltrichloroethane) that have provided inconclusive evidence associating its levels of exposure with increased breast cancer risk. The inconclusive results from these studies may be attributed to a multitude of confounding variables surrounding DDT, such as its endocrine disrupting actions, age of exposure, environmental persistence and bioaccumulation in breast adipose tissue. DDT exists in humans and the environment as a mixture of DDT, dichlorodiphenyldichloroethylene (DDE), and dichlorodiphenyldichloroethane (DDD). The DDE component has two isomers, para, para´ DDE (p,p´ DDE) and ortho, para´ DDE (o,p´ DDE) . The p,p´ DDE isomer possesses strong anti-androgenic activity (Kelce et al., 1995) while o,p´ DDE has very weak estrogenic activity (Ociepa-Zawal et. al., 2010). The concern with DDT exposure and breast cancer is that these isomers, that are hormonal mimics, are highly lipid-soluble and able to persist and therefore bioaccumulate in lipid-rich environments, such as the breast. These congeners are also very prevalent in the environment and the human body due to their resistance to degradation/metabolism, which account for their long half-lives and ubiquitous nature.

In human studies, age of exposure is usually unknown, but can occur as early as *in utero* in amniotic fluid and cord blood (Foster et al., 2000; Tan et al., 2008; Barraza-Vazquez et al., 2008). Thus, having a true negative control in human studies is impossible.

In a tumor study conducted by Davis and colleagues, it was shown that p,p´ DDE has the ability to accelerate tumor formation in an MMTV-*neu* mouse model that mimics $HER2^{+}$ breast cancer. The earlier tumor onset is postulated to be associated with changes in gene expression that occur due to chronic exposure and accumulation of p,p´ DDE in the mammary gland of MMTV-*neu* mice. Gaining insight into the genes that are being modified by these congeners may suggest directions to pursue regarding potential mechanisms of action of p,p´ DDE *in vivo* that result in earlier tumor onset. The results from this pre-clinical study will provide important information regarding DDT-induced responses *in vivo*. If p,p´ DDE modulates the expression of similar genes in cultured normal human breast cells, then it may have similar actions in women, and thus, may lead to identification of marker genes useful as novel screening tools for breast cancer risk assessment with DDT exposure. Identification of potential actions of p,p´ DDE in mammary tissue may also lead to testing of novel therapies to block these actions, such as to inhibit androgen action or to prevent the early onset of mammary cancer. Furthermore, these finding may be used to provide vital information that could aid in future policy decisions and risk assessment, as those involved in Superfund sites, the Stockholm Convention on Persistent Organic Pollutants and the World Health Organization, that all continue to support the production and use of DDT for malaria vector (mosquito) control (van den Berg 2009).

BACKGROUND AND SIGNIFICANCE

DDT

The organochlorine pesticide DDT gained popularity during WWII when it was used to combat malaria and typhoid fever. As DDT use continued to increase, it quickly began being used in worldwide proportions, with peak production reaching an estimated 400 million pounds globally in the late 1960s and early 1970s. However, DDT was banned in the United States in 1972 after the release of the book *Silent Spring* by Rachel Carson. This publication revealed disturbing revelations about the harmful impact of DDT on the environment and ignited growing concerns about human health effects.

Today, approximately 14 countries currently use DDT for the control of malaria and leishmaniasis and several other countries are preparing to reintroduce DDT (van den Berg 2009). Although the quantity is deemed unacceptable by the Stockholm Convention on Persistent Organic Pollutants, 160 metric tons of DDT is still being produced each year in India, China, and North Korea for vector control (with India being the largest producer and consumer). There has been no reported use of DDT for vector control in the Americas (van den Berg 2009). There is still insufficient information on the safety profile of DDT and questions regarding global public policy remain unanswered. Moreover, agencies like the World Health Organization (WHO) and the United States Agency for International Development (USAID) continue to endorse the use of DDT (Eskenazi et al., 2009).

Breast Cancer Biology

Breast cancer is a multifactorial disease. It occurs when normal healthy breast cells undergo multiple processes and stages that cause them to change into abnormal cells that rapidly proliferate uncontrollably. Initiation of the disease is caused, for example, when genes are mutated by harmful agents/chemicals in the environment. However, not all chemical- induced carcinogens have genotoxic effects. Several chemicals, including pesticides like DDT, are non-genotoxic and therefore may be considered cancer promoters (Bagga et al., 2000). Promotion is characterized by an increase in the number of initiated cells through long-term epigenetic mechanism (Mukherjee et al., 2006). The final stage in carcinogenesis is progression, which is associated with chromosomal/karotypic changes (Ludewig et al., 2008).

Breast tissue is particularly sensitive to developing cancer because estrogen stimulates breast cell division. Increases in breast/mammary cancer have been widely studied in association with environmental contaminants known to display estrogenic and/or endocrine disruption, such as DDT, bisphenol A, deildrin, heptachlor epoxide and oxychlordane (Weber and Keri 2011; Vo et al., 2008; Mukherjee et al., 2006; Cassidy et al., 2005). Furthermore, these environmental contaminants and many others persist in the environment and human tissue because of their resistance to metabolism. Slow metabolism then leads to bioaccumulation in adipose tissue due to their lipid solubility. Bioaccumulation is the increase in concentration of the chemical with increased time and exposure. Therefore, girls and young women are especially susceptible from exposure to

carcinogens due to not having fully matured breast cells, which are more efficient at repairing DNA damage (Cohn et al., 2011; Snedeker and Allyger 2001). It has been hypothesized that lipid-soluble carcinogens in the adipose tissue in human breast may influence ductal epithelial cells, from which breast tumors commonly arise. Therefore, deposition of these chemicals in the adipose tissue of the breast has been linked to neoplastic transformation (Mukherjee et al., 2006).

DDT Mechanisms Involved in the Pathological Process of Breast Cancer

Postulated mechanisms involved in breast cancer have been extensively studied. Some include, DNA methylation associated with initiation and progression of the disease (Rusiecki et al., 2008), inhibition of gap junctional intercellular communication, which has tumor promoting potential (Kang et al., 1996), genotoxic potential (Ennaceur et al., 2008), free-radical mediated stress (Iscan et al., 2002), apoptosis (Perez-Maldonado et al., 2006), changes in aromatase activity (Holloway et al., 2005) and perhaps most notably, its endocrine disrupting potency (Li et al., 2008).

p,p´ DDE is an environmental anti-androgen (Kelce et al., 1995; 1997). The role of androgens in breast cancer remains largely unclear. However, over 70% of human breast cancer biopsies from untreated women are positive for the androgen receptor (AR) (as reviewed by Liao and Dickson 2002), which suggests an androgen-mediated role in normal and malignant breast tissue. Endogenous androgens are purported to normally inhibit estrogen-induced mammary epithelium proliferation and, therefore, act as a protector of the mammary gland (Aboghe et al., 2008). Therefore, AR antagonists

compete with endogenous androgens for the receptor and prevent downstream activation of androgen-dependent gene expression (Wilson et al., 2008). However, although this is the most commonly accepted mechanism, there are other ways in which anti-androgens can interfere with androgen-signaling cascades. These alternative mechanisms include modifying the production or metabolism of endogenous hormones, modifying the number of hormone receptors, decreasing mRNA of steroidogenic enzymes and/or decreasing testosterone synthesis (Wilson et al., 2008). Furthermore, Yang and colleagues (2010) found the chaperone role of heat shock proteins to be significantly modified following p,p´ DDE exposure, due to a decrease in expression of heat shock proteins 70 and 90.

DDT and several of its isomeric forms are also environmental estrogens, meaning they can interact with the estrogen receptor (ER) similar to estrogen and modulate transcription of responsive genes to result in estrogenic responses (Jaga et al., 2000). Similar to anti-androgens, environmental estrogens in breast adipose tissue function by other mechanisms besides receptor interaction. Due to their weak affinity to estrogen receptors, they likely act by affecting the expression of cytochrome P450 genes encoding cytochromes P450 engaged in the metabolism of environmental as well as endogenous estrogens (Ociepa-Zawal et al., 2010). Specifically, DDE has been found to be an inducer of rat hepatic cytochrome P450 2B1 and 3A1 and therefore may have an impact on events such as the metabolism of endogenous and exogenous substrates (Wyde et al., 2003). Considering all of these possible actions, it is feasible that the androgen receptor antagonist p,p´ DDE may be able to disrupt the estrogen-androgen balance regulating the

growth of hormone-dependent breast cancer cells (Aube et al., 2007), thus leading to tumorigenesis.

Also, in addition to estrogen and androgen modulation of gene transcription by exerting their direct actions via their cognate nuclear receptor, both receptor types may have non-classical intracellular locations such as the plasma membrane and the mitochondria, which add to the complexity of their functionality (Vasconsuelo et al., 2011).

DDT and Endocrine Disruption

Technical grade DDT is a mixture of three isoforms of DDT, p,p´ DDT , o,p´ DDT and o,o´ DDT (Nuñez et al., 2002). The main metabolite of DDT is the highly lipophilic, highly persistent p,p´ DDE. Para, para´ DDE has been shown to have potent anti-androgenic affects (Kelce et al., 1995; 1997), while its estrogenic isomer, o,p´ DDE, is relatively weak (Kelce et al., 1995). With time and exposure, these DDE isomers bioaccumulate in humans and the environment due to their long half-lives (Snedecker et al., 2001). Disruption of endocrine responses has long been associated with DDT congeners and metabolites in wildlife. Endocrine disruptors are any chemical that has the ability to interfere with the body's own natural endocrine system, possibly resulting in adverse effects. These systems are usually involved in the regulation of growth, development, and maturation as well as many other functions. In concordance with *Silent Spring*, DDT has been shown to have an effect on reproductive systems (i.e., sexual development and fertility) of many wildlife vertebrates, including birds and amphibians

(Bernanke and Köhler 2009). These findings imply that DDT does indeed elicit hormonal effects and should be considered an endocrine disruptor.

Hormones are also known to have a critical impact on the etiology of human cancers such as in the breast, uterus, ovary, prostate, testis and thyroid (Dees et al., 1997; reviewed in Cocco 2002). Abnormal stimulation of the breast with hormones, like estrogen, has been associated with uncontrolled growth and neoplasia. Androgens also play a role in breast development, growth and carcinogenesis (Liao et al., 2002; Suzuki et al., 2010) and these effects may be potentiated by compounds with hormonal influences, such as the anti-androgenic p,p´ DDE and estrogenic o,p´ DDE. Thus, different isomers of DDT may induce abnormal hormone effects in human breast tissue.

DDT and Breast Cancer

Breast cancer is the most common cancer among women in almost all developed countries (Shakeel et al., 2010); however, there remains inconclusive evidence regarding the safety of DDT and its relationship with increased breast cancer risk. There has been supporting evidence (Wolff et al., 1993; Charlier et al., 2003) as well as studies that refute (Kriger et al., 1994; Gatto et al., 2007) the association between DDT and elevated breast cancer risk. The ambiguous conclusions drawn from these studies may be due to variables associated with age or the exposure used for analysis (blood vs. adipose tissue). Para, para´ DDE is often the only congener measured in human blood and adipose tissue because it is the most abundant and persistent of all DDT isomers. However, many of the human studies that have investigated p,p´ DDE use serum of older, post-menopausal

women. Age of exposure to DDT is an important factor to consider due to the prepubertal and pubertal years were found to be critical in the development of breast cancer (Cohn et al., 2011), since developing mammary tissue is more sensitive to hormonal or carcinogenic exposures. The older women in these studies were initially exposed to DDT and its isomers at unknown times and therefore may have decreased or increased levels of p,p´ DDE in their serum and mammary tissue with age or changes in exposure at the time of testing, when they would be considered less vulnerable to exposure. This decrease or increase in levels may also be the result of changes in exposure, changes in type of exposure (parental DDT or dietary p,p´ DDE from environmental exposures), prior lactations, and metabolic processes over decades. Thus, studies that measure exposure in older women may have missed this critical period (Eskenazi et al., 2009). As such, all of the aforementioned variables may have contributed to the lack of solid evidence linking DDT and breast cancer risk.

In 2007, Cohn and colleagues concluded exposure to higher concentrations of p,p´ DDT in young women was associated with a 5-fold increase in breast cancer risk later in life (odds ratio 5.52; confidence interval 1.4-19.1). This study was the first of its kind to investigate age of DDT exposure and its effects on breast cancer risk. This study identified p,p´ DDT as being associated with an increased breast cancer risk when the women were exposed to peak levels before the age of 14. DDT came into widespread use in 1945 in the United States and this year was used in the study as the first possible time a woman could be exposed to DDT. The average age of study participants was 26 years and women who were 14 years or older by 1945 showed little risk. These findings may

disagree with many of the prior studies investigating DDT and breast cancer because the previous studies investigated DDT levels in older women with and without breast cancer who were already adults in 1945. Importantly, this finding also suggest women born after 1945, who would be less than 14 years old when exposed would have a greater risk of breast cancer. This also implies that these women are now reaching ages associated with higher incidences of breast cancer and could potentially initiate an increase in new breast cancer cases over the next decade. According to the Cohn et al (2007) report, women exposed to DDT beginning as adults would be at a low risk, which may explain the inconclusive results in previous epidemiology studies. Similar to the study by Cohn and colleagues (2007), the MMTV-*neu* female mice in the present study were exposed to DDT prior to puberty. The similarity in age of exposure between the two studies may ultimately lead to better designs in epidemiology studies to accurately assess DDT and its isomers influence on breast cancer risk in women.

DDT and the Immune System

Decreased immunosurveillance and immune dysfunction are key mechanisms underlying carcinogenesis. Several DDT congeners have been shown to modulate the immune system, which could be a means for DDT to promote carcinogenesis. For example, the immunosuppressive effects of DDT have been shown to decrease the function of human natural killer cells, which are first-line immune defense against tumor cells (Udoji et al., 2010). Banerjee and colleagues (1996) showed that DDT exposure produced marked suppression of humoral and cell-mediated responses in experimental animals. Other immunomodulatory effects of DDT include activation of the complement system (Dutta

et al., 2008) and production of tumor necrosis factor-α and nitric oxide production in macrophages (Nunez et al., 2002). These effects of DDT on the immune system contribute to inflammatory reactions, cytokine imbalance, and overall immune dysregulation seen following exposure (Dutta et al., 2008). Moreover, DDT affects interleukin secretion from lymphocytes (Beach and Whalen 2006) and relatively low levels of DDE have been associated with statistically significant changes in immune markers (Vine et al., 2001). The strong implications of DDT and its metabolites impact on the immune system imply these compounds may influence multiple aspects of immune function.

DDT and Mixtures

Another aspect of DDT exposure on breast cancer that must be considered is the effect of DDT as a mixture. That is, multiple congeners, like p,p´ DDT, p,p´ DDE , and o,p´ DDE, are detected simultaneously in people worldwide exposed to organochlorine pesticides (Valerón et al., 2009). Most human case-control studies that investigate an association between DDT and breast cancer risk measure serum levels of a single chemical (Cohn et al., 2007), which is generally p,p´ DDE, with negative or inconclusive results (Payne et al., 2001). Kortenkamp (2006) states that epidemiologist assessing breast cancer risk should take into account the recent evidence showing that pollutants, present at low levels, can act together to add to the internal estrogenic load. Combined effects of p,p´ DDT, p,p´ DDE, o,p´ DDT and β-hexachlorocylohexane were also found to be synergistic and/or additive in their capacity to enhance human breast cancer cell proliferation (Payne et al., 2001). Additionally, it was shown that exposure of normal

human mammary epithelial cells exposed to environmentally relevant mixtures that included p,p´ DDE, o,p´ DDE and p,p´ DDD (among others), sharply up-regulated the mRNA expression of a number of protein kinases associated with the transformation process of human breast cells at concentrations close to those detected in human populations (Valerón et al., 2009). Shekhar and colleagues (1997) found that a combination of p,p´ DDT and o,p´ DDT enhanced proliferation in MCF-7 human breast cancer cells. Mixtures are a very relevant concern that needs to be considered for assessing DDT and breast cancer risk.

MMTV-*neu* MOUSE MODEL

The present study used the unactivated mouse mammary tumor virus (MMTV)-*neu* mouse model, which overexpresses the wild-type rat *neu* (*erb*B2) gene. HER2, the human homologue of *neu*, is commonly elevated in 20-30% of human breast cancer cases (Slamon et al., 1987). This gene codes for a member of the epidermal growth factor receptor family of receptor tyrosine kinases. In the transgenic mice, the *neu* proto-oncogene requires spontaneous activation, similar to humans, to an oncogene to result in the formation of mammary adenocarcinomas that resemble human tumors (Guy et al., 1992). Additionally, the majority of mice develop mammary tumors that frequently metastasize to the lungs (Taneja et. al., 2009; Hutchinson and Muller, 2000), which is also a common site for breast cancer metastasis. Although androgens have been shown to act directly on the regulatory region of the long terminal repeat (LTR) of MMTV and thereby possibly influence gene expression (Darbre et al., 1986; Cato et al., 1987), the MMTV promoter has been successfully used for studying the control of gene expression

by steroid hormones (Reviewed in Ringold 1985; Yamamoto 1985). The hormone response element (HRE) located between -202 and -59 upstream of the start of transcription in the LTR region is required for this induction (Cato et al.,1987), but does not contain an estrogen response element (ERE) and therefore, is not regulated by estrogens (Otten et al., 1998). The MMTV-*neu* mouse model was used in the present study because it: 1) contains the unactivated form of *neu* and transgenic models that contain the activated form of *neu* are not good for studying mammary carcinogenesis (Andrecheck et al., 2000); 2) overexpresses *neu*, of which the human homologue, HER2, is overexpressed in human breast cancer cases; 3) requires spontaneous activation like human cancers and produces tumors that resemble human tumors; 4) forms tumors that frequently metastasize to the lungs, similar to breast cancer metastasis and 5) it is the same one used in the tumor study, which correlates with the mouse model with documented influence by DDE on mammary cancer.

ELVAX 40P IMPLANTS

In order to replicate human exposure to DDT in the breast and to remain consistent with the tumor study conducted by Davis and colleagues, the treatments in this study were all delivered locally to the mammary gland through the use of ELVAX 40P implants. These implants are made of an ethylene vinyl-acetate copolymer that does not cause an inflammatory response (Silberstein and Daniel, 1982). The implants mimic human exposure because they allow for the slow, sustained (approximately 100 days), local release of a large variety of macromolecules *in vivo* as well as for the assessment of primary (non-systemic) effects of biologically active molecules on developing tissue

(Silberstein and Daniel, 1982), such as the mammary gland. This material/controlled delivery system has been successfully used in the mammary gland previously (Haslam 1998; Andrapp et al., 1998; Wang et al., 1990; VanderPloeg et al., 1992) and elicits no adverse local or systemic response over extended periods *in vivo* (Shastri 2002).

Ethylene-vinyl acetate copolymer is synthesized by free-radical polymerization of ethylene gas and vinyl acetate using a peroxide catalyst. The physical properties can be tuned between a rigid and rubbery material by altering the ratio of the co-monomers (Shastri 2002). Ethylene-vinyl acetate pellets (40% by weight vinyl acetate) are the first biocompatible polymeric delivery system capable of continuously delivering macromolecules (Rhine et al., 1980). In addition, it did not induce any inflammatory responses and showed no foreign cells in the region surrounding the polymer or in other areas of the cornea when aseptically implanted into the avascular cornea of rabbits (Langer and Folkman 1976). Mechanism of release does not appear to be simple diffusion and substances deep in the polymer may be driven to the surface by osmotic pressure or capillary action (Langer and Folkman 1976). Release rates were also shown to decrease due to increases in diffusion distance through the pores of the polymer matrix as time increased. However, constant release rates have been achieved with appropriate geometric design (Brown et al., 1983). Also, the ethylene-vinyl acetate copolymer has had reproducible release kinetics using different proteins and particle sizes (Rhine et al., 1980).

PREVISOUS STUDIES ON p,p´ DDE AND MAMMARY CANCER DEVELOPMENT

The major goals of the studies conducted by Davis and colleagues were to investigate tumor outcomes induced by exposure to the anti-androgen, p,p´ DDE, beginning in the prepubertal mammary gland of MMTV-*neu* mice. The purpose of the tumor study was to explore localized effects of the prevalent anti-androgen p,p´ DDE and its estrogenic isomer, o,p´ DDE on the tumor outcomes of incidence, latency, and metastasis. The effects of a combination treatment of p,p´ DDE and o,p´ DDE in a 2:1 ratio were also investigated (Table 1).

Table 1. Treatment Groups for the Previous Tumor Study (n=72-80)

Treatment	Control	p,p´ DDE	o,p´ DDE	p,p´ DDE + o,p´ DDE (2:1)
Dose per pellet	0	5 µg	5 µg	3.3 µg + 1.7 µg

In these studies, ELVAX 40P pellets (5 µg/pellet) were bilaterally implanted into the axillary and inguinal mammary fat pads (20 µg total) which allowed DDE to be directly delivered to the mammary adipose tissue, in close proximity to the mammary epithelial cells, very similar to how DDT is stored in the adipose tissue of the human breast. The tumor study results revealed that the maximal incidence of mammary tumors was unaffected by localized exposure of 5 µg p,p´ DDE via ELVAX pellets compared to the

control group (Fig.1). The unaffected incidence suggests DDE acts as a tumor promoter rather than initiating cancer. However, p,p′ DDE did significantly (p=0.008, t-test) accelerate tumor formation compared to control mice (vehicle pellet) (Fig. 2). Age of tumor onset was shortened by 1.5 months, where the earliest tumor was detected in the p,p′ DDE group at age 90 days and in the control group at day 147. Survival curves of the percent of tumor-free animals with age also showed p,p′ DDE exposure resulted in earlier tumor formation (Fig. 3). The estrogenic isomer, o,p′ DDE, did not demonstrate similar effects as p,p′ DDE. Both latency and maximal incidence were not significantly different from control (Fig. 1 & 2). Additionally, Cohn and colleagues (2007) observed a correlation between DDT levels and breast cancer in women before the age of 50. As breast cancer before age 50 precedes the average age of diagnosis, the increased risk with higher levels of p,p′ DDT suggests an effect on latency similar to that observed with p,p′ DDE in the MMTV-*neu* mice. The age of onset profoundly impacts breast cancer; early-onset (premenopausal) cancer (≤ 50 years) is more aggressive and has a worse prognosis than in postmenopausal years (Benz et al., 2008). Inducing an earlier onset of breast cancer can increase the likelihood of women developing ER-negative tumors (Benz et al., 2008; Vetto et al., 2009), including HER2-positive breast cancer, which the mouse model used in these studies mimics.

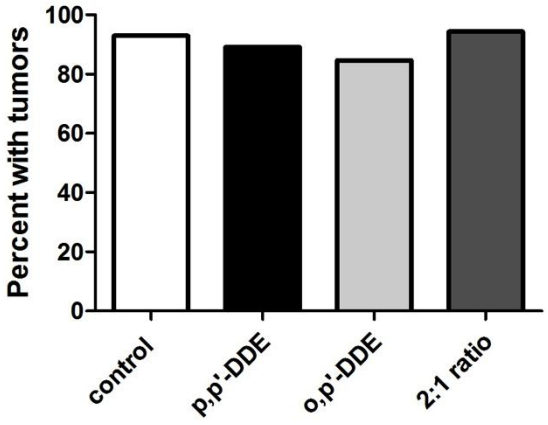

Figure 1. No significant difference in maximal tumor incidence was detected in > 9 month old MMTV-*neu* female mice. The number of tumors formed by p,p´ DDE (n=65), o,p´ DDE (n=59) and 2:1 ratio (n=54) groups was not significantly different from control (n=57) by chi-square test, p> 0.05.

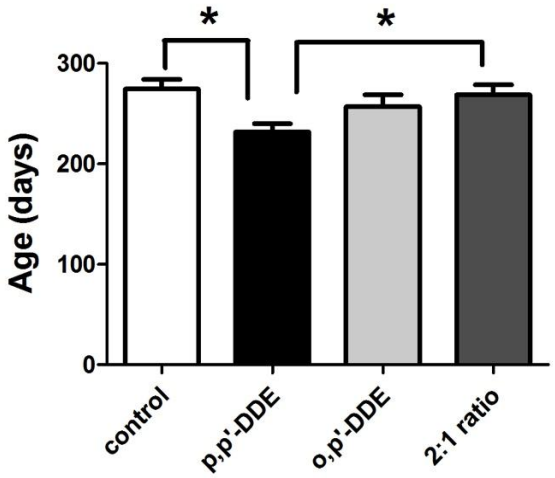

Figure 2. Localized delivery of p,p´ DDE accelerated tumor formation in > 9 month old MMTV-*neu* female mice. Para,para´ DDE significantly decreased the age of tumor onset by an average of 1.5 months compared to control. Also, when p,p´ DDE was co-administered with o,p´ DDE in a 2:1 ratio (total concentration of 5 µg/pellet), latency effects resembled control. One-way ANOVA with Tukey's post test used for statistical analysis ($p<0.05$) n= 50-58.

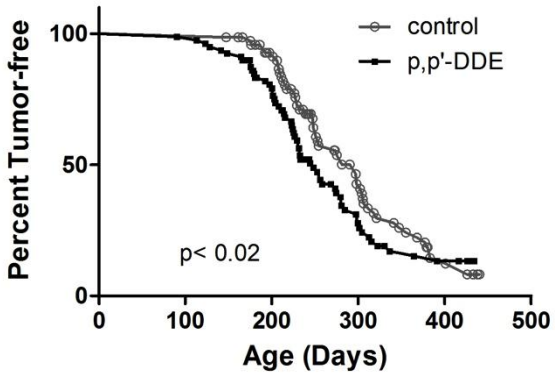

Figure 3. p,p´ DDE consistently caused tumors to develop earlier versus control. The first mammary tumor was detected almost two months earlier in the p,p´ DDE group (at 91 days of age) compared to the control animals on day 147 of age. Gehan-Breslow-Wilcoxon test used for statistical analysis ($p<0.05$).

Also, as DDT occurs in women as a mixture, an additional group examining the combined effects of p,p´ DDE and o,p´ DDE in a 2:1 ratio (5 µg/pellet total) was also investigated. The concentration of p,p´ DDE included in the combined treatment was higher than o,p´ DDE due to it being the most prevalent metabolite in humans. The o,p´ DDE concentration was higher than found in humans, but may represent the total of estrogenic congeners in some women. Surprisingly, although p,p´ DDE significantly accelerated tumor formation, when combined with o,p´ DDE at a 2:1 ratio (p,p´ DDE:o,p´ DDE), no change in tumor latency occurred (Fig. 2). Similar to its latency, the survival curve of this group also resembled control, indicating that the addition of o,p´ DDE mitigated p,p´ DDE's tumor latency accelerating effects (Fig. 4). These findings imply that the ratio of DDE isomers may influence tumor development differently than the single p,p´ DDE congener. The incidence of mammary tumor formation was similar between groups, suggesting that these congeners, p,p´ DDE and o,p´ DDE , do not cause an increase in mammary tumor formation (Fig. 1). Collectively, these findings may

suggest that p,p´ DDE has the ability to remove many cancer-free years from a woman's life.

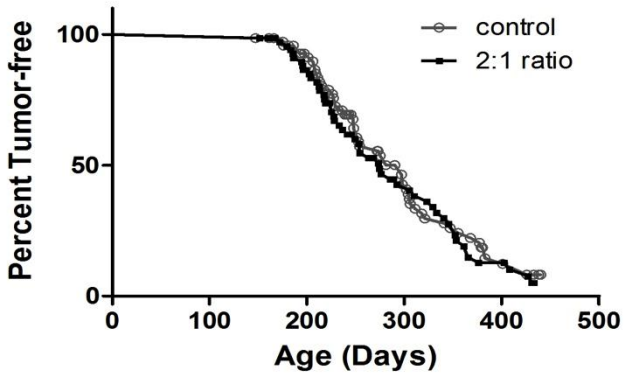

Figure 4. Co-administration of p,p´ DDE and o,p´ DDE in a 2:1 ratio resembled control. When p,p´ DDE was combined with o,p´ DDE, the accelerated tumor onset was not observed, as the results from this combination treatment group resembled those of the control group. Gehan-Breslow-Wilcoxon test used for statistical analysis ($p>0.05$).

PREVIOUS STUDY RELEVANCE

The previous study demonstrated that **p,p´ DDE caused mammary tumors to develop at an earlier age in MMTV-*neu* female mice**. If levels and timing of exposure are similar in women, p,p´ DDE may have similar results in women. As mice have a short lifespan, the 1.5 months earlier tumor onset could equate to the loss of years or decades of cancer-free years from a woman's life. The importance of DDE ratios was also demonstrated when the anti-androgenic p,p´ DDE was co-administered with the estrogenic isomer o,p´ DDE in a 2:1 ratio. With this combination, p,p´ DDE lost its ability to cause earlier tumor onset. As a lower dose was tested for p,p´ DDE, the effects with the 2:1 ratio may be due to dose or to the effects of the combined isomers. Thus, the doses or ratios of p,p´ DDE with other DDT components within the mammary gland may be involved in tumor development and was tested within this study.

As the result in the tumor study show an effect on latency, the studies to identify markers of p,p′ DDE action are needed to look for effects of p,p′ DDE prior to tumor onset. Therefore, these studies used animals exposed to the treatments for 2 months. The animals examined included the pretumor study that was performed with the tumor study by Davis and colleagues to examine the same treatments after 2 months of exposure in the normal, adult mammary gland prior to tumor detection. In addition, additional groups (DDT study 01 and 02) were developed to test central questions regarding the local effects of p,p′ DDE in the mammary gland, as well as possible systemic effects.

HYPOTHESIS

Delivery of p,p´ DDE to the mammary glands of MMTV-*neu* female mice results in concentrations applicable to human exposure, which induce local and systemic gene expression associated with cancer promotion. The p,p´DDE-induced differential gene expression identified by microarray analysis is partially caused by the anti-androgenic actions of p,p´DDE, as well as distinct doses, ratios and immune properties not shared with o,p´ DDE or p,p´ DDT congeners that could be related to the cancer-promoting ability of p,p´ DDE.

Specific Aims

1. *To validate the translational relevance of the MMTV-neu mouse model by assessing the impact of treatment with DDT congeners on the MMTV promoter and the levels of DDE in serum and mammary adipose tissue*

   Androgens regulate MMTV transcription (Darbe et al., 1986; Cato et al., 1987). Therefore, in order to directly correlate the decreased latency in the tumor study to p,p´ DDE treatment, the possibility of aberrant stimulation of the promoter needed to be excluded, as this promoter would not be associated with breast cancer development in women. If *neu* levels were strongly increased, its expression, and not p,p´ DDE actions in the mammary tissue, could be related to the earlier tumor formation.

Additionally, lipid-adjusted levels of DDE delivered by ELVAX 40P pellets to the mammary gland were measured in serum and mammary gland tissue, identical to the way it is measured in women in epidemiological studies, for correlating to human exposure.

2. *To identify candidate genes that are differentially expressed in the mammary gland of MMTV-neu mice after chronic exposure to* p,p´ *DDE*

The findings of Davis and colleagues showed tumors develop earlier when p,p´ DDE was delivered to the mammary gland beginning prior to puberty. Normal mammary tissue prior to tumor detection was examined to identify genes modified by 2 months of exposure to p,p´ DDE. Using microarray technology, genes that were differentially expressed in the mammary tissue of mice treated with p,p´ DDE were compared to control (vehicle pellets) mice. Genes (markers) selected for further testing had previously reported roles in cancer or were hormonally regulated or immune-related.

3. *To investigate the potential anti-androgenic actions of p,p´ DDE, its dose-dependence, and its similarity to or influence by other DDT congeners in the mammary tissue using the identified p,p´ DDE -regulated genes.*

The genes identified in Aim 2 were further analyzed in mammary tissue in other treatment groups to characterize p,p´ DDE actions. *In vitro* and in

male rats, p,p′ DDE has been demonstrated to act as an anti-androgen, but whether it has this activity in the female mammary gland has not been tested. This study will test whether p,p′ DDE mimics responses of a known anti-androgen (hydroxyflutamide). The lack of effect in the tumor study by the 2:1 ratio suggests either the dose of p,p′ DDE or its combination with o,p′ DDE caused the loss of accelerated tumor onset. Therefore, varying doses of p,p′ DDE will be tested to observe the resulting effects on the identified p,p′ DDE markers. Additionally, ratios with varying levels of o,p′ DDE combined with a constant concentration (5 µg/pellet) of p,p′ DDE were tested. As p,p′ DDT has correlations to increased breast cancer risk (Cohn et al. 2007) and anti-androgenic action (Kelce et al., 1995), this DDT congener will also be investigated alone and in combination with p,p′ DDE on the same markers.

4. *To investigate p,p′ DDE effects from systemic exposure by examining its influence on the hormonally-responsive uterus and cytokine gene expression in splenic leukocytes*

Although p,p′ DDE was delivered locally to the mammary gland, detectable levels of DDE isomers were measured in serum (see Chapter 4), thereby confirming systemic exposure. Cytokine gene expression was measured in both splenic leukocytes and lymph node positive mammary gland tissue to determine and compare the systemic and local effects of

p,p′ DDE. Uterine weights were also used as an endpoint to assess systemic hormonal effects of p,p′ DDE.

Collectively, these specific aims seek to answer five key central questions:

CENTRAL QUESTIONS

1. What genes are differentially expressed after localized exposure to the same dose of p,p′ DDE that resulted in the earlier onset of mammary tumors?

2. Is modulation of the identified p,p′ DDE markers in the mammary gland dose dependent?

3. Does p,p′ DDE action mimic a known anti-androgen locally within the mammary gland or systemically?

4. Does combining p,p′ DDE with its estrogenic isomer o,p′ DDE influence the pattern of gene expression of p,p′ DDE-regulated markers in the mammary gland?

5. Does p,p′ DDT, which also has anti-androgen activity, induce similar or different responses, locally or systemically, as p,p′ DDE in the identified markers?

Chapter 2

Testing the Translational Relevance of the MMTV-neu Mouse Model

INTRODUCTION

The first goal for Aim 1 was to confirm that the MMTV-*neu* mouse model is relevant for examining p,p´ DDE exposure on mammary tumor development. Even though this model is commonly used to study breast cancer, the concerns reside in the regulation of the MMTV promoter by androgens and other steroid hormones (Darbe et al., 1986). Therefore, it is important to ensure this promoter driving expression of the *neu* transgene was not significantly regulated by p,p´ DDE and its anti-androgenic activity. Although p,p´ DDE was observed to act like an androgen *in vitro* (Kelce et al., 1995), its action in the mammary gland is unknown. It binds to the androgen receptor, and some anti-androgens have weak androgenic activity. The MMTV sequences that direct hormonally-regulated transcription are contained within a region of long terminal repeats (LTR) between nucleotides -202 and -50 relative to the start of transcription (+1) (Gunzburgh and Salmons, 1992). Glucocorticoids, progestins and, most importantly for this study, androgens have all proven to regulate MMTV transcription (Darbre et al., 1986; Cato et al., 1987). Therefore, if p,p´ DDE abnormally stimulates the MMTV promoter and, consequently, rat *neu* transgene expression, the decreased latency may not be due to treatment. In that case, the early tumor formation could be an artifact of the model. As women have the HER2/*neu* gene regulated by its own promoter and not MMTV, then HER2/*neu* would not be similarly regulated in women to increase their risk of early breast cancer with p,p´ DDE exposure. If the transgene is not differentially stimulated

compared to control mice, then the p,p´ DDE treatment versus the *neu* regulation by the MMTV promoter would be responsible for any responses detected for this treatment in the mammary gland; this is the desired result for using this model for analyzing tumor outcomes from the tumor study and for the subsequent investigations in Aims 2-4 within this study. As increased expression of *neu* would need to precede tumor detection to influence tumor formation, transgene expression was examined in the normal mammary glands in adult mice treated for 2 months (Pretumor study).

METHODS

Total RNA Isolation

For gene expression investigations total RNA was isolated from lower mammary tissue and splenic leukocytes using the Absolutely RNA® Miniprep Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Due to the mammary gland being mainly adipose, RNA yields from 20 mg of tissue were consistently low. However, after completing a small series of tests, using approximately 30, 40, 50, 90 and 110 mg of mammary tissue, it was discovered that RNA yields could be increased by increasing tissue weight without clogging the filter or the need for additional amounts of reagents (Table 2).

Table 2. Increasing RNA Yields with Same Amount of Reagents

Tube #	Tissue	Weight (mg)	Lysis Buffer (µl)	β-ME (µl)	260 Abs (nm)	280 Abs (nm)	260/280 Ratio	Dilution	Yield (µg)
1	LM	30	600	4.2	.188	.089	2.113	1:5	5.63
2	LM	43	600	4.2	.428	.246	1.742	1:5	12.84
3	LM	55	600	4.2	.501	.246	2.040	1:5	15.03
4	LM	90	600	4.2	.548	.260	2.104	1:5	16.44
5	LM	110	1000	7	1.131	.549	2.078	1:5	33.93

LM= lower mammary; β-ME=beta mercaptoethanol

Therefore, tissue sample weights were modified and 40-90 mg of tissue was homogenized with 600 µl of lysis buffer (which contains guanidine thiocyanate, a strong protein denaturant) and 4.2 µl of β-mercaptoethanol. The homogenate was pre-spun to remove particles and reduce DNA by transferring the tissue homogenate to a 2 mL receptacle with a fiber matrix spin cup that was provided in the kit and centrifuged at 13,200 rpm for 5 minutes. An equal amount of 70% ethanol was added to the filtrate, vortexed for 5 seconds and transferred to a fresh 2 mL receptacle with an RNA binding spin cup and microcentrifuged at 13,200 rpm for 60 seconds. Next, samples were washed with 600 µl of low-salt wash buffer to remove any remaining DNA and microcentrifuged at 13,200 rpm for 60 seconds. After the filtrate was discarded, the receptacle and RNA binding spin cup were centrifuged for 2 minutes in order to dry the fiber matrix. Samples were then treated with the DNase solution and incubated at 37°C for 20 minutes. After the incubation period, the samples were washed once with high-salt buffer and centrifuged at 13,200 for 60 seconds. The high-salt wash was followed by two subsequent low-salt buffer washes centrifuged at 13,200 rpm for 60 and 120 seconds,

respectively. To elute the highly pure RNA from the fiber matrix, the RNA binding spin cups were transferred to new RNase-/DNase-free tubes. Seventy five microliters of elution buffer warmed to 60°C was added directly to the fiber matrix and incubated at room temperature for two minutes, followed by centrifugation at 13,200 rpm for 60 seconds. The elution process was repeated a second time before RNA quality/integrity was determined using the 260:280 nm ratio on the Beckman DU®530 Life Science UV/Vis spectrophotometer. High quality RNA used for subsequent real-time RT-PCR possessed a ratio of 1.8-2.2.

cDNA Reverse Transcription (RT) Reactions

RT reactions for real-time RT-PCR were made using the Reverse-iT™ 1st Strand Synthesis Kit by ABgene (Epsom, Surrey, UK) according to the manufacturer's protocol. In the applied two-step protocol, 500 ng of RNA was combined with random decamers and sterile water in thin-walled reaction tubes and heated at 70°C for 5 minutes to remove any secondary structures. Samples were then placed on ice. Next, 5x First Strand Synthesis Buffer, dNTP mix (5mM each), 100 mM DTT and *Reverse*-iT™ RTase Blend were added to each tube and incubated at 47°C for 50 minutes for strand synthesis in the Eppendorf Mastercycler® ep (Brinkman Instruments, Westbury, NY). This step was followed by an incubation at 75°C for 10 minutes to inactivate the reverse transcriptase. In addition to RT reactions being made, "No RT" reactions were also made. These reactions consisted of all the same components as the RT reactions with the exception of the *Reverse*-iT™ RTase Blend, which was substituted by an equal amount of sterile RNase/DNase-free water. Total volume for all samples was 20 µl. "No RT" reactions are

used as a control in order to identify possible DNA contamination. After the incubations, several tubes of the same sample were often pooled in order to decrease variability within cDNA synthesis levels, because multiple genes were examined per sample. Pooling produces a more accurate representation of the sample. All samples were then stored at -20°C until ready to use for real-time RT PCR analysis of gene expression.

Subsequent to the Reverse-iT™ 1st Strand Synthesis Kit by ABgene, RT reactions for gene analysis from the second microarray were made using the qScript™ cDNA Synthesis Kit, by Quanta Biosciences (Gaithersburg, MD) due to the ABgene/BioRad product relationship being terminated . Although very similar to the ABgene cDNA kit described above, the qScript cDNA kit contained all the necessary components for cDNA synthesis (including random and oligo(dT), 5X optimized buffer, and dNTPs) all in one reaction mix, only enzyme and the RNA template needed to be added. This master mix is particularly beneficial considering it has both random and oligo dTs. Oligo (dT) are specific to the poly-A tail of the RNA and may not reach the desired region of amplification. However, random dTs are not specific to the poly-A tail and can produce cDNA from all regions of the RNA. When making RT reactions, all components were added to 0.2 mL thin-walled PCR tubes on ice. For a single reaction, the only component amounts that varied were the RNA template (250 ng) and nuclease-free water. qScript reaction mix remained constant at 4.0 µl as did 1.0 µl of reverse transcriptase; for a total RT volume of 20 µl. Once all components were added together they were place in the Eppendorf Mastercycler® ep (Brinkman Instruments, Westbury, NY) programmed as follows: 1 cycle: 22º C for five minutes, 1 cycle: 42º C for 30 minutes, 1 cycle: 85º C for

five minutes followed by a 4º C hold. After cDNA synthesis was complete, samples were stored at -20°C until ready to use for real-time RT PCR analysis of gene expression.

Real-Time RT-PCR

Real-time detection of RNA levels was performed on the iCycler (Bio-Rad, Hercules, CA) using SYBR Green Supermix (Quanta Biosciences, Gaithersburg, MD) detection. All primers (see appendix) were designed to span at least 1 intron/exon junction, in order to eliminate possible contamination of amplified genomic DNA. PCR protocols were as follows: Absolute QPCR SYBR Green Fluorescein Mix (ABgene, Rochester, NY) PCR protocol: enzyme activation at 95 º C for 15 minutes, followed by 50 cycles of denaturation for 30 seconds at 95 º C and annealing/extension at 60 º C for 30 seconds. Quanta B-R SYBR Green Supermix: initial activation 95 º C for 3 minutes, followed by 50 cycles of denaturation at 95 º C for 15 seconds and annealing/extension at 60 º C for 45 seconds. Melt curves were also performed at the end of each run to ensure the presence of a single product for the sample. Prior to any real-time RT-PCR being conducted, primer efficiencies were also conducted to ensure the primer would work. These efficiencies entailed assigning arbitrary concentrations of the RT using a 10-fold serial dilution that was then used to make a standard curve, with a perfect fit having a correlation coefficient of 1.00. An acceptable PCR efficiency was considered 100% ± 20. Additionally, gel electrophoresis was conducted on a small subset of samples tested for each gene used, in order to visualize that the amplified product had the appropriate base pair size (Fig. 5).

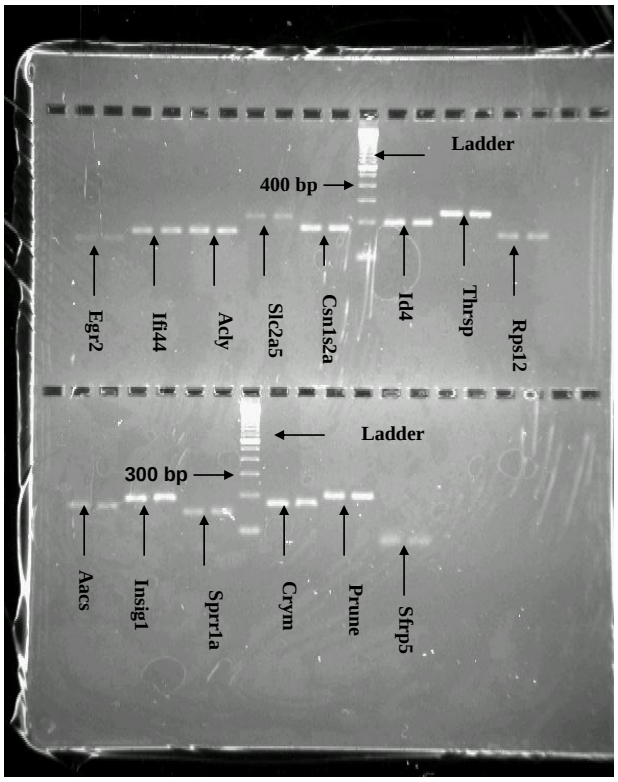

Figure 5. Example of gel electrophoresis used to confirm product size of primers. All genes that were investigated had their amplified product visualized to ensure proper base pair size. Primers were designed to be less than 300 base pairs in length.

Relative Gene Expression

In order to analyze the data from the real-time RT-PCR, relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with cyclophilin A (*ppia*) used as the housekeeping gene for normalization. Briefly, the ΔCt value was obtained by subtracting the cycle threshold (Ct) of the housekeeping gene from the Ct value of the gene of interest for each sample. The Ct is the number of cycles it takes for the fluorescent signal to cross a specific threshold value. Therefore, the more cycles needed to cross the threshold would be indicative of lower amounts of transcript of that gene. The ΔΔCt value was obtained by subtracting the average ΔCt of the control group from

the average ΔCt of the treated group. The relative gene expression is expressed by a fold change using the equation $2^{-\Delta\Delta Ct}$. Statistical analysis was performed using ΔCt.

Statistical Analysis

Analyses were performed using GraphPad® Prism 5.0 software. A Mann-Whitney U test was used to determine the differences between two groups. This non-parametric test has no requirement that the two populations have a normal (Gaussian) distribution or any other particular distribution. One-way analysis of variance (ANOVA) was also used when analyzing sample variances of three or more populations that are independent of each other. This method is robust against variances that are not equal, meaning it works well unless the population variances differ by large amounts (Triola and Triola, 2006). Additionally, Tukey's HSD (honestly significant difference) post ANOVA tests were performed in order to find what means were significantly different from one another. In other words, this test measures the means of every treatment to the means of every other treatment. Analysis by two-way ANOVA with Bonferroni post test was employed to determine variances between samples that are categorized two ways. Similar to a one-way ANOVA, this method is also robust against unequal variances. The Bonferroni test is a multiple comparison test that identifies differences in pairs of means but makes adjustments to overcome for the problem of having a significance level that increases as the number of individual tests increases (Triola and Triola, 2006). Statistical significance was considered $p<0.05$.

RESULTS

To investigate regulation of *neu* expression in the MMTV-*neu* mice, rat *neu* (transgene) levels were measured in RNA prepared from mammary tissue of 3-month-old MMTV-*neu* female mice from the Pretumor study. Six animals from each group, including the control, p,p´ DDE, o,p´ DDE, and the 2:1 ratio (p,p´ DDE:o,p´ DDE) groups, were examined by real-time RT-PCR using a specific primer set for rat *neu* transgene, which does not detect the endogenous mouse *neu* gene. None of the treatments significantly increased the expression of the transgene in mammary tissue (Fig.6). These findings demonstrate that p,p´ DDE and the other treatments used in the study did not cause abnormal stimulation of the promoter in normal, adult mammary tissue prior to tumor onset. As p,p´ DDE was the only treatment group that accelerated tumor development in the MMTV-*neu* mice, the lack of stimulated transgene expression by this DDT metabolite was an important finding for interpreting translational relevance of this tumor outcome.

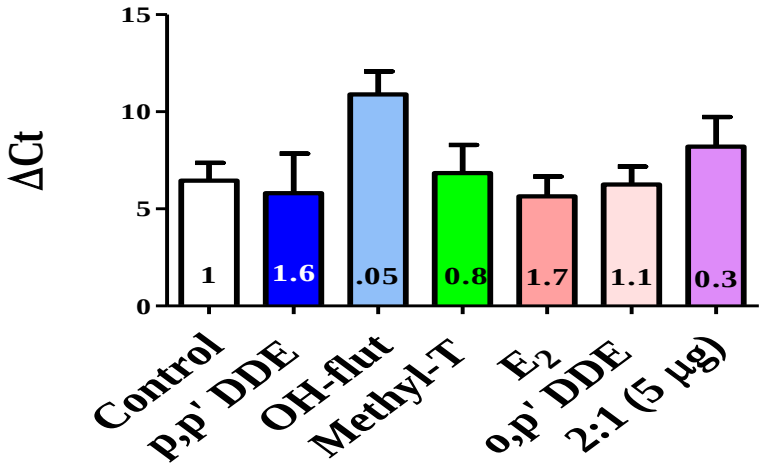

Figure 6. Treatment groups did not modify expression of the rat *neu* transgene in mammary tissue of 3-month-old MMTV-*neu* female mice. Regulation of the MMTV promoter was not affected by the treatments as demonstrated by there being no significant difference in expression of the rat *neu* transgene in any group compared to control mice. RNA levels were determined by real-time RT-PCR from random mammary tissue sections from mice in the Pretumor study. ΔCt expression represents the normalized levels of the rat *neu* transgene to *ppia* (cyclophilin A). A lower ΔCt value indicates the gene is expressed at a higher level. Numbers displayed within each bar represent the fold-change compared to control using the $2^{-\Delta\Delta Ct}$ method. One-way ANOVA with Tukey's post test was used for statistical analysis ($p>0.05$). n=6.

As the MMTV promoter has known regulation by several hormones, its regulation by other treatment groups in the Pretumor study were compared to the control and p,p´ DDE groups. For example, comparing p,p´ DDE to the known anti-androgen, hydroxyflutamide (OH-flut), will determine if these two anti-androgens have similar effects on this androgen-regulated promoter. OH-flut is the active metabolite of flutamide, a pharmaceutical anti-androgen used for treating prostate cancer. With oral delivery in men, the drug would pass through the liver and be metabolized to its active form. However, as little metabolism would be expected to occur in mammary tissue, OH-flut is the preferred compound for localized delivery to the mammary glands. OH-flut was tested to determine if p,p´ DDE influences responses similarly to this known anti-

androgen, even though not all anti-androgens may not stimulate or repress the exact same gene profile as another. In tissue culture, p,p´ DDE was equipotent to OH-flut at inhibiting an androgen-regulated reporter gene (Kelce et al., 1995), so the same dose of both compounds was tested in the mice (5 μg/pellet).

The anti-androgenic activity of OH-flut was evident on the MMTV promoter, since it significantly down-regulated rat *neu* transgene expression compared to control ($p=0.02$, Mann-Whitney test). However, p,p´ DDE was not significantly different than the control group. Examination of the expression of rat *neu* transgene within the pretumor animals, revealed that the OH-flut and p,p´ DDE did not produce similar effects on rat *neu* gene expression (Fig. 7), with a difference approaching significance ($p=0.06$, Mann-Whitney test). Although the disparity between the anti-androgens was unexpected, it demonstrated p,p´ DDE did not act like this known anti-androgen on rat *neu* regulation.

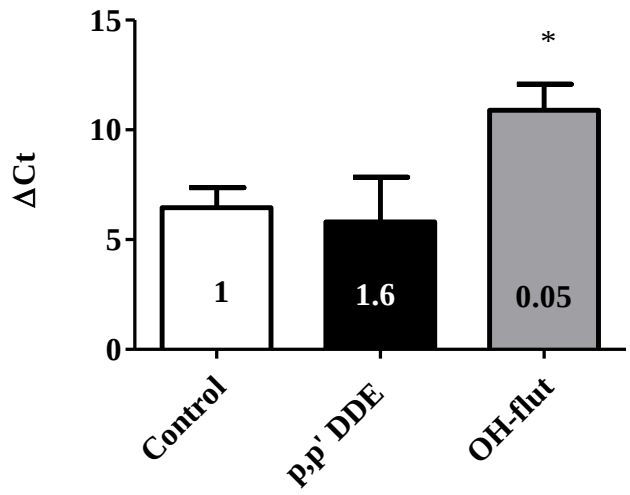

Figure 7. Anti-androgenic treatments did not uniformly impact the expression of the rat *neu* transgene after 2 months of exposure in 3-month-old MMTV-*neu* female mice. Unlike OH-flut, the 1.6-fold *neu* levels compared to control mice show an up-regulation in expression of the transgene by p,p´ DDE although not significant by one-way ANOVA. ΔCt expression represents the normalized levels of the *neu* transgene to *ppia* (cyclophilin A). A lower ΔCt value indicates the gene is expressed at a higher level. Numbers displayed within each bar represent the fold-change compared to control using the $2^{-\Delta\Delta Ct}$ method. One-way ANOVA with Tukey's post test was used for statistical analysis ($p<0.05$). n=6. OH-flut= hydroxyflutamide, * denotes significance compared to control by Tukey's test.

Para, para´ DDE was also compared to methyl-T (50 ng/pellet), an androgen that is not converted to estrogen by the enzyme aromatase. Due to the androgenic nature of methyl-T, it might be expected to stimulate *neu* gene expression, however, this effect was not observed (Fig. 8). The lack of significant change in transgene expression compared to the control mice suggests androgen regulation of the MMTV promoter by methyl-T did not occur at the dose tested in the Pretumor study.

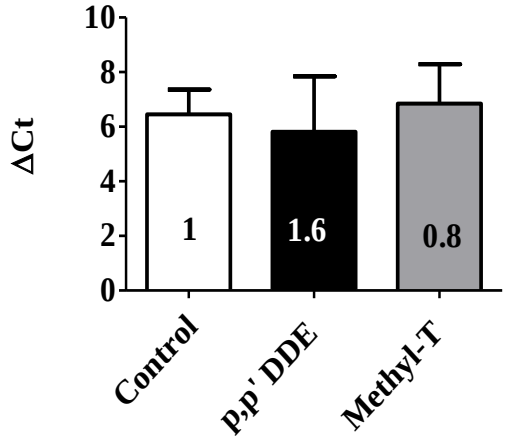

Figure 8. The expression levels of rat *neu* with a non-aromatizable androgen and p,p´ DDE were similar to the control group in mammary tissue of 3-month-old MMTV-*neu* females. When compared to control, p,p´ DDE (5 µg/pellet) and methyltestosterone (methyl-T; 50 ng/pellet) expression of the rat *neu* transgene resembled control. ΔCt expression represents the normalized levels of the *neu* transgene to *ppia* (cyclophilin A). A lower ΔCt value indicates the gene is expressed at a higher level. Numbers displayed within each bar represent the fold-change compared to control using the $2^{-\Delta\Delta Ct}$ method. One-way ANOVA with Tukey's post test was used for statistical analysis ($p>0.05$). n=6.

Next, comparisons in rat *neu* expression were also made with the estrogenic treatments (Fig. 9). The o,p´ DDE isomer with weak estrogenic activity was compared to 17β-estradiol (E_2), the most potent endogenous estrogen. Neither o,p´ DDE nor E_2 (2.5 ng/pellet) were significantly different from control or one another. These data support the findings of Otten et al., (1987) and Glover and Darbre (1989) that report MMTV hormone response elements (HRE) of the long terminal repeat (LTR) is not regulated by estrogens, because it does not contain an estrogen response element (ERE). Additionally, although p,p´ DDE has weak estrogenic effects (Ociepa-Zawal et al., 2010), weakly binds to the estrogen receptor (Kelce et al., 1995), and had similar expression levels as o,p´ DDE, the possibility of p,p´ DDE having estrogenic action in the mammary glands

cannot be determined due to the inability of o,p´ DDE and E_2 to induce a response via the MMTV promoter.

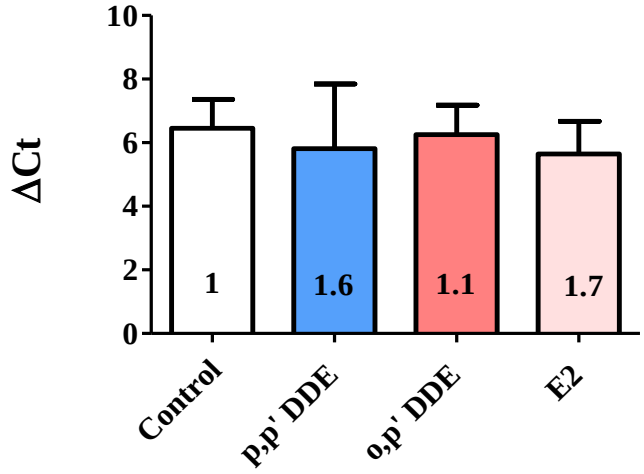

Figure 9. Estrogenic treatments did not significantly alter rat *neu* expression. The estrogenic treatments of o,p´ DDE (5 µg/pellet) and 17β-estradiol (E_2, 2.5 ng/pellet) did not significantly influence rat *neu* expression as expected. Additionally, p,p´ DDE displayed very similar expression patterns with both o,p´ DDE and E_2, having fold-changes ranging from 1.1-1.7. ΔCt expression represents the normalized levels of the *neu* transgene to *ppia* (cyclophilin A). A lower ΔCt value indicates the gene is expressed at a higher level. Numbers displayed within each bar represent the fold-change compared to control using the $2^{-\Delta\Delta Ct}$ method. One-way ANOVA, with Tukey's post test were used for statistical analysis ($p>0.05$). n=6.

Lastly, when examining the co-administration of p,p´ DDE (3.3 µg) and o,p´ DDE (1.7 µg) in a 2:1 ratio (5 µg total), a modest decrease in expression (0.3-fold) was observed, though this effect was not significant (Fig. 10). These data correlate with the lack of regulation of the *neu* transgene by the individual DDE isomers in normal, adult mammary tissue.

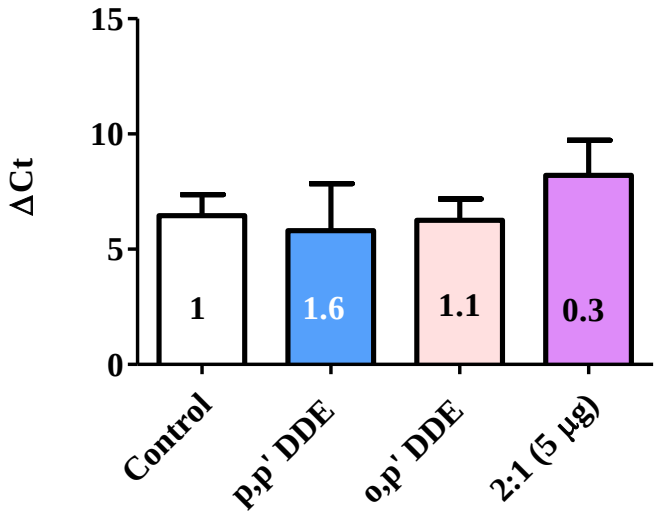

Figure 10. Co-administration of p,p´ DDE and o,p´ DDE in a 2:1 ratio did not significantly affect *neu* transgene expression. Similar to the individual expression of p,p´ DDE (5 µg /pellet) and o,p´ DDE isomers (5 µg/pellet), the expression of the combination treatment of p,p´ DDE (3.3 µg/pellet) and o,p´ DDE(1.7 µg/pellet) in a 2:1 ratio (total of 5 µg/pellet) [2:1 (5 µg/pellet)] was also not significantly different from control. ΔCt expression represents the normalized levels of the *neu* transgene to *ppia* (cyclophilin A). A higher ΔCt is indicative of the gene being poorly expressed, thus requiring more amplification cycles before it crosses the threshold. One-way ANOVA with Tukey's post test was used for statistical analysis ($p>0.05$). n=6.

Expression of the endogenous mouse *neu* was also measured in the same animals by real-time RT-PCR using a primer set that is selective for the mouse version of the endogenous *neu* gene. Investigating how the six treatment groups impacted the normal mouse *neu* gene expression by its endogenous promoter may suggest how the human HER2/*neu* gene may respond to these DDT congeners and hormones in breast tissue. If it is increased strongly by p,p´ DDE, these results could suggest the potential for this upregulated gene to influence proliferation or tumor development in breast tissue exposed to this prevalent DDT metabolite. However, although 20-30% of primary human breast cancers express elevated levels of HER2/*neu*, which is associated with poor clinical

outcome (as reviewed by Hutchinson and Muller 2000), this elevation is due to genomic amplification of the HER2 protooncogene (Slamon, 1987) and not simple upregulation by regulating factors.

Mouse *neu* expression was measured by real-time RT-PCR using specific primers for mouse *neu* that do not recognize the rat *neu* transgene in six animals per group from the Pretumor study in the control, DDT congeners (p,p´ DDE , o,p´ DDE , 2:1 ratio) and hormonal controls (E_2 , methyl-T and OH-flut). Endogenous mouse *neu* expression for all treatments was not significantly different from control (Fig. 11). Not surprisingly, mouse *neu* expression was also lower than rat *neu* transgene expression (ΔCt at least 5 cycles later, or >30-fold, than for the transgene, see Fig. 6 & 11), because it is not under the control of the strong MMTV promoter. These data demonstrate that all six treatment groups did not cause an increase in *neu* expression compared to mice treated with control (vehicle) pellets.

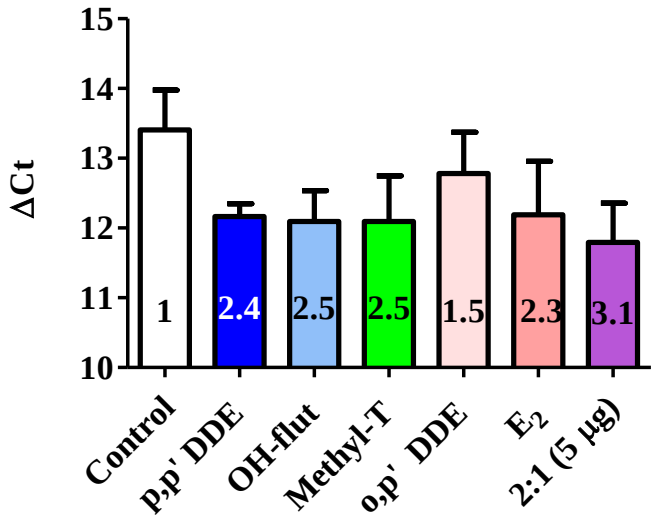

Figure 11. Endogenous mouse *neu* expression was unaffected by DDE or hormonal treatments in mammary tissue of MMTV-*neu* mice treated for 2 months. Regulation of endogenous mouse *neu* was also not affected by treatment as demonstrated by there being no significant difference in expression of the gene in any treatment group compared to control using real-time RT-PCR. ΔCt expression represents the normalized levels of endogenous *neu* to *ppia* (cyclophilin A). A lower ΔCt value indicates the gene is expressed at a higher level. Numbers displayed within each bar represent the fold-change compared to control using the $2^{-\Delta\Delta Ct}$ method. One-way ANOVA with Tukey's post test was used for statistical analysis ($p>0.05$). n=6. OH-flut = hydroxyflutamide; Methyl-T = methyltestosterone; E_2 = 17β estradiol; 2:1 = 3.3 μg p,p´ DDE + 1.7 μg o,p´ DDE.

DISCUSSION

The MMTV-*neu* (c-*erb*B-2) mouse model was used for these studies due to it being an important oncogene in human breast cancer, which is amplified in 20-30% of all human breast cancers (Slamon et al., 1987). Its appearance in women correlates with poor prognosis and minor perturbations in amplified *neu* expression are sufficient to alter mammary development and induce malignant transformation (Weinstein et al., 2000). Furthermore, mouse mammary tumor virus (MMTV)-driven transgenic mice allow for the targeted expression of the *neu* oncogene to examine its effects on neoplastic transformation of mammary tissue (Taneja et al., 2009). The MMTV-*neu* model also possesses other similarities to human breast cancer, such as requiring spontaneous activation (Hutchinson and Muller 2000) and result in mammary tumors that frequently metastasize to the lungs after a long latency (Guy et al., 1992). However, although the MMTV-*neu* model had been successfully used to study breast cancer for decades, there are limitations of the model. For example, the oncogene utilizes an extremely strong viral promoter that allows for high levels of transcriptional activity. This promoter among others, are of necessity and not physiologic; causing elevated levels of oncogene expression, not regulated in a physiologic manner (Weinstein et al., 2000); therefore tumor development in this model does not represent an accurate recapitulation of human disease (McDermott and Wicha, 2010). However, the MMTV-*neu* mouse model has been proven to be a useful tool for preclinical studies on breast cancer. One aspect of this model that had to be tested to ensure that the tumor-inducing effects of p,p´ DDE were not due to an artifact of the model is the hormonal regulation of the MMTV promoter.

The long terminal repeat (LTR) region of the MMTV promoter has a hormone response element (HRE) located between nucleotides -202 and -50 relative to the transcription initiation site (reviewed in Günzburgh and Salmons 1992). This HRE has been documented as being induced by androgens (Darbe et al., 1986; Cato et al., 1987; Otten et al., 1987; Glover et al., 1989). Since several treatment groups in the pretumor study contained steroid hormones or possessed hormonal activity, it is possible that the treatments could alter the expression of the *neu* transgene. However, the group of concern would be p,p´ DDE since it was the only group to significantly shorten tumor latency. An increase in expression by the promoter could result in aberrant increases in *neu* expression and, possibly, earlier tumorigenesis. An increase in *neu* expression would then be considered an artifact of the model, making any potential findings by p,p´ DDE less relevant as it suggests the treatment effects on tumor development could be on the MMTV promoter and not the mammary tissue. In women, the endogenous HER2 promoter is not under the control of a viral promoter (MMTV), so translational relevancy would be reduced. However, as expected, the androgenic p,p´ DDE did not significantly modify expression of the transgene compared to the control group. Therefore, the decreased latency observed in the tumor study was, in fact, due to p,p´ DDE treatment effects on mammary tissue and not due to the design of the mouse model. These data imply the MMTV-*neu* mouse model is an appropriate model to use for subsequent investigations.

Comparisons between p,p´ DDE-induced effects versus OH-flut, which is an androgen receptor antagonist, revealed that the treatments did not have the same effect on the rat

neu transgene. OH-flut resulted in significantly down-regulated *neu* expression (0.05-fold) compared to control (Fig. 7). As an anti-androgen, OH-flut should block the stimulation of the MMTV promoter via the HRE in its LRT by physiological levels of androgen (Glover and Darbre 1989). Therefore, its effect on rat *neu* expression is the expected response. As the strongest regulation of the *neu* transgene was by OH-flut (Fig. 7), these data indicate that its dose was sufficient to inhibit the androgen-regulated promoter, even without the ability to bioaccumulate like p,p´ DDE in the mammary gland. Although p,p´ DDE and OH-flut are both anti-androgens, the lack of correlation in rat *neu* expression suggests that p,p´ DDE is not acting like an anti-androgen on the MMTV promoter.

Methyl-T, which is a non-aromatizable androgen, would be expected to stimulate *neu* expression by the androgen-regulated MMTV promoter. However, the levels of *neu* transgene expression were similar to the control group; these results suggest that the dose of methyl-T was insufficient to regulate expression from the MMTV promoter. Due to its lack of effect on the androgen-regulated MMTV promoter, no conclusions can be drawn about other potential androgen actions in the mammary tissue that could have been mediated by methyl-T.

Similar to the testing of the rat *neu* transgene, for aberrant modulation, the endogenous mouse *neu* gene was also examined to ascertain whether or not various treatments induced its expression. The expression of endogenous *neu* was considerably lower than the rat transgene (Fig. 6 & 11), as expected, since the endogenous *neu* gene is not under

the control of the strong MMTV promoter. None of the treatment groups significantly modified the expression of mouse *neu*. These data demonstrate the endogenous mouse *neu* gene is not significantly regulated by any of the hormonal treatments tested. This lack of regulation is a desired effect, which implies, if extrapolated to the human population, the treatments may not influence HER2-positive breast cancer development by modifying HER2 expression.

The only treatment group to induce significant regulation of the transgene was the androgen receptor antagonist OH-flut, which did not cause a decrease or increase in latency (data not shown). Since rat *neu* expression does not correlate to the tumor outcomes, the decreased latency by p,p´ DDE would be caused by the treatment effects on mammary tissue. Collectively, these data suggest that the MMTV-*neu* mouse model is an acceptable model to use in the current investigations of p,p´ DDE actions in mammary tissue (Aims 2 and 3).

Chapter 3

DDE L

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ls in Mammary Adipose Tissue and Serum

INTRODUCTION

Lipid-adjusted DDT congener levels were measured in both serum and mammary adipose tissue of young (Pretumor study) and old (Tumor study) MMTV-*neu* female mice to determine how the concentrations delivered by ELVAX compare to human DDE concentrations and the tumor outcomes in the mice. Although these ethylene vinyl-acetate copolymer pellets have previously been used to study hormone effects in the mammary gland (Haslam et al., 1988; Andrapp et al., 1998; Wang et al., 1990), it was important to demonstrate in our hands, that this implant material released DDE. However, the main purpose for these assays is to correlate levels with human exposure and with the tumor outcomes in the mice. Pretumor study animals were examined which were treated simultaneously with the Tumor study mice.

In women, p,p´ DDE is the most commonly measured congener of DDT due to its ability to persist and bioaccumulate (Jaga and Dharmani 2003). Therefore, in the young mice treated for 2 months, levels measured in mammary glands would reflect DDE released over the treatment periods that would persist in the mammary fat due to its stability, slow metabolism, and its lipophilic properties, that all aid in increased exposure and bioaccumulation with time until pellet depletion. In the aged mice, at least 4 months after pellet depletion, no additional exposure would occur to add to the existing levels. Thus,

the levels would be expected to slowly decline in line with the half-lives of p,p´ DDE and o,p´ DDE. Therefore, both young animals from the Pretumor study after 60 days of pellet release and aged mice from the Tumor study over 9-months-old and > 4 months after pellet depletion, based on the reported 100 days for ELVAX (Silberstein and Daniel 1982) were analyzed.

Although directly delivered into the mammary fatpads, some redistribution of p,p´ DDE and o,p´ DDE was expected. Therefore, serum levels were measured to assess circulating levels. The lipid-adjusted serum levels were also compared to adipose tissue levels to determine local and systemic exposures. Using lipid-adjusted p,p´ DDE levels reduces the variation between adipose and serum levels in order that serum or plasma can be analyzed in women instead of invasive procedures to obtain breast adipose tissue. Thus, to be able to compare levels in serum and adipose tissue to current epidemiology studies, the DDE measurements were performed as for human tissues. These analyses of DDE levels in mammary adipose tissue and serum address part of Aim 1 on translational relevance of the DDE exposures.

METHODS

DDE Isomer Analysis in Serum and Mammary Adipose Tissue

Epidemiological studies measure DDT congener concentrations in both serum and adipose tissue. In order to be consistent with current practices, DDE isomer concentrations were determined by gas chromatography-mass spectroscopy by the Centre

de Toxicologie, Quebec Canada, which used total serum lipids and percent lipids in the mammary fatpads to calculate the lipid-adjusted levels (ng/g lipid). Lipid-adjusted levels are what change the limits of detection among pools. Mammary gland tissue and serum from <3-month-old (Pretumor study) and >9-month-old (Tumor study) MMTV-*neu* females were used from previous studies conducted by Davis and colleagues. Due to a minimum requirement of 1 g of mammary tissue and 2 mL of serum, samples had to be pooled to meet specifications. For the Tumor study, 15 animals (5 animals per pool) were used for both lipid-adjusted serum and mammary adipose tissue analysis. Identical analysis was also performed in the Pretumor study animals. However, due to the limited number of mice in the study and the smaller weight of mammary tissue and serum available per mouse, only 1 group per treatment could be analyzed, consisting of 12 animals.

RESULTS

To prepare tissues for assessing DDE levels, roughly 1/3 of one inguinal mammary gland from 15 animals/group in the Tumor study and 12 animals/group in the Pretumor study were weighed and pooled for analysis by the Centre De Toxicologie, Quebec Canada. Due to a minimum requirement of 1 g of mammary tissue and 2 mL of serum, samples had to be pooled in order to meet the desired specifications. For the older mice (> 9 months) in the tumor study, three pools were prepared for analysis, consisting of 5 mice/pool (Table 3). The same animals in each pool were used to analyze DDE levels for both the serum and mammary tissue. Due to the limited number of mice in the Pretumor

study and the smaller weight of the mammary tissue and serum available per mouse, only one group of serum and mammary tissue was sent to examine concentrations in these younger mice (<3 months old, Table 4).

Table 3. Tumor Study Pools for DDE Serum and Mammary Gland Concentrations

Tumor Treatment Group	Number of Animals Per Group	Pools
Control	5	3 (5 animals each)
p,p´ DDE	5	3 (5 animals each)
o,p´ DDE	5	3 (5 animals each)
p,p´ DDE : o,p´ DDE (2:1)	5	3 (5 animals each)

Table 4. Pretumor Study Pools for DDE Serum and Mammary Gland Concentrations

Pretumor Treatment Group	Number of Animals Per Group	Pools
Control	12	1
p,p´ DDE	12	1
o,p´ DDE	12	1
p,p´ DDE : o,p´ DDE (2:1)	12	1

Lipid-adjusted p,p´ DDE and o,p´ DDE concentrations in both serum and mammary gland tissue from young MMTV-*neu* mice treated for 2 months demonstrated detectable DDE levels, suggesting the predicted release of the treatments from the ELVAX 40P pellets into the local environment of the mammary gland (Tables 5 & 6). The levels of other DDT congeners not included in the treatments, p,p´ DDT, o,p´ DDT, p,p´ DDD and o,p´ DDD, were below the limits of detection, as expected (Tables 5 & 6).

Table 5. Lipid-adjusted DDT congener concentrations in serum of young MMTV-*neu* female mice treated for 2 months

Pretumor Treatment Group n=1 (12/pool)	p,p´ DDE Serum µg/kg	o,p´ DDE Serum µg/kg	p,p´ DDT Serum µg/kg	o,p´ DDT Serum µg/kg	p,p´ DDD Serum µg/kg	o,p´ DDD Serum µg/kg
Control	<14	<1.6	<7.8	<7.8	<7.8	<7.8
p,p´ DDE	**230**	<1.6	<7.9	<7.9	<7.9	<7.9
o,p´ DDE	<15	**8.8**	<8.2	<8.2	<8.2	<8.2
p,p´ DDE : o,p´ DDE (2:1)	**150**	**3.4**	<9.5	<9.5	<9.5	<9.5

Table 6. Lipid-adjusted DDT congener concentrations in mammary tissue of young MMTV-*neu* female mice after 2 months of treatment

Pretumor Treatment Group n=1 (12/pool)	p,p´ DDE Mammary Gland µg/kg lipid	o,p´ DDE Mammary Gland µg/kg lipid	p,p´ DDT Mammary Gland µg/kg lipid	o,p´ DDT Mammary Gland µg/kg lipid	p,p´ DDD Mammary Gland µg/kg lipid	o,p´ DDD Mammary Gland µg/kg lipid
Control	**12**	<2.4	<6	<6	<6	<3.6
p,p´ DDE	**1800**	<2.2	<5.5	<5.5	<5.5	<3.3
o,p´ DDE	**11**	**1200**	<6.2	<6.2	<6.2	<3.7
p,p´ DDE: o,p´ DDE (2:1)	**1600**	**590**	<5.5	<5.5	<5.5	<3.3

Serum DDE isomer concentrations in young animals show o,p´ DDE and p,p´ DDE concentration differences of 8.8 µg/kg and 230 µg/kg, respectively, even though the same dose was delivered to the glands (5 µg/pellet). Both isomers were also detected in the combined group, p,p´ DDE:o,p´ DDE(2:1) (Table 5 & Fig. 12), albeit not in proportion to the original 2:1 ratio. Neither isomer was detected in the control group, as expected (Table 5). Similarly, the same trend of higher p,p´ DDE relative to o,p´ DDE was

observed in mammary tissue of the young mice (Table 6 & Fig. 13). However, given the fact that only one pool was analyzed due to tissue limitations, no statistical analysis could be performed to compare these concentrations. Additionally, when lipid-adjusted p,p´ DDE levels were compared in serum versus mammary adipose tissue after 2 months of treatment, levels were consistently higher in the mammary tissue for all groups (Fig. 14). The higher levels were expected since p,p´ DDE is known to accumulate in fat tissue and due to localized delivery to the mammary glands. Detectable levels were also measured in the mammary fatpads in the control and o,p´ DDE groups (Table 6).

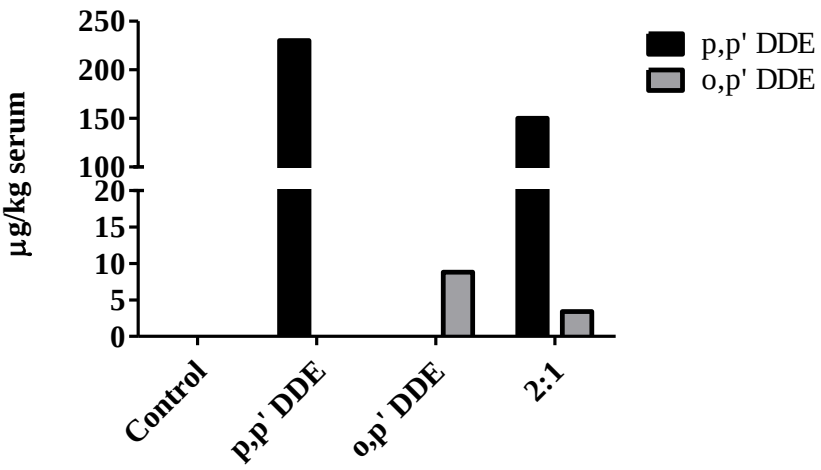


Figure 12. Lipid-adjusted p,p´ DDE levels in serum are higher in comparison to o,p´ DDE in young mice. p,p´ DDE was detected in its corresponding group and 2:1 ratio groups at levels of 230 and 150 µg/kg, respectively. Whereas, o,p´ DDE was detected in its corresponding group as well as the 2:1 ratio at levels of only 8.8 and 3.4 µg/kg, respectively. n=1 (pool consisted of 12 animals).

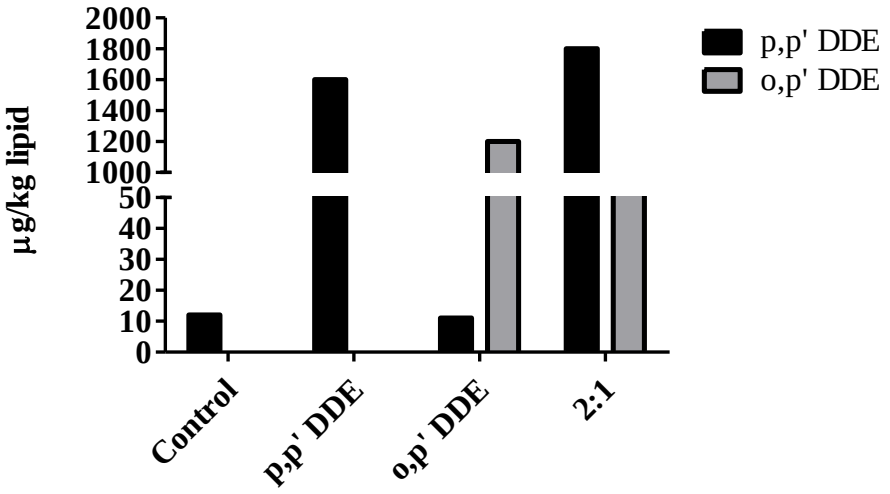


Figure 13. Lipid-adjusted levels of p,p´ DDE in mammary tissue were detected in all groups and at higher levels than o,p´ DDE in young animals. At approximately 3 months of age p,p´ DDE had noticeably higher concentrations than o,p´ DDE. Its ubiquitous nature was also demonstrated by there being detectable levels in the control and o,p´ DDE groups (groups without p,p´ DDE treatment) at concentrations of 12 and 11 µg/kg, respectively. 2:1 = 3.3 µg p,p´ DDE + 1.7 µg o,p´ DDE.

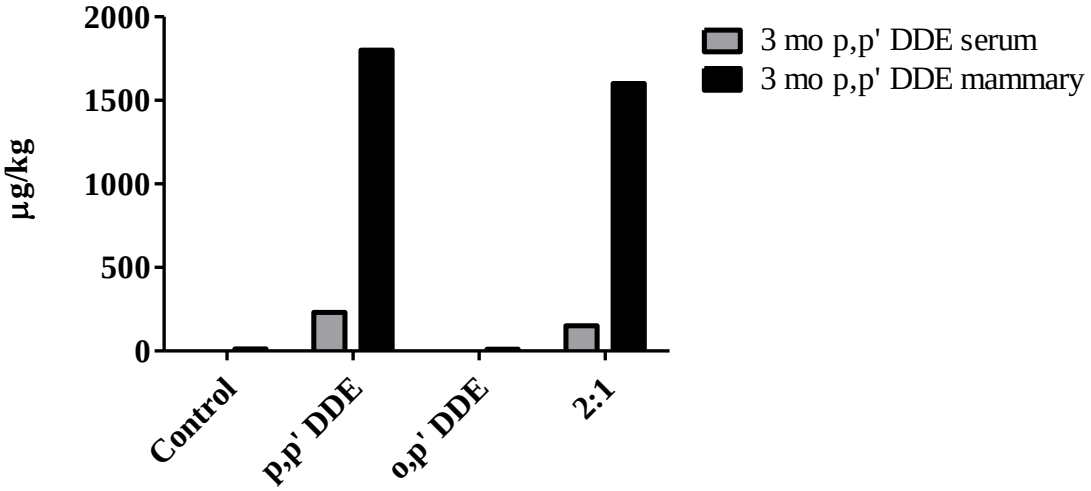


Figure 14. Lipid-adjusted levels of p,p´ DDE in the mammary gland of young animals was consistently higher than serum concentrations in all groups. At 3 months of age, p,p´ DDE had detectable levels in all treatment groups in mammary gland tissue. Conversely in serum, p,p´ DDE was either below the limits of detection (control and o,p´ DDE groups) or at very low concentrations (p,p´ DDE and 2:1 ratio groups). 2:1= 3.3 µg p,p´ DDE + 1.7 µg o,p´ DDE.

Similar to the measurements taken in young females, lipid-adjusted levels of DDE, DDT, and DDD were also measured in serum and mammary gland tissue of aged MMTV-*neu* female mice, >9 months of age from the Tumor study (Tables 7 & 8). Due to the Tumor study having more animals per treatment group and larger mammary glands and serum volumes due to their age, multiple samples with sufficient tissue and serum were available for measurement. For DDT congener levels in both serum and mammary adipose tissue, three pools were made, consisting of five animals each (Table 3). The average age (in months) for all three pools for the control, p,p´ DDE , o,p´ DDE, and 2:1 ratio group were 13.0, 11.6, 12.0, and 11.6, respectively (Table 9).

Table 7. Lipid-adjusted DDT congener concentrations in serum of Aged MMTV-*neu* female mice

Tumor Treatment Group n=3 (15/pool)	p,p´DDE Serum µg/kg	o,p´DDE Serum µg/kg	p,p´DDT Serum µg/kg	o,p´DDT Serum µg/kg	p,p´DDD Serum µg/kg	o,p´DDD Serum µg/kg
Control	<18	<2	<9.8	<9.8	<9.8	<9.8
Control	<10	<1.1	<5.6	<5.6	<5.6	<5.6
Control	<8.1	**1.2**	<4.5	<4.5	<4.5	<4.5
p,p´ DDE	<18	<2	<9.9	<9.9	<9.9	<9.9
p,p´ DDE	<10	<1.1	<5.5	<5.5	<5.5	<5.5
p,p´ DDE	<12	<1.3	<6.6	<6.6	<6.6	<6.6
o,p´ DDE	<10	<1.1	<5.6	<5.6	<5.6	<5.6
o,p´ DDE	<8.7	**1.2**	<4.9	<4.9	<4.9	<4.9
o,p´ DDE	<10	<1.1	<5.7	<5.7	<5.7	<5.7
p,p´ DDE: o,p´ DDE (2:1)	<9.5	<1.1	<5.3	<5.3	<5.3	<5.3
p,p´ DDE: o,p´ DDE (2:1)	<12	**1.4**	<6.6	<6.6	<6.6	<6.6
p,p´ DDE: o,p´ DDE (2:1)	**12**	<1.2	<6	<6	<6	<6

Table 8. Lipid-adjusted DDT congener concentrations in mammary glands of Aged MMTV-*neu* female mice

Tumor Treatment Group n=3 (15/pool)	p,p´ DDE Mammary Gland µg/kg lipid	o,p´ DDE Mammary Gland µg/kg lipid	p,p´ DDT Mammary Gland µg/kg lipid	o,p´ DDT Mammary Gland µg/kg lipid	p,p´ DDD Mammary Gland µg/kg lipid	o,p´ DDD Mammary Gland µg/kg lipid
Control	**5.7**	<2.5	<6.3	<6.3	<6.3	<3.8
Control	**5.6**	<2.1	<5.3	<5.3	<5.3	<3.2
Control	**3.9**	<2.3	<5.7	<5.7	<5.7	<3.4
p,p´ DDE	**6.7**	<2.2	<5.6	<5.6	<5.6	<3.3
p,p´ DDE	**11**	<2.1	<5.2	<5.2	<5.2	<3.1
p,p´ DDE	**19**	<2.2	<5.6	<5.6	<5.6	<3.4
o,p´ DDE	**4.2**	<2.3	<5.7	<5.7	<5.7	<3.4
o,p´ DDE	**3.4**	<2.1	<5.3	<5.3	<5.3	<3.2
o,p´ DDE	**4.1**	<2.2	<5.5	<5.5	<5.5	<3.3
p,p´ DDE: o,p´ DDE (2:1)	**18**	<2.2	<5.4	<5.4	<5.4	<3.2
p,p´ DDE: o,p´ DDE (2:1)	**27**	<2.2	<5.6	<5.6	<5.6	<3.4
p,p´ DDE: o,p´ DDE (2:1)	**20**	<2.2	<5.4	<5.4	<5.4	<3.3

Table 9. Average age of Tumor study animals used for DDE levels

Tumor Study Treatment Group	Pool Number (5 animals/pool)	Average Age (months)	Age Range (months)
Control	1	12.28	9.5-14
Control	2	12.52	9.5-14.4
Control	3	14.22	14-14.4
p,p´ DDE	1	12.2	9.6-14.2
p,p´ DDE	2	10.12	9.6-10.9
p,p´ DDE	3	12.52	10.0-14.1
o,p´ DDE	1	11.92	9.8-13.5
o,p´ DDE	2	11.2	9.2-14.1
o,p´ DDE	3	12.94	11.9-14.0
2:1	1	11.44	9.1-14.2
2:1	2	11.64	9.2-14.0
2:1	3	11.82	10.113.3

While analyzing the serum concentration differences between p,p´ DDE and o,p´ DDE in the older animals, it was discovered that o,p´ DDE was detectable in one of the three pools for all groups treated with this isomer (1.1 -1.4 µg/kg), but not in the p,p´ DDE group, with detection limits ranging between 1.1 and 2 µg/kg. Para, para´ DDE was only detectable in one pool which was in the 2:1 group at 12 µg/kg; this concentration was at, near, or below the detection limits in the other pools of all four groups. Collectively, p,p´ DDE had detection limits ranging from < 8.1- 18 µg/kg, indicating higher levels of p,p´ DDE are required for detection compared to o,p´ DDE (Table 7). The undetectable levels of p,p´ DDE in the serum of older animals in comparison to some pools with detectable o,p´ DDE may be due to these differences in detection limits.

Opposite to what was observed in the serum of older animals, p,p´ DDE levels in mammary adipose tissue were detected in all groups, unlike o,p´ DDE, which was undetectable in all pools of every treatment group (Figure 15). These results suggest the lower detection limits of p,p´ DDE in the mammary fatpad allow for its detection at levels as low as 3.4 µg/kg lipids and were within the detectable range (Table 8). These data also show that p,p´ DDE persists in the mammary tissue, unlike o,p´ DDE. Also, levels of p,p´ DDE in serum and mammary glands were compared in older MMTV-*neu* female mice from the Tumor study, >9 months of age (Fig. 16).

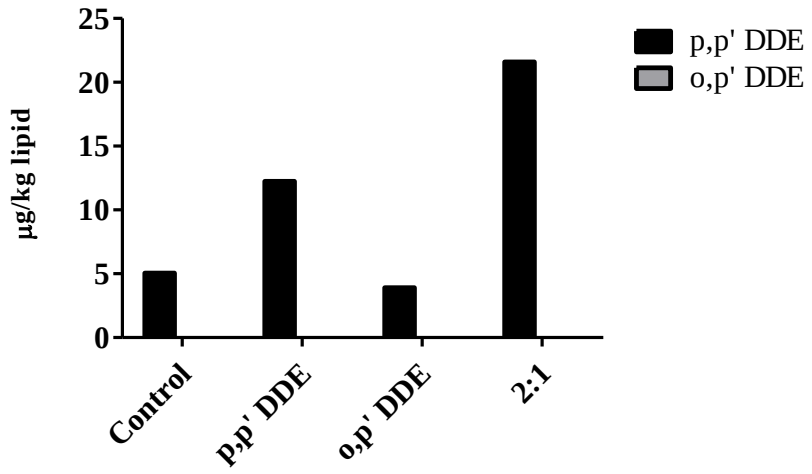


Figure 15. p,p´ DDE was the only isomer detected in mammary gland tissue after 9 months of age in MMTV-*neu* female mice. Concentrations of o,p´ DDE were below the limits of detection in all three pools of all treatment groups. In contrast, p,p´ DDE levels were present in the control, p,p´ DDE, o,p´ DDE and 2:1 ratio groups, having average concentrations of 5.0, 12.2, 3.9, and 21.7 µg/kg lipid, respectively.

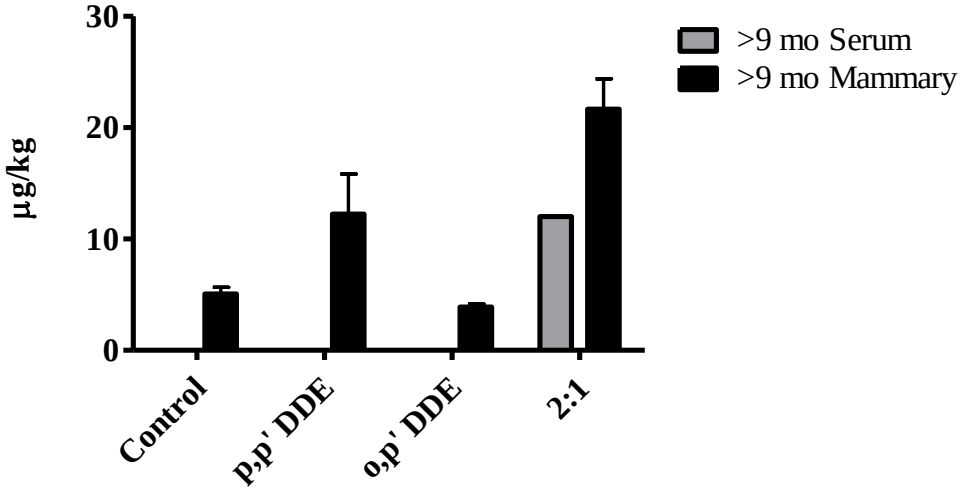


Figure 16. Mammary adipose tissue concentrations of p,p´ DDE in aged MMTV-*neu* animals were detected in all groups. Collectively, p,p´ DDE had a dominant presence in mammary gland tissue at >9 months of age compared to serum, in which it was only detected in the 2:1 ratio group.

Table 10. Serum and Mammary Adipose Tissue DDE Concentrations in Young and Old Animals

Treatment Group n=1 (12/pool)	Pretumor Serum p,p´ DDE µg/kg	Pretumor Serum o,p´ DDE µg/kg	Pretumor Mammary p,p´ DDE µg/kg	Pretumor Mammary o,p´ DDE µg/kg
Control	ND	ND	**12**	ND
p,p´ DDE	**230**	ND	**1800**	ND
o,p´ DDE	ND	**8.8**	**11**	**1200**
p,p´ DDE + o,p´ DDE (2:1)	**150**	**3.4**	**1600**	**590**

Treatment Group n=3 (15/pool)	Tumor Serum p,p´ DDE µg/kg	Tumor Serum o,p´ DDE µg/kg	Tumor Mammary p,p´ DDE µg/kg	Tumor Mammary o,p´ DDE µg/kg
Control	ND	ND	**5.06**	ND
p,p´ DDE	ND	ND	**12.23**	ND
o,p´ DDE	ND	ND	**3.9**	ND
p,p´ DDE + o,p´ DDE (2:1)	ND	ND	**21.67**	ND

DISCUSSION

ELVAX 40P has been successfully used for decades to study the *in vivo* effects of drugs in rodent models, especially for studying the mammary gland. (Silberstein and Daniel 1982; Ankrapp et al., 1998; Haslam et al., 1988; Wang et al., 1990). Until the studies in the Tumor, Pretumor, and current studies, DDT congeners had not been tested in this controlled drug delivery system within the mammary gland. This implant material/method is ideal for the following reasons: 1) It does not induce an inflammatory response (Silberstein and Daniel 1982); 2) there is no toxicity and the prognosis for ELVAX use *in vivo* is excellent (Shastri 2001); 3) it is capable of a sustained (approximately 100 days), gradual release of a remarkable variety of molecules

(Silberstein and Daniel 1982); and 4) it greatly reduces systemic effects, which, therefore, allows the study of direct, primary, local effects of drug treatments in the mammary gland.

The functionality of ELVAX 40P pellets to deliver the treatments was evidenced by measurable DDE isomer levels in mammary adipose tissue in young mice (Pretumor study). Para, para DDE is the most prevalent, lipophilic and persistent congener that exists in the environment and humans (Cocco et al., 2000; Snedeker 2001; Eskenazai et al., 2009). For these reasons, it is also able to bioaccumulate in human adipose tissue (Jaga and Dharmani 2003). The ubiquitous nature of DDT in the environment was also evident in the present study as p,p´ DDE was detected in mammary adipose tissue of the control and o,p´ DDE groups in young and old animals, even though these animals were not treated with this isomer via the ELVAX pellets and the environment was controlled. Turusov and colleagues (2002) stated that no living organism may be considered DDT-free. In addition, DDT and its congeners, such as p,p´ DDE, have been detected *in utero* in amniotic fluid (Foster et al., 2000) and in cord blood samples (Tan et al., 2009) demonstrating exposure can occur even before birth. The presence of this isomer in control mice and in women as reported in epidemiological studies in young women from, for example, the United States (Cohn et al., 2007) and from developing countries (Shakeel et al., 2010), reinforces that there are no true negative controls when studying the impact of DDT exposures.

The persistence and lipophilic properties of p,p´ DDE allow it to bioaccumulate in the body. The more stable isomer, p,p´ DDE, remains detectable in the mammary adipose tissue, despite the cessation of exposure due to pellet depletion, which occurred at approximately 100 days (Silberstein and Daniel 1982). These results support the reported half-life of p,p´ DDE in rats of 80-120 days (Muhlebach et al., 1991). However, the levels in the p,p´ DDE group (6.7-19 µg/kg lipids) are only slightly higher than the levels in control (3.9-5.7 µg/kg lipids) and o,p´ DDE groups (3.4-4.2 µg/kg lipids) and, thus, may be partially due to the treatment. In contrast, o,p´ DDE levels were below detection limits in mammary fatpads of aged mice. In addition, its lower serum levels in young mice compared to mammary adipose tissue were considerably lower due to its rapid metabolism, unlike the more stable p,p´ DDE isomer.

The lipid-adjusted p,p´ DDE levels obtained in this study are also consistent with ranges found in human epidemiological studies. For example in adipose, reported concentrations were: 6.66 ng/g (López-Carrillo et al., 1996) 45.5-17,387 ng/g (López-Carrillo et al., 1999), 3.5-3229 ng/g (Smeds et al., 2001), 396- 60,980 ng/g (Jaga and Dharmani 2003) and 0.593 - 0.642 mg/kg (Ociepa-Zawal et al., 2010). Furthermore, reported concentrations found in human serum/plasma were: 0.1-88.5 ng/g (López-Carrillo et al., 1999), 0.768 (cases) and 0.817 µg/g (controls) (Laden et al., 2001), ≤ 0.44 µg/g (Gatto et al., 2007) and 0.06- 8.0 µg/g (Eskenazi et al., 2009). These results indicate that p,p´ DDE influences mammary cancer development in levels relevant to human exposure. However, most of the epidemiology studies did not find a positive correlation between high p,p´ DDE levels and increased breast cancer risk; however, the incidence of

mammary cancer also was not modified in the mice. Tumor onset was accelerated in mice, but this endpoint was not studied in women, which may be the reason for the lack of correlation between DDE levels and breast cancer risk.

Breast cancer epidemiology studies used older women (at time of breast cancer diagnosis) to investigate the relationship between DDT levels and breast cancer risk. Brody and colleagues (2004) have also stated that individual differences in metabolism and excretion result in measures that may not represent original exposure levels, particularly when the assessment is many years after the exposure; such is the case in postmenopausal women. The levels in the present study reveal very low to non-detectable levels of these congeners in aged mice which did not correlate with the earlier tumor onset in the mice. Only in younger mice were the levels of p,p´ DDE associated with the shorter latency, similar to what was reported for the young women with early-onset breast cancer (prior to age 50) with p,p´ DDT(Cohn et al., 2007). These findings suggest that age of onset versus incidence may need to be investigated in epidemiology studies examining DDT exposure and breast cancer risk.

The 230 µg /kg lipids of p,p´ DDE in serum after a 2 month exposure was detected only approximately a week prior to the detection of the first palpable tumor (age 90 days). The timing of these events implies that lower levels of p,p´ DDE than were detected after 2 months of exposure may be able to promote tumorigenesis, since it takes weeks for tumors to develop and grow to a detectable size and p,p´ DDE levels accumulate with time (lower doses at younger ages). The lipid-adjusted level of p,p´ DDE in serum after 2

months of exposure is within or below the first tertile/quartile/quintile of several epidemiology studies investigating the link between higher p,p´ DDE levels and breast cancer risk (Dorgan et al., 1999; Laden et al., 2001; Gatto et al., 2007; Cohn et al., 2007). As this reference group (lowest p,p´ DDE levels used to compare to higher levels and breast cancer incidence) includes a level that was able to influence mammary tumor development in the mice, these data suggest lower levels of p,p´ DDE may need to be used as the reference category in future epidemiology studies.

As expected and shown in younger animals, DDE concentrations in mammary adipose tissue were expectedly higher than serum concentrations because of the lipophilic nature of the compound and localized delivery to the mammary gland. Overall, the lipid-adjusted DDE levels in serum and mammary gland suggest: 1) the ELVAX 40P pellets were functional, which allowed the mammary gland to be primarily exposed to DDE; 2) the persistence of p,p´ DDE , as is demonstrated by measurable levels of this congener in the aged mice after pellet depletion, which is reported to be approximately 100 days (Silberstein and Daniel 2000), unlike o,p´ DDE; 3) the lipophilic nature of DDE as supported by the higher concentrations in mammary adipose tissue and 4) the redistribution of the locally delivered DDE isomers by the presence of their levels in serum of young mice; 5) the metabolism and/or excretion of DDE as represented by their decreased levels in the aged versus young mice; 6) the levels of p,p´ DDE associated with the early onset of mammary cancer are relevant to human exposure; and 7) systemic as well as local responses may influence mammary tumorigenesis since p,p´ DDE and o,p´ DDE were detected in the serum of young animals.

The data from the mouse study clearly demonstrate that p,p′ DDE and o,p′ DDE levels are present in the mammary glands and serum in mice treated for 2 months. The MMTV-*neu* mice treated with the same or other concentrations of p,p′ DDE, o,p′ DDE, and p,p′ DDT as well as combinations of the congeners with p,p′ DDE were tested in the subsequent studies for Aims 2-4 in the next chapters. Accordingly, the mice treated for the same length of time (2 months) would be expected to have proportional exposure levels relating to their dose. As serum levels and mammary adipose tissue of p,p′ DDE were detected in the mice, local (Aims 2 and 3) and systemic effects (Aim 4) will be investigated for responses induced by p,p′ DDE exposure.

Chapter 4

Identification of Differentially Expressed Genes in the Mammary Gland with Localized p,p´ DDE Exposure

INTRODUCTION

The next goal was to identify the effect of p,p´ DDE exposure on mammary tissue gene expression through the use of microarray analysis. Microarray analysis provides an effective and efficient means to screen differential expression between control and treated groups. Each chip contained over 20,000 well characterized mouse genes that allow for fast and easy selection of genes with modified expression by p,p´ DDE. The predetermined selection criteria used to identify the chosen genes were based upon whether the genes were androgen- or immune-regulated or cancer related. All genes were then verified by real-time RT-PCR. The significant, differentially expressed candidate genes will then be used in subsequent experiments to provide potential correlations to p,p´ DDE action and possibly tumor outcomes. The identification of such markers may help in the development of additional tumor studies that can ultimately provide a direct correlation to tumor outcomes. The experiments outlined in this chapter apply to Aim 2 and Central Question 1.

METHODS

Microarray Analysis

The first microarray was conducted to identify differential expression of genes in control, hydroxyflutamide (OH-flut), o,p´ DDE and p,p´ DDE lower mammary tissue of Pretumor study animals. Microarray analysis was performed by the Functional Genomics

Core at Columbus Children's Research Institute, Columbus, Ohio. Six individual mammary gland RNA samples (RNA obtained by using the Absolutely RNA miniprep kit as outlined in the total RNA isolation method, p. 26) from each group were sent. Once received by the Functional Genomics Core, the individual RNA samples were pooled (3 animals per pool), to account for variability among samples. RNA quality and integrity were also checked before hybridization by the Functional Genomics Core. Each pool was run on an Affymetrix GeneChip® Mouse Genome 430 2.0 Array (Affymetrix Inc., Santa Clara, CA). This gene chip has over 45, 000 probes that are biased to the 3' end of the gene and which represent roughly 21,000 well characterized mouse genes (Fig. 17). It contains 8-15 probes made out of 25-mer oligonucleotides. Briefly, the RNA samples are converted to cDNA, which then have fluorescent nucleotides incorporated in it. After, the synthesized cDNA is bound to its complement/specific probes (hybridized) on the genechip. The genechip is then scanned and the amount of bound target is measured by its fluorescent intensity. Properly designed probes and stringent hybridization allow for only message of a specific gene to bind, therefore the relative fluorescence of the probe can infer gene expression.

The second microarray was performed by Vanderbilt Functional Genomics Shared Resources, Nashville, TN using the Affymetrix Genechip® Mouse Gene 1.0 ST (Affymetrix Inc., Santa Clara, CA), similar to the first array, in order to identify genes that were differentially expressed by p,p´ DDE . However, due to limited tissue being available from the pretumor study, three animals per group from the control and p,p´ DDE groups were used from the present study. Unlike the genechip used in the first

array, this chip covers the entire length of the gene, providing whole-transcript coverage and a more accurate account of gene expression. Additionally, this chip has nearly 29,000 genes, each of which are divided into roughly 27 probes made out of 25-mer oligonucleotides (Fig. 17).

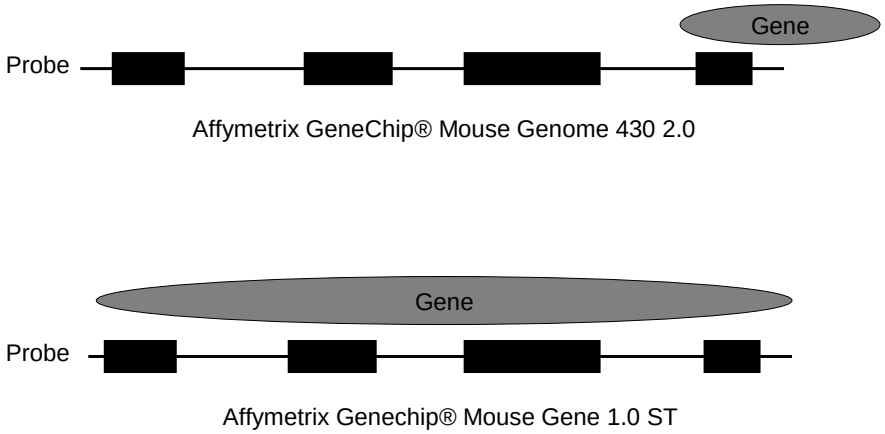


Figure 17. Schematic Representation of Genechips used for Microarrays. On the mouse genome 430 2.0 chip, probes are biased to the 3'end of the gene, whereas on the Mouse Gene 1.0 ST chip, the entire length of the gene is covered. Both chips contain 25-mer oligonucleotides.

Chapter 2 includes the methods on total RNA isolation, cDNA reverse transcriptase reactions, real time RT-PCR, relative gene expression and statistical analysis that apply to the studies within this chapter.

RESULTS

Differential Expression of Mammary Gland Genes

RNA was isolated from random portions of the mammary gland from six animals from the Pretumor study in the control and p,p´ DDE groups. The six samples were assigned to 1 of the 2 pools consisting of three animals each (Table 11).

Table 11. First Microarray Pool Designations

Treatment Group	Number of Animals	Pool Designation
Control	3	Pool #1
	3	Pool #2
p,p´ DDE	3	Pool #1
	3	Pool #2

Pretumor study animals were tested in ordered to elucidate the early changes that result from exposure to p,p´ DDE prior to tumor formation that may have a role in the early tumor onset. The RNA from each animal was sent to the Functional Genomics Core at Columbus Children's Research Institute, Columbus, OH., where they confirmed the quality of the RNA before pooling the samples with equal amounts of RNA. The microarray analysis used the Affymetrix GeneChip® Mouse Genome 430 2.0 Array (Affymetrix Inc., Santa Clara, CA). Initially, five genes shown to be differentially expressed by p,p´ DDE compared to control pools were chosen for further investigation based upon their correlation to cancer or androgen-regulation as shown in Table 12. These two characteristics were chosen due to the documented hormonal action of p,p´

DDE (Kelce et al., 1995) and its ability to accelerate tumor formation, as shown by Davis and colleagues in the previous data.

Table 12. First five candidate genes selected from the microarray

Gene	Abbrev	Function	Reference	Criteria Category	Microarray Regulation	Fold Change
Histone deacetylase 3	hdac3	Responsible for transcriptional events, leading to events such as proliferation & cell survival	Wilson et al., 2005	Cancer	Up	1.55
Serum Amyloid A	saa1	Acute responses to various tissue insults including inflammation and cancer; Overexpression of SAA genes accelerates apoptosis of mammary epithelial cells; elevated 100-1000-fold as part of the response to neoplasia, trauma, infection, inflammation	Kho et al., 2008; Urieli-Shoval et al., 1998	Cancer	Up	4.28
Tropomyosin -2	tpm2	Contributes to cell morphology and integrity of the actin cytoskeleton. Cell growth and adhesion properties	Varga et al., 2005; Li et al., 2006	Cancer	Down	0.60
Interleukin 18	il18	Activates immune response/pro-inflammatory. Well known anti-tumor activity; also has been shown to have pro-cancer activity.	Park et al., 2009	Cancer	Down	0.48
Solute carrier organic anion transporter 3a1	slco3a1	Androgen regulated. Involved in glucose metabolism and tumorigenesis	Villaamil et al., 2011	Androgen-regulated/ Cancer	Down	0.57

In order to confirm the expression patterns of the microarray, the same animals designated for the microarray pools were then tested individually, in addition to four

more animals per group using real-time RT-PCR with SYBR-Green detection. The results revealed that of the ten individual animals tested, none of the p,p´ DDE-treated animals was significantly different from control, with fold changes ranging from 0.4-1.7 (Fig.18).

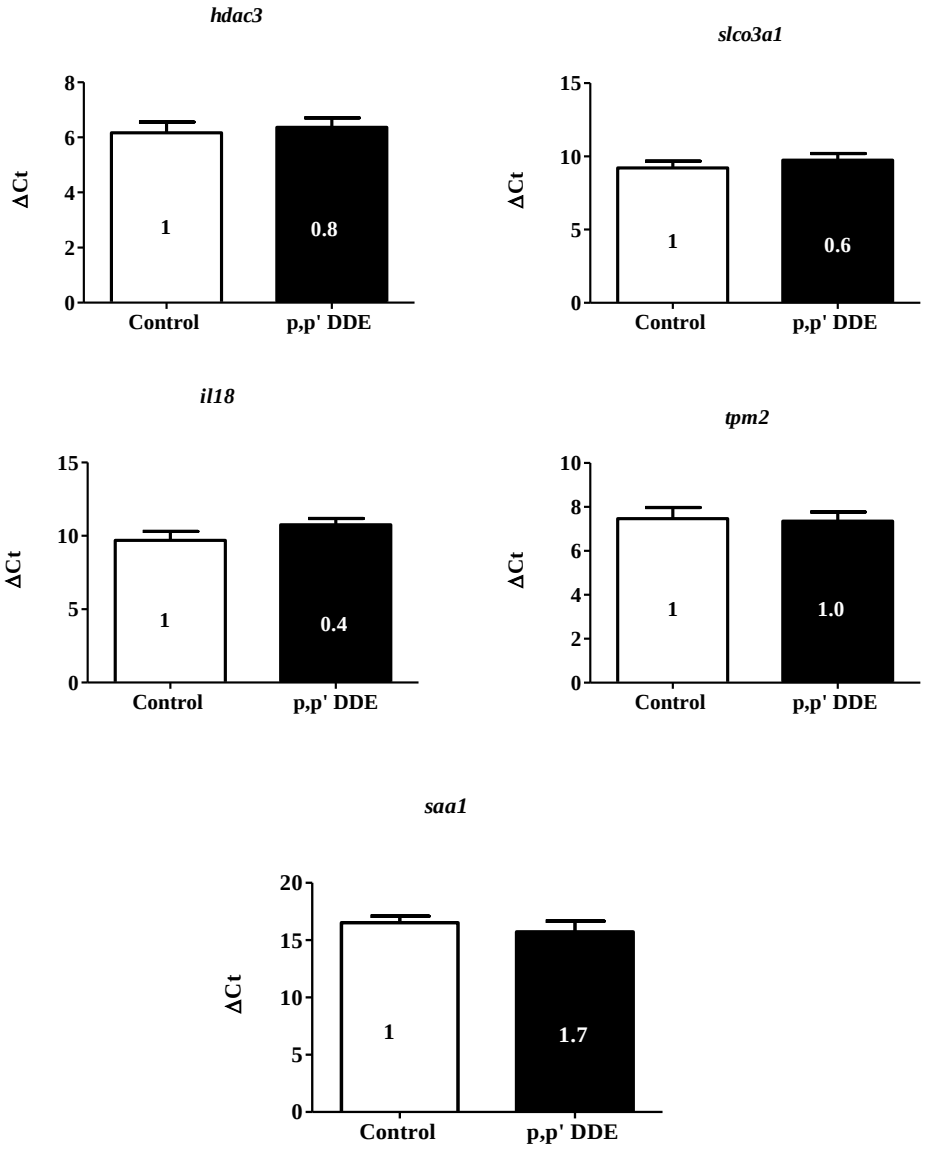


Figure 18. Cancer-related and androgen-regulated genes tested in the individual animals of the microarray pools did not show a significant difference in expression. Using specific primers for *hdac3*, *slco3a1*, *il18*, *tpm2* and *saa1*, expression levels were measured by real-time RT-PCR. Expression of the 5 µg p,p´ DDE treatment group was not significantly different compared to control. A lower ΔCt value indicates the gene is expressed at a higher level. Numbers displayed within each bar represent the fold change compared to control using the $2^{-\Delta\Delta Ct}$ method. The genes were not significant by the Mann-Whitney test ($p > 0.05$). n=10.

Table 13. Additional Candidate Genes Not Pursued from the Microarray

Gene Name	Abbreviation	Function	Reference	Criteria Category
Bcl2-like 11	Bl2l11	Involved in apoptotic cell death	Hoshino et al., 2011	Cancer
Inhibitor of differentiation/ DNA binding	Id1	Involved in tumor growth, invasiveness, metastasis and angiogenesis; shown to be up-regulated in a wide range of cancers and its expression correlates to disease stage and poor prognosis.	Mern et al., 2010; Yap et al., 2010	Cancer
Thioredoxin 2	Tnx2	An antioxidant defense enzyme. ROS are mitogenic and capable of tumor promotion	Cebrian et al., 2006	Cancer

Other candidate genes initially chosen were *txn2, bcl2l11* and *id1* (Table 13). However, *bcl2l11, txn2* and *id1* had opposing expression between the microarray and the individual animals (similar to *hdac3*) (data not shown). In total, 8 genes were initially selected, but found unsuitable for further testing due to lack of statistical significance or not matching the microarray (different effect on expression).

Immune-related Markers and Lymph Node Involvement in Mammary Gene Expression

To attempt to identify additional candidate genes that were differentially expressed by p,p´ DDE, it was discovered that many immune-related genes in the microarray were modulated by the well-known anti-androgen OH-flut. Correspondingly, only one of the tested pools from the p,p´ DDE group had similar regulation as both OH-flut pools, while one pool did not. Upon further investigation, one pool of RNA was found to have strong

modulation of immune-related genes. DDT has been shown to affect macrophages and contribute to inflammatory reactions, cytokine imbalance, and immune dysregulation (Dutta et al., 2008). Moreover, Vine and colleagues (2000) concluded that relatively low levels of plasma DDE are associated with statistically significant changes in immune markers, which supported further investigation of the immune markers on the microarray. Therefore, the immune-regulated genes that had similar regulation in the p,p´ DDE and OH-flut pools were examined for differential regulation in the individual animals.

Twenty immune gene markers and one non-immune related gene marker were selected from the OH-flut microarray according to their fold increase or decrease in expression values compared to control animals and that were correspondingly modified in the one p,p´ DDE pool. Using specific primers for real-time RT-PCR with SYBR-Green detection, the initial genes tested were: glycosylation dependent cell adhesion molecule 1(*glycam),* chemokine (C-C motif) ligand 19 (*ccl19),* chemokine (C-C motif) ligand 21B *(ccl21b),* selectin (*sell),* POU domain, class 2, associating factor 1 (*pou2af1),* interleukin 7 receptor (*il7r),* chemokine (C-C motif) ligand 5 (*ccl5),* chemokine (C-C motif) ligand 7 (*ccr7),* CD52 antigen (*cd52),* interleukin 2 receptor, gamma chain (*il2rg),* IL2-inducible T-cell kinase (*itk),* lymphocyte protein tyrosine kinase (*lck),* schlafen 2 (*slfn),* tumor necrosis factor receptor superfamily, member 11 (*rankl*), brain expressed gene 1 (*bex1),* T-cell receptor alpha chain (*tcra),*tumor necrosis factor receptor superfamily, member 13c (*tnfrsf13c),* CD8 antigen, alpha chain (*cd8a),* CD8 antigen, delta polypeptide (*cd3d),* membrane-spanning 4 domains, subfamily A, member 1 *(ms4a1)* and aldo-keto reductase family 1, member c18 (*ark1c18).* *Akr1c18* is not an immune-related gene, but

codes for a protein involved in steroid metabolism which could therefore potentially influence androgen-regulated genes but would have minimal effects on immune function. Pools of genes from control, p,p´ DDE and OH-flut treated animals were analyzed, using the three individual animals used for the microarray pool that had the differential expression for p,p´ DDE, as well as in three additional animals not tested in the microarray analysis, for a total n=6. Expression of the first set of genes by p,p´ DDE and OH-flut was not significantly different from control (Fig. 19). The remaining 14 other genes were also not significantly different (data not shown).

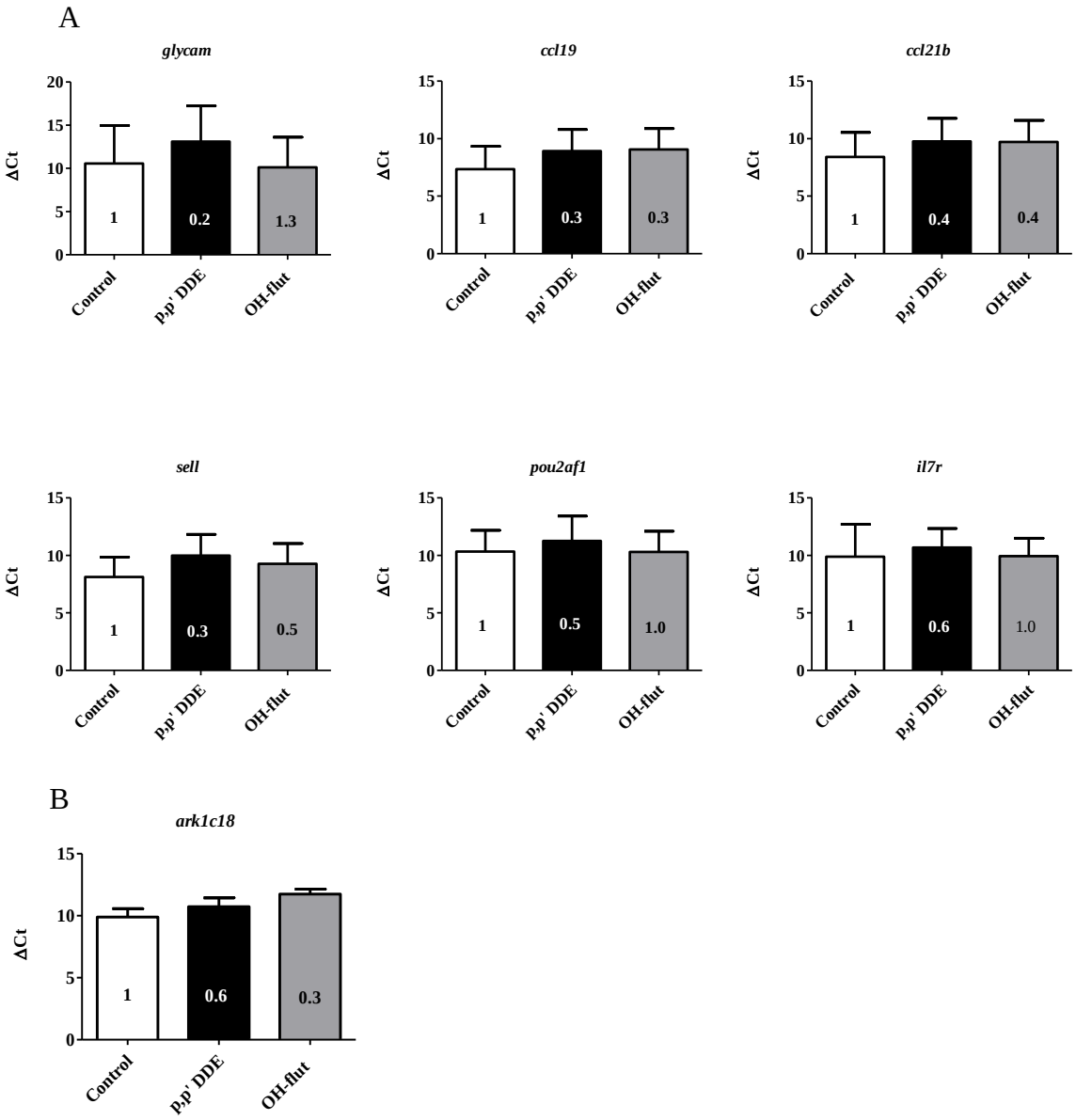


Figure 19. Immune-related genes were not significantly modified by p,p´ DDE or OH-flut treatment. (A) Immune genes *glycam1*, *ccl19*, *ccl21b*, *sell*, *pou2af1*, *il7r* did not induce significant changes in expression in individual pretumor microarray and non-microarray animals. (B) p,p´ DDE also did not induce significant effects different from control in *akr1c18*, a non-immune related gene, however, there was much less variability in expression in this gene. A lower ΔCt value indicates the gene is expressed at a higher level. Numbers displayed within each bar represent the fold-change compared to control using the $2^{-\Delta\Delta Ct}$ method. Analysis by one-way ANOVA did not find significance ($p>0.05$), n=6. OH-flut = hydroxyflutamide.

It was concluded that the wide range of ΔCt values between the RNA from animals within each group made it difficult to obtain significance as demonstrated by scatter plots from the control and p,p´ DDE groups (Fig. 20). Also, it was observed that *akr1c18*, a non-immune-related gene did not display the large variation observed in the other immune-related genes. Due to this discovery, the pools of the microarray were recreated (using the same animals and RNA in equivalent amounts) and tested in the first set of genes (Fig. 21).

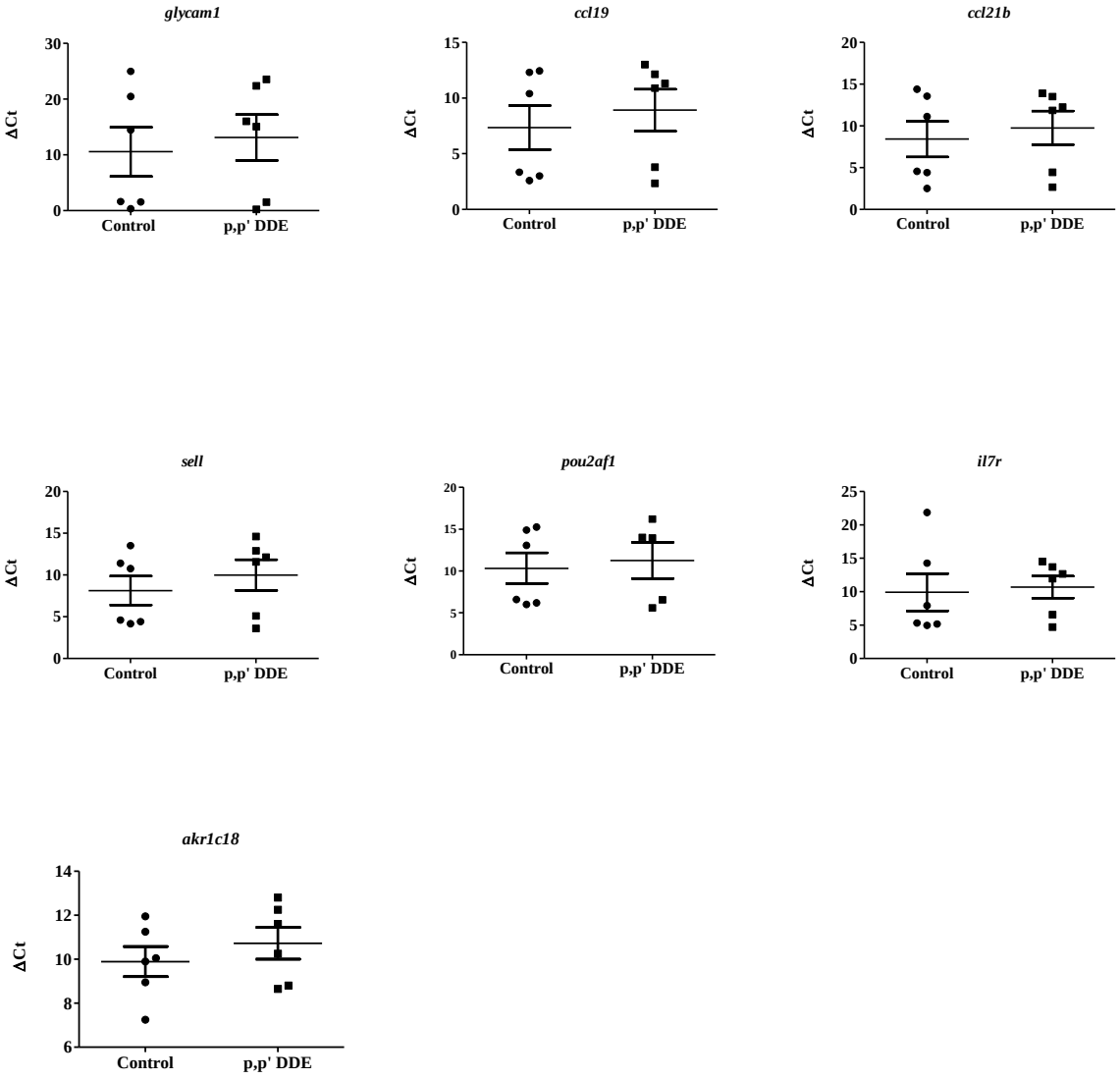


Figure 20. Wide ΔCt variation among immune-related genes in control and p,p´ DDE animals in pretumor MMTV-*neu* females, unlike the non-immune gene *akr1c18*. Scatter plots displayed the large variation that occurred between the three animals of the microarray and the three non-microarray animals in both control and p,p´ DDE groups. However, the distinct separation in expression values were not observed with *akr1c18*, a non-immune regulated gene. A Mann-Whitney test was used for statistical analysis ($p>0.05$). n=6.

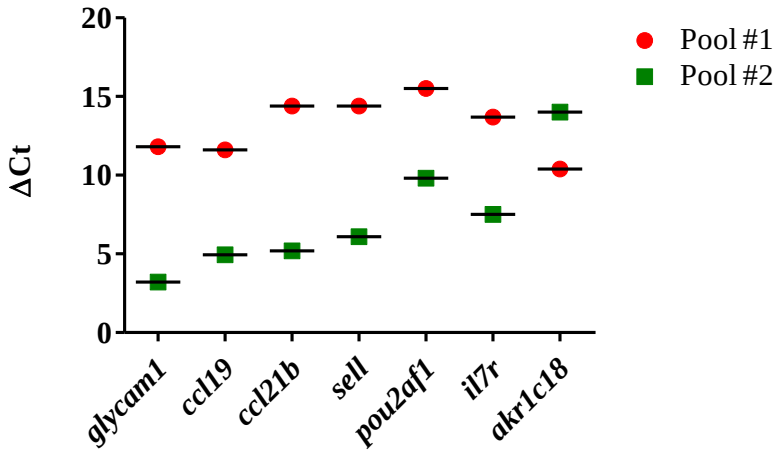


Figure 21. Microarray p,p´ DDE pools #1 and #2 were markedly different in immune-regulated genes. A distinct separation pattern was discovered between the microarray pools of the p,p´ DDE groups. Genes in p,p´ DDE pool #1 had ΔCt values ranging from 11.6-15.5 whereas genes in pool #2 had values ranging from 3.2-9.8. The non-immune-regulated gene *akr1c18* did not display as large a variation and its expression values were opposite of the immune-related genes, with pools #1 and #2 having values of 10.4 and 14, respectively. A lower ΔCt value indicates the gene is expressed at a higher level. Numbers displayed within each bar represent the fold-change compared to control using the 2-ΔΔCt method. Two-way ANOVA was used for statistical analysis ($p>0.05$). n=6.

A significant difference between p,p´ DDE microarray pool 1 and pool 2 was detected among the immune-related genes (Table 14). In contrast, the expression of *akr1c18* did not fit the pattern of the immune genes, as pool 2 had lower expression than pool #1. These data confirmed the results observed in the microarray and indicate the difference in expression was not due to errors in pooling the samples or labeling groups as control or treated.

As a result of the inconsistency between the p,p´ DDE pools within the immune markers, the possible role of the lymph node in the mammary gland became an area of interest since it would contain an abundance of leukocytes that express these cytokines.

Therefore, if the presence of the lymph node in the mammary gland has the ability to alter expression of immune-related genes and not all samples used to prepare the RNA would contain the lymph node, it would be more difficult to select p,p´ DDE-regulated genes that are immune-related. The presence of the lymph node may also affect other unknown gene populations as well, also possibly contributing to gene selection error and/or skewed expression.

Table 14. Immune-related Gene Expression Differences Between Microarray Pools

	glycam1	*ccl19*	*ccl21b*	*sell*	*pou2af1*	*il7r*	*akr1c18*
Pool #1 (Fold change)	0.0019	0.0084	0.0016	0.0031	0.0066	0.0186	0.6598
Pool #2 (Fold change)	0.9330	0.8409	0.8409	0.7846	Not available	0.8409	0.0412

The Influence of Lymph Node Presence on Mammary Gene Expression

To test whether or not the lymph node influenced gene expression within the mammary gland, a marker was sought to definitively discriminate between lymph node-positive (LN-positive) from lymph node-negative (LN-negative) mammary tissue. To accomplish this, specific mammary gland sections from two wild-type (FVB/N) mice with and without lymph node (as identified by visual inspection), one kidney (to serve as a negative control) and an individual lymph node (LN alone, to serve as a positive control) dissected from a mammary gland, were used to screen lymph node markers. RNA was isolated and then converted to cDNA by reverse transcriptase. Specific primers for lymphatic vessel endothelial receptor-1 (*lyve-1*), fms-related tyrosine kinase 4 (*flt4*), podoplanin (*pdpn*), prospero homeobox 1(*prox-1*) and *neu*ropilin-2 (*nrp2*) were examined

using real-time RT-PCR. Genes *cd3d* and *ms4a1*(cd20) were also employed as they are markers of T- and B-cells, respectively, since these leukocytes are densely populated within the lymph node. Gene names and functions are listed in Table 15. Finding an acceptable lymph node marker was important to distinguish between the presence (LN-positive) or absence (LN-negative) of lymph node in mammary gland tissue. Having a suitable lymph node marker will help to determine if the presence of the lymph node is responsible for the large variations in gene expression with the immune-related genes between the treatment groups.

Table 15. Potential Lymph Node Markers

Gene Name	Abbreviation	Function	Reference	Criteria Category
Membrane-spanning 4-domains, subfamily A member 1	cd20	Expressed on the surface of premature and mature B-lymphocytes	Deirickx et al., 2011	LN marker
CD3 antigen	cd3d	T-cell related marker	Denkert et al., 2009	LN marker
FMS-like tyrosine kinase 4	flt4	Found in lymphatic vessels	Huang et al., 2001	
Lymphatic vessel endothelial hyaluronan receptor 1	lyve-1	Lymphatic vessel marker	Vigl et al., 2011; Ji et al., 2010	LN marker
*Neu*ropilin-1	nrp2	A transmembrane receptor for the lymphangiogenic vascular endothelial growth factor C (Vegf-C), plays an important role in lymphatic vessel sprouting and lymph node metastasis of human cancers	Xu et al., 2010; Yasuoka et al., 2009	LN marker
Podoplanin	pdpn	Lymphatic marker; expressed in lymphatic endothelium	Ji et al., 2010; Cimpean et al., 2007; Vandenput et al., 2010	LN marker
Prospero-related homeobox 1	prox-1	Lymphatic vessel marker	Vigl et al., 2011	LN marker

As shown in the table above, seven immune-related markers were selected and screened in mammary tissue with (LN-positive) or without (LN-negative) lymph node, as well as in kidney (no lymph node). As shown in Figures 22 and 23, *cd20* was identified as a possible immune marker as it demonstrated a higher expression in lymph node-positive tissue and a lower expression in lymph-node negative tissue. The other tested genes did not have this same pattern or had more variation among the node-negative samples and were not significant. Therefore, due to the large difference in Ct values between node-

positive and node-negative mammary tissue, the presence of the lymph node was easier to identify with the cd20 marker of B-cells.

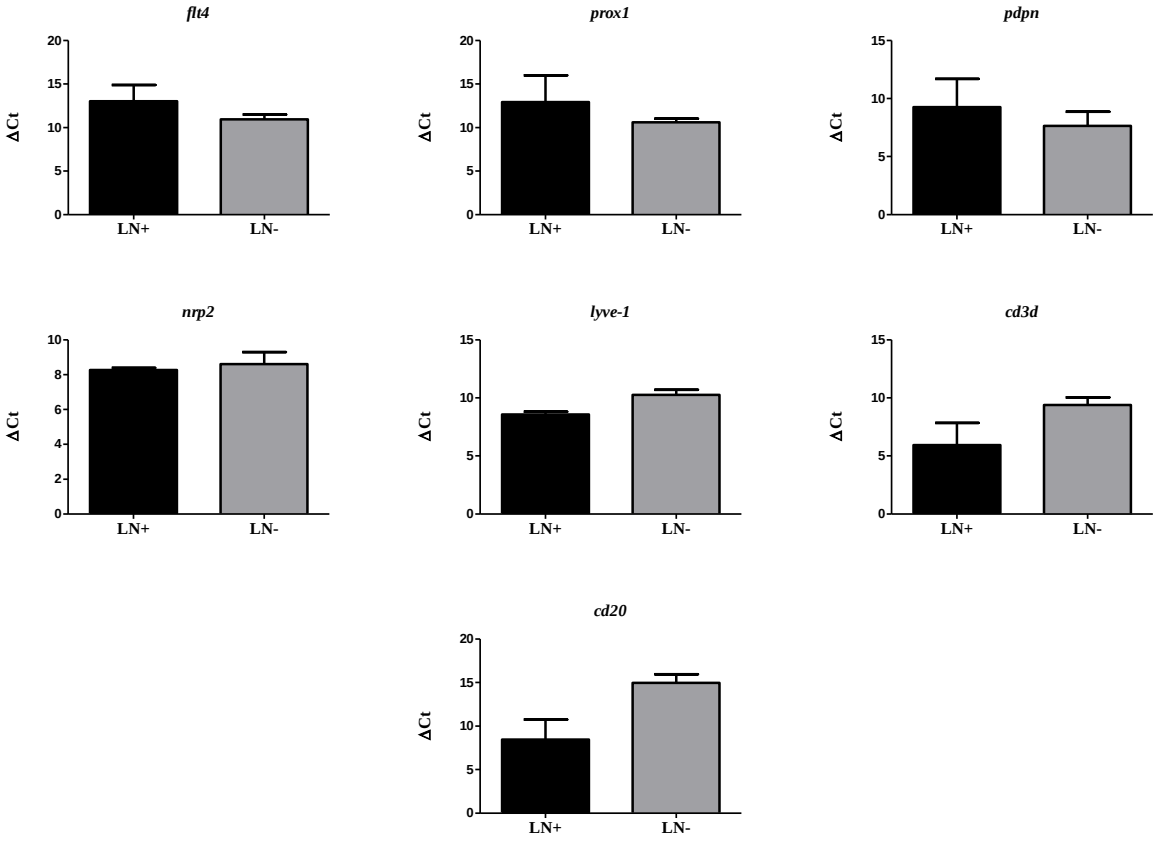


Figure 22. Lymph node markers in lymph node-positive and lymph node-negative mammary gland tissue showed varying expression. Lymph node endothelial cell markers *flt4, prox1, pdpn, nrp2,* and *lyve-1*, as well as T- and B-cell markers *cd3d* and *cd20* did not yield consistent expression. The majority of the markers did have lower ΔCts in lymph node positive mammary, indicative of higher expression compared to the remaining three. A t-test was used for statistical analysis ($p>0.05$). n=2

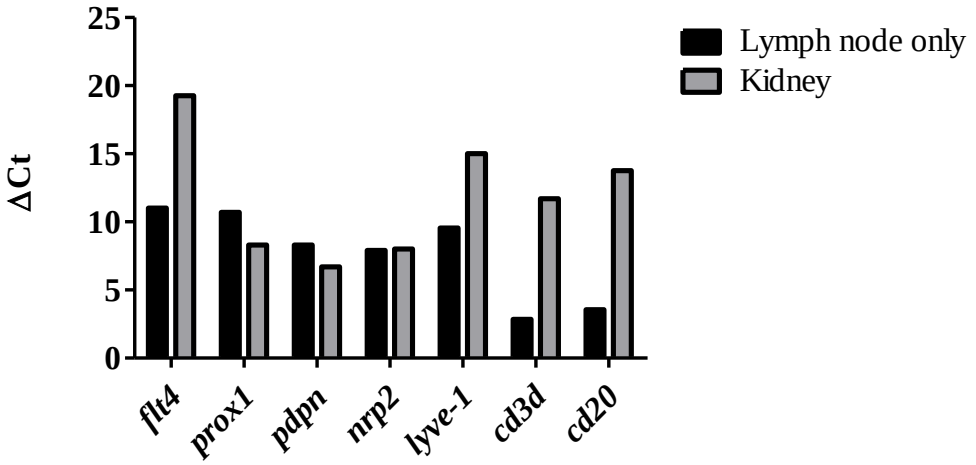


Figure 23. Lymph node marker expression varied between lymph node only and kidney tissue. *Flt4*, *lyve-1*, *cd3d*, and *cd20* were more highly expressed in a lymph node isolated from mammary gland (lymph node only) than the negative control kidney tissue. Oppositely, *prox1* and *pdpn* had lower expression in lymph node only tissue, whereas *nrp2* appeared to have equal expression in both positive and negative control tissues. A lower ΔCt value indicates the gene is expressed at a higher level. Statistics were not performed since single samples were examined.

The presence or absence of the lymph node within the tissue may affect our gene expression of immune-related genes, therefore, only lymph node-negative mammary tissue needed to be used in all future RNA analyses. As such, designated pieces of the mammary gland were prepared for RNA isolation to identify samples with and without the lymph node and to select sections with similar amounts of mammary tissue (Fig. 24).

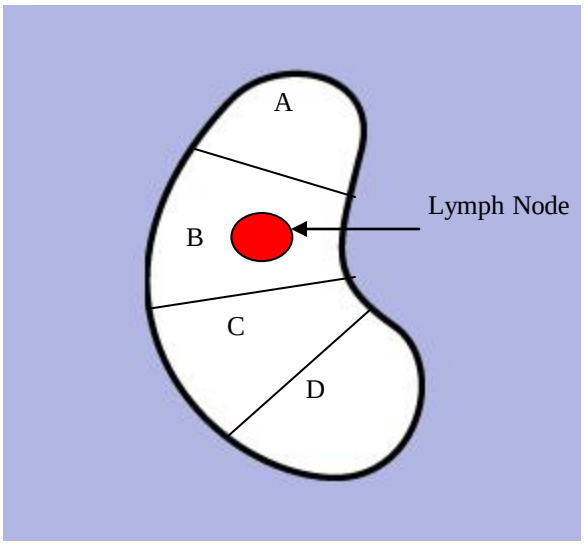


Figure 24. Schematic of Mammary Gland with Designated Sections. In order to avoid mammary gland tissue samples that contained the lymph node and to ensure consistency between samples, the mammary glands were sectioned A, B, C and D. Unless otherwise noted, section B was determined to be the lymph node positive region.

To ensure mammary gland sections without lymph node had different expression of the three potential lymph node markers compared to section B that contained the lymph node, inguinal mammary glands from five wild-type (FVB/N) mice were harvested and visual confirmation of the lymph node was performed. Section B was designated as being the lymph node positive region prior to the gland being sectioned (A, B, C, and D). Real-time RT-PCR for the selected marker, *cd20*, as well as 2 other lymph node markers was performed to confirm the findings in Figure 22 with more samples from the designated mammary gland sections. Expression of *flt4* and *lyve-1* in sections C and D (lymph node-negative) were similar, but were not significantly different from lymph node-positive section B. In contrast, *cd20* did show a significant ($p<0.0001$, one-way ANOVA) difference in expression between lymph node-positive (section B) and lymph node-negative (sections C and D) mammary gland sections (Fig. 25). Therefore, *cd20* was

used to determine which RNA preparations were node-negative for subsequent RNA analyses.

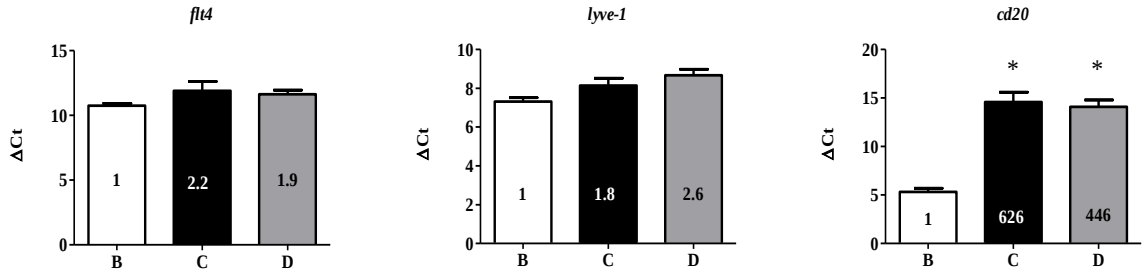


Figure 25. Lymph node screening revealed only *cd20* expression differed between lymph node-positive or lymph node-negative mammary gland sections. Real-time RT-PCR demonstrated expression of *flt4* and *lyve-1* in sections C and D were similar, but were not significantly different from lymph node-positive section B. In contrast, *cd20* did show a significant ($p<0.0001$) difference in expression between lymph node-positive (section B) and lymph node-negative (sections C and D) mammary gland sections. A higher ΔCt value indicates the gene is expressed at a lower level and requires more amplification cycles before it can be detected. Numbers displayed within each bar represent the fold-change compared to control using the $2^{-\Delta\Delta Ct}$ method. One-way ANOVA was used for statistical analysis ($p<0.05$). n=5.

All animals in the Pretumor study, including those used for the microarray analysis, had their mammary gland RNA screened for *cd20* in order to identify which samples were lymph node positive and negative. Table 16 shows the random distribution of the lymph node in control and p,p´ DDE animals (Pretumor study) used for the microarray, which probably confounded the interpretation of the data from the microarray analysis, especially for cytokines and other immune-related genes. When the initial six immune-related genes had their expression measured again by real-time PCR in confirmed lymph node-negative mammary tissue, p,p´ DDE treatment was not significantly different from

control (Fig. 26). Therefore, no genes were identified to be differentially regulated from this microarray analysis.

Table 16. Lymph Node Determination by Cd20 Screen in Microarray Pools

Animal	Group	cd20 ΔCt	Lymph Node	No Lymph Node	Microarray Pool #
A	Control	14.3		X	1
B	Control	3.7	X		1
C	Control	15.2		X	1
D	Control	14.1		X	2
E	Control	4.9	X		2
F	Control	13.75		X	2
A	p,p´ DDE	3.05	X		1
B	p,p´ DDE	13.55		X	1
C	p,p´ DDE	13.55		X	1
D	p,p´ DDE	14		X	2
E	p,p´ DDE	4.3	X		2
F	p,p´ DDE	14.4		X	2

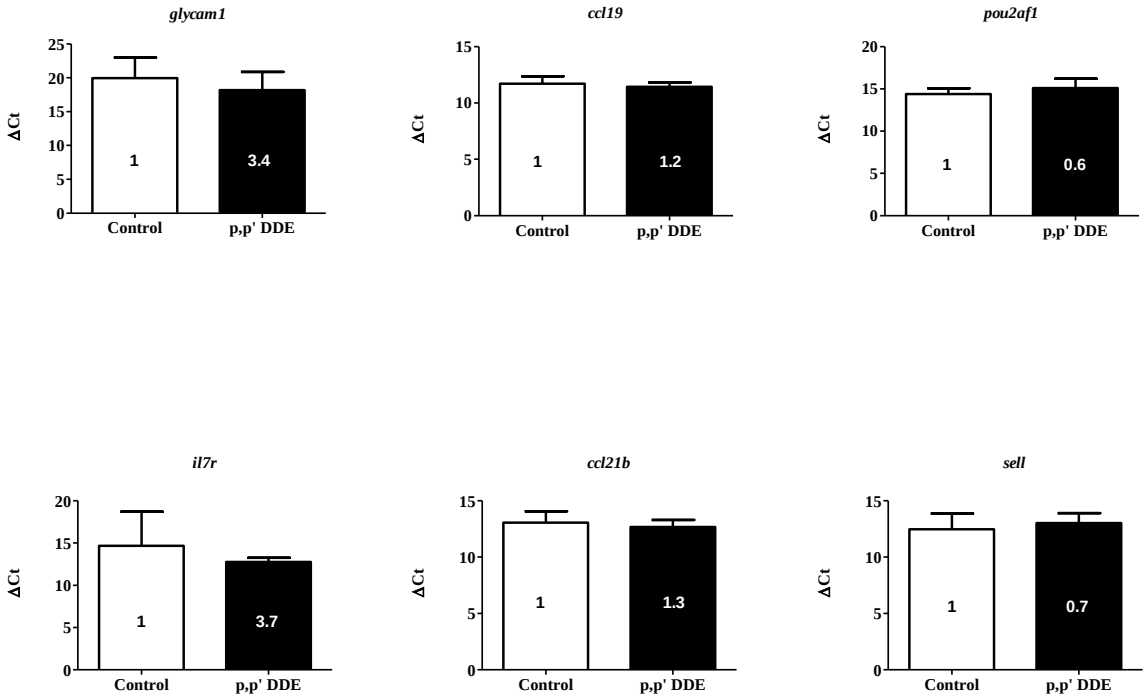


Figure 26. The microarray genes examined in the p,p´ DDE group were not significantly different from control in confirmed lymph node-negative mammary tissue. When expression of immune-related genes *glycam1, ccl19, pou2af1,il7r, ccl21b and sell* was re-examined by real-time RT-PCR with SYBR Green detection in lymph node-negative mammary gland tissue, p,p´ DDE effects were similar to control. A higher ΔCt value indicates the gene is expressed at a lower level and requires more amplification cycles before it can be detected. Numbers displayed within each bar represent the fold-change compared to control using the $2^{-\Delta\Delta Ct}$ method. A Mann-Whitney test was used for statistical analysis ($p>0.05$). n=3.

Preparation of Samples for Performing a New Microarray Analysis

Since no candidate genes were identified, it became evident that a second microarray analysis needed to be prepared. Thus, for the new microarray, only lymph node-negative mammary tissues were used to avoid the problems with the first microarray analysis, including 1) the unexpectedly low number of differentially expressed genes by p,p´ DDE, 2) immune genes not being identified because of the presence of the lymph node in some

samples, and 3) the inability to identify any genes differentially expressed between the p,p´ DDE and control groups.

As a result of the tissue weight requirements needed to measure DDT congener levels and the RNA preparations and analyses already performed, there were insufficient amounts of mammary gland tissue and RNA remaining to replicate and analyze the new microarray analysis with the Pretumor study tissues. Therefore, in order to conduct another microarray analysis and to further characterize any identified genes, additional animals implanted with vehicle control or p,p´ DDE (5 mg) ELVAX pellets were needed. Additional treatment groups for testing expression of the identified genes for Aim 3 are described in the next chapter.

Ten prepubertal MMTV-*neu* females per group were bilaterally implanted with ELVAX 40P pellets in the axillary and inguinal mammary fat pads (4 pellets/mouse) and euthanized after two months of treatment. RNA was isolated from the inguinal mammary glands and screened for low *cd20* levels to ensure the absence of the lymph node. Candidate genes that were differentially regulated by p,p´ DDE were identified by microarray analysis by the Functional Shared Genomics Core, Vanderbilt University, Nashville, TN using the Affymetrix Genechip 1.0 ST. The second microarray used only tissues from individual animals from the DDT-01 study instead of pools from three mice. Three animals were tested per group to allow the genomics core to perform statistical analyses for differential expression. Only the control and p,p´ DDE groups were

examined since the goal was to identify genes that were differentially expressed by p,p´ DDE treatment.

Candidate Gene Selection

Candidate genes with significant differential expression by p,p´ DDE were further selected based on the selection criteria for genes with known associations with cancer or immune functions or androgen regulation as these actions have been previously related to DDT. Approximately 15 genes were initially selected from the microarray based upon these criteria and confirmed by real-time RT-PCR using SYBR-Green detection. Expression trends of the microarray matched that of the genes tested by real-time RT-PCR in the individual animals used for the microarray, with the exception of *rsp12*. (Table 17). These data indicate stronger correlations between the second microarray and the individual animals tested in the microarray than were observed with the first microarray. The genes tested and their functions are listed in Table 18.

Table 17. Expression comparison between microarray analysis and real-time RT-PCR in animals used for the second microarray

Gene	Microarray Expression n=3	Fold Change p,p´ DDE vs control	Individual Expression n=3	Fold Change p,p´ DDE vs control
aacs	↑	2.1	↑	5.9
acly	↑	2.6	↑	9.7
csn1s2a	↑	6.5	↑	9.5
crym	↑	2.1	↑	4.5
egr2	↑	2.4	↑	6.0
id4	↓	-2.1	↓	0.8
ifi44	↑	2.5	↑	4.4
insig1	↑	2.1	↑	7.4
prune2	↑	2.0	↑	6.7
rsp12	↑	2.9	↓	.08
sfrp5	↓	-2.7	↓	0.4
slc2a5	↑	3.5	↑	55.0
sprr1a	↑	3.9	↑	9.1
thbs1	↓	-2.1	↓	0.8
thrsp	↑	2.5	↑	3.9

Table 18. Candidate Genes Selected From the Second Microarray Analysis

Gene Name	Abbreviation	Function	Reference	Criteria Category
Acetoacetyl-CoA	aacs	Involved in lipogenesis	Aguilo et al., 2010	Cancer
ATP citrate lyase	acly	Involved in the lipogenesis metabolic pathway. Activated at a relatively early stage in various types of tumors; plays a role in tumorigenesis and tumor cell survival. Plays a role in the aggressive phenotype seen in African American women.	Furuta et al., 2010; Yancy et al., 2007	Cancer
Casein gamma	csn1s2a	Milk protein related gene. Involved in mammary differentiation	Hilvo et al., 2011	Cancer
Mu-crystallin	crym	Androgen regulated in human cancer	Malinowska et al., 2009	Androgen-regulated
Early growth response 2	egr2	Up-regulated during ErbB2/*neu* tumor induction	Dillon et al., 2007	Cancer
Inhibitor of differentiation/DNA binding 4	id4	Involved in numerous cell processes including cell proliferation, differentiation and tumorigenesis; proangiogenic	Candia et al., 2006; Dell'Orso S et al., 2010	Cancer
Interferon-induced protein 44	ifi44	Mediates the effects of hormones during tumorigenesis in	Peng et al., 2010	Cancer, Androgen-regulated

		the breast		
Insulin-induced gene 1	insig1	Involved in lipid biosynthesis and adipocyte differentiation; often up-regulated in BC	Li et al., 2003; Einbond et al., 2007; Hilvo et al., 2011	Cancer
Prune homolog 2	prune2	Implicated in programmed cell death;	Valencia et al., 2007	Cancer
Ribosomal protein S12	rsp12	Involved in the pathological process of BC and may be a useful biomarker	Deng et al., 2006	Cancer
Secreted frizzled-related protein 5	sfrp5	Inhibitor of the Wnt pathway. Often down-regulated in BC; also methylated at high frequencies	Suzuki et al., 2008;Veeck et al., 2008;	Cancer
Solute carrier family 2, member 5	slc2a5 (Glut-1)	Glucose transporter that regulates glucose uptake; up-regulated in metabolic dependence of tumors	Chen et al., 2010; Schmidt et al., 2010	Cancer
Small proline-rich protein 1A	sprr1a	Involved in epithelial differentiation. Used to determine malignant hyperproliferation from benign hyperplasia.	Marshall et al., 2000; Haider et al., 2006	Cancer
Thromobspondin-1	thbs1	Androgen regulated. A multifunctional extracellular matrix protein. Affects cell adhesion, migration, proliferation,	Bertin et al., 1997; Mattila et al., 2006	Cancer and androgen-regulated

		survival, apoptosis and differentiation by mediating cell-matrix interactions. Modulates tumor growth, angiogenesis and metastasis;		
Thyroid hormone-responsive protein	thrsp	Involved in lipid metabolism; highly expressed in clinical BC samples	Hilvo et al., 2011	Cancer

Of the fifteen genes identified in Table 18 with p,p´ DDE-induced differential expression, seven candidate genes (*slc2a5, ifi44, csn1s2a, acly, thrsp, prune2* and *crym*) were chosen for analysis in the Pretumor study mammary tissues for the control, p,p´ DDE groups as well as for OH-flutamide and the 2:l ratio groups. The goal of this analysis was to determine how the DDT-01 and Pretumor studies compare on genes identified from the second microarray analysis. As shown in Figure 27, no change in expression of any of the genes occurred in the treatment groups compared to control. The lack of significant regulation by p,p´ DDE may be due to the differences between the original Pretumor study and the DDT-01 animals used for the two microarray analyses. These differences include animals and treatments were performed in different environments (Duquesne University and Cedars-Sinai Medical Center) and use of random regions of the mammary glands with and without lymph nodes for RNA for the first microarray analysis.

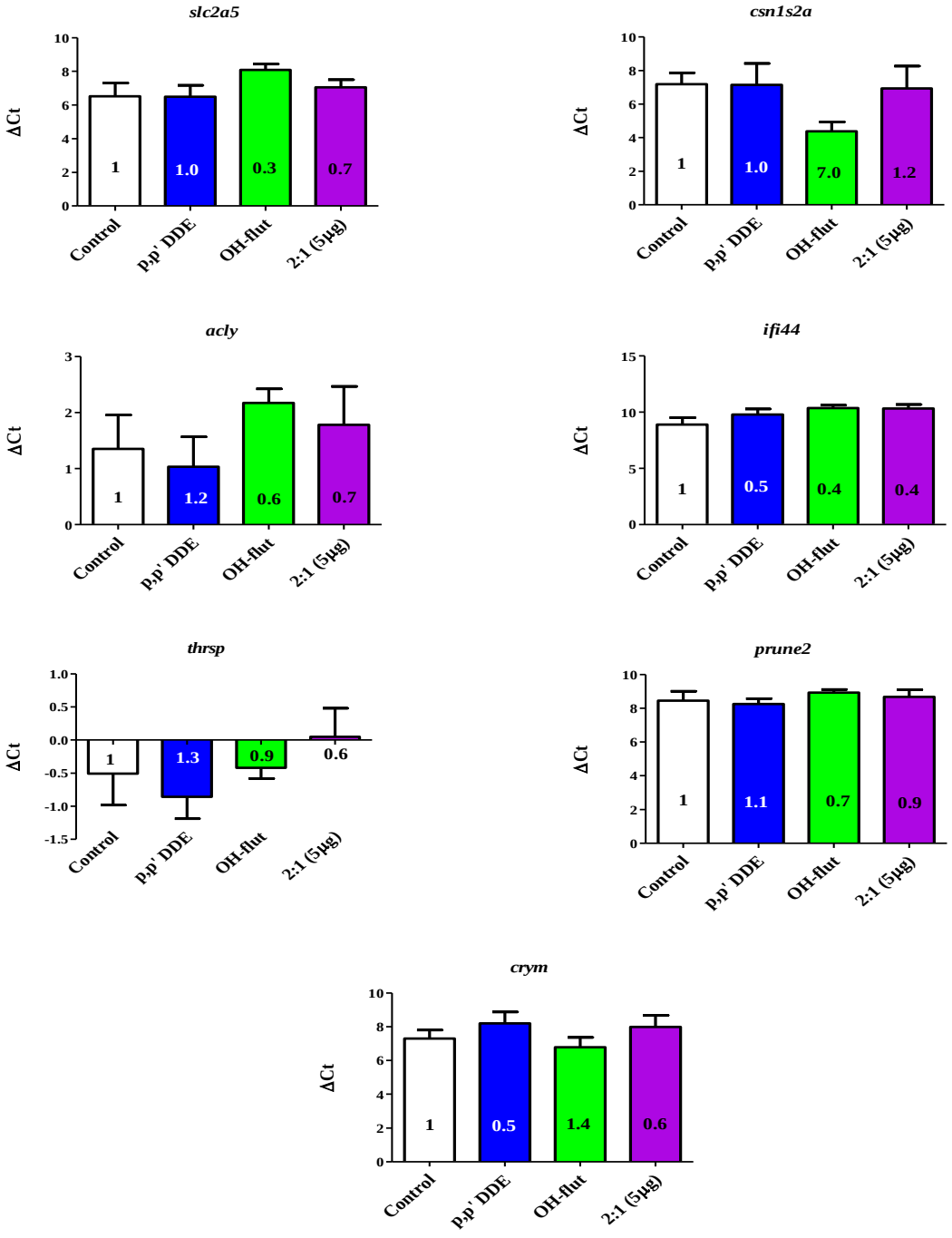


Figure 27. Candidate genes from the second microarray were not differentially expressed in Pretumor study animals. Selected genes *slc2a5*, *csn1s2a*, *acly*, *ifi44*, *thrsp*, *prune2* and *crym* had their gene expression tested by real-time RT-PCR with SYBR-Green detection. These genes in the p,p´ DDE , OH-flut, and the 2:1 ratio groups were not significantly different from control. A higher ΔCt value indicates the gene is expressed at a lower level and requires more amplification cycles before it can be detected. Numbers displayed within each bar represent the fold-change compared to

control using the $2^{-\Delta\Delta Ct}$ method. Mann-Whitney test was used for statistical analysis ($p>0.05$). n=6. OH-flut = hydroxyflutamide; 2:1 = 3.3 µg/pellet p,p´ DDE + 1.7 µg/pellet o,p´ DDE.

Wide variability was evident among animals from the Pretumor and DDT-01 studies within the treatment groups, particularly between the control groups. Figure 28 displays the scatter plots of the seven candidate genes by real-time RT-PCR in both Pretumor and DDT-01studies (3 animals/group). Expression in the Pretumor study was predominately higher (lower ΔCt) than the DDT-01 study, with the following ΔCt averages for the Pretumor study and DDT-01 studies, respectively, for the tested genes: *slc2a5* (5.91 and 15.75), *csn1s2a* (6.38 and 9.45), *acly* (0.86 and 6.91), *ifi44* (8.36 and 12.23), *thrsp* (-1.00 and 2.21), *prune2* (7.81 and 11.73), and *crym* (6.98 and 8.56). Accordingly, none of the expression differences between the p,p´ DDE and control groups was statistically significant for any gene.

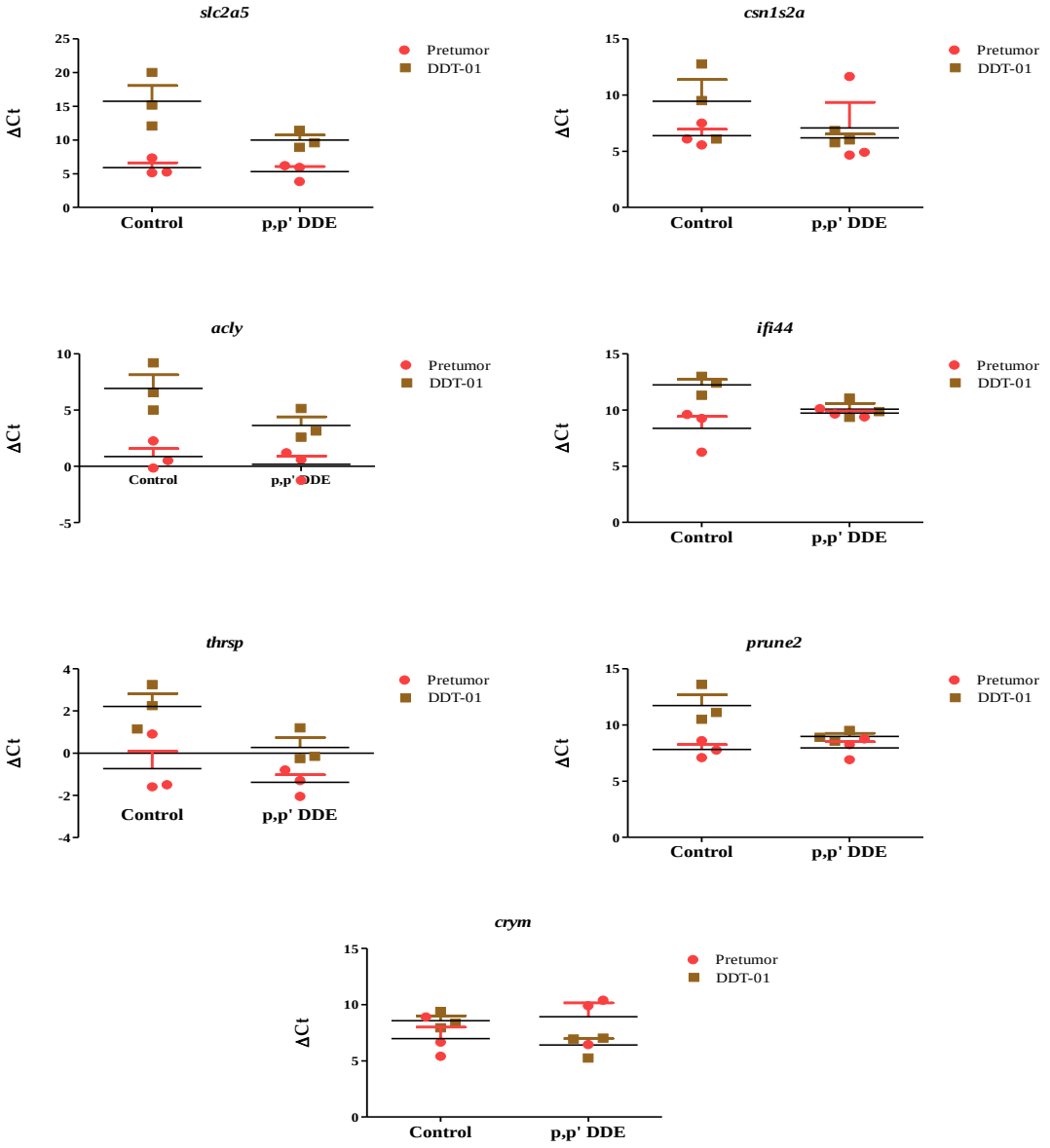


Figure 28. Variability between the control groups in the Pretumor and DDT-01 studies may contribute to the lack of noticeable effect by p,p´ DDE . Real-time RT-PCR showed the control group of the Pretumor study had consistently higher expression levels among all candidate genes in comparison to the DDT-01 study controls. p,p´ DDE regulation among the genes was also not significantly different, with all genes exhibiting less variability than controls with the exception of *crym*. A higher ΔCt value indicates the gene is expressed at a lower level and requires more amplification cycles before it can be detected. Numbers displayed within each bar represent the fold-change compared to control using the 2-ΔΔCt method. A Mann-Whitney test was used for statistical analysis ($p>0.05$). n=3.

With the disparate expression results observed between the Pretumor and DDT-01 studies, it was speculated that the amount of mammary tissue within the samples may be inconsistent between the two studies. One reason for this could be that the region of mammary gland used in the Pretumor study for RNA preparation was not controlled. The Pretumor study used randomly selected pieces since the glands were broken and quick frozen together in one tube, during necropsy. In contrast, the DDT-01 study used designated sections of the gland as shown in Figure 24. Perhaps, some sections of mammary gland had more fat and less mammary tissue in one study compared to a section that was lower in fat and higher in mammary tissue, such as the epithelium. To address if consistent amounts of mammary tissue were in the different samples, expression of an epithelial marker, cytokeratin 18 (*krt18*) and a differentiation marker, casein beta (*csn2*) were compared in control and p,p´ DDE treatment groups in both Pretumor and DDT-01 studies by real-time RT-PCR. Figure 29 shows no substantial differences in gene expression between control and p,p´ DDE groups in either study, indicating that the amounts of epithelial tissue between the studies was consistent. Therefore, the amount of mammary epithelium in the samples in the Pretumor and DDT-01 studies may not be the cause of variation between the Ct values in the two studies. Consequently, the disparity in expression was likely due to other variables related to the study sites, such as 1) the facilities where the studies were conducted, 2) different staff prepared the ELVAX 40P pellets and performed the surgeries, and 3) different generations of breeders were used to produce the study mice. Accordingly, for the subsequent analyses, only animal studies conducted at Duquesne University were used.

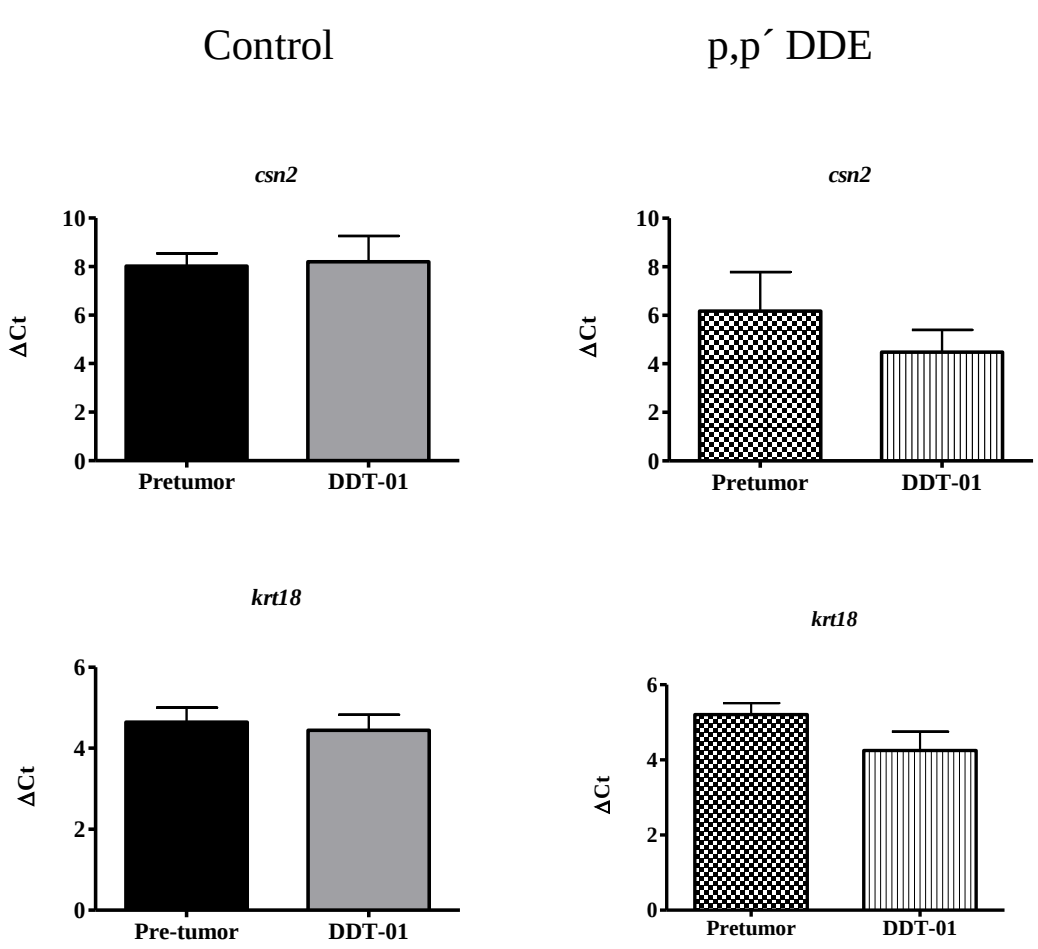


Figure 29. Epithelial markers casein beta (*csn2*) and keratin 18 (*krt18*) show the mammary tissues are consistent between Pretumor and DDT-01 studies. Mammary gland epithelium expression was measured by real-time RT-PCR with SYBR-Green detection in both Pretumor and DDT-01 studies. Results demonstrated no distinguishable difference in expression in either control or p,p´ DDE treatment groups. A Mann-Whitney test was used for statistical analysis ($p>0.05$). n=6.

The initial seven candidate genes (Fig. 28) were tested in animals from the control and p,p´ DDE groups in the DDT-02 study in lymph node-negative mammary tissue from section C (Fig. 24); additional DDT-01 study animals from these groups were also tested. Expression of these genes was expected to be similar in the two studies since both were conducted at Duquesne University. The expression of the candidate genes in DDT-01 and DDT-02 studies were more comparable than observed with the Pretumor and DDT-01 studies (Fig. 30 & 31). Expression of *slc2a1*, *csn1s2a*, *ifi44*, *prune2* and *crym*

expression in the control groups was not significantly different between DDT-01 and DDT-02 studies; a similar effect was observed for the p,p´ DDE groups in both studies (Fig. 30 and 31). Whereas *acly* and *thrsp* displayed significant differences between the control groups (p=0.03, Mann-Whitney test).

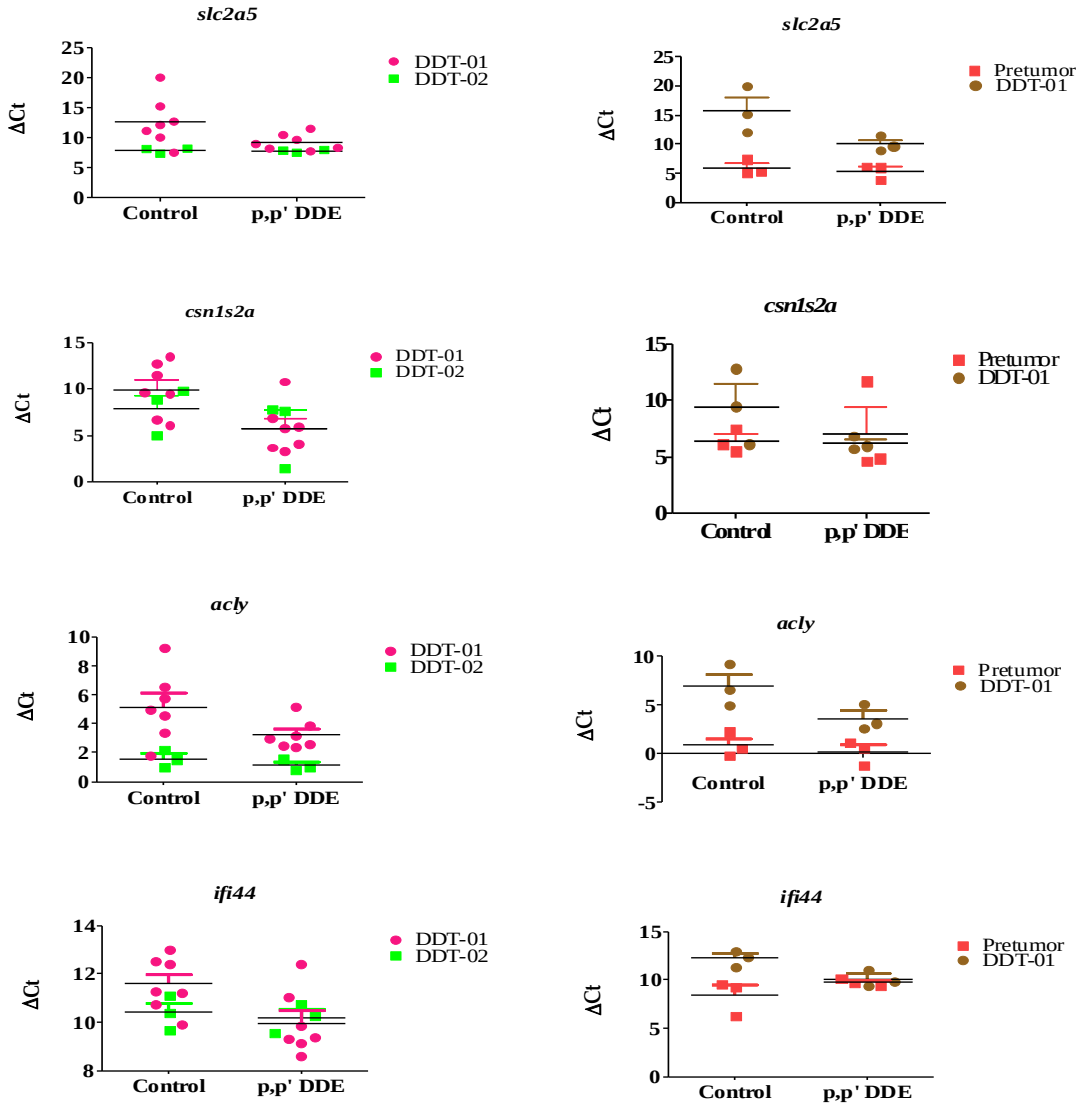


Figure 30. Expression variability between DDT-01 and DDT-02 was reduced in comparison to DDT-01 and Pretumor studies. When the expression of the seven candidate genes was measured by real-time RT-PCR in the DDT studies, the ΔCt values of DDT-01 and DDT-02 studies displayed less variation than those in comparison to the DDT-01 and Pretumor studies. A higher ΔCt value is indicative of the transcript being expressed at lower levels, therefore requiring more amplification cycles before it can be detected. A Mann-Whitney test was used for statistical analysis. $p>0.05$. Pretumor vs. DDT-01, n=3; DDT-01 vs. DDT-02, n=6 and 3, respectively.

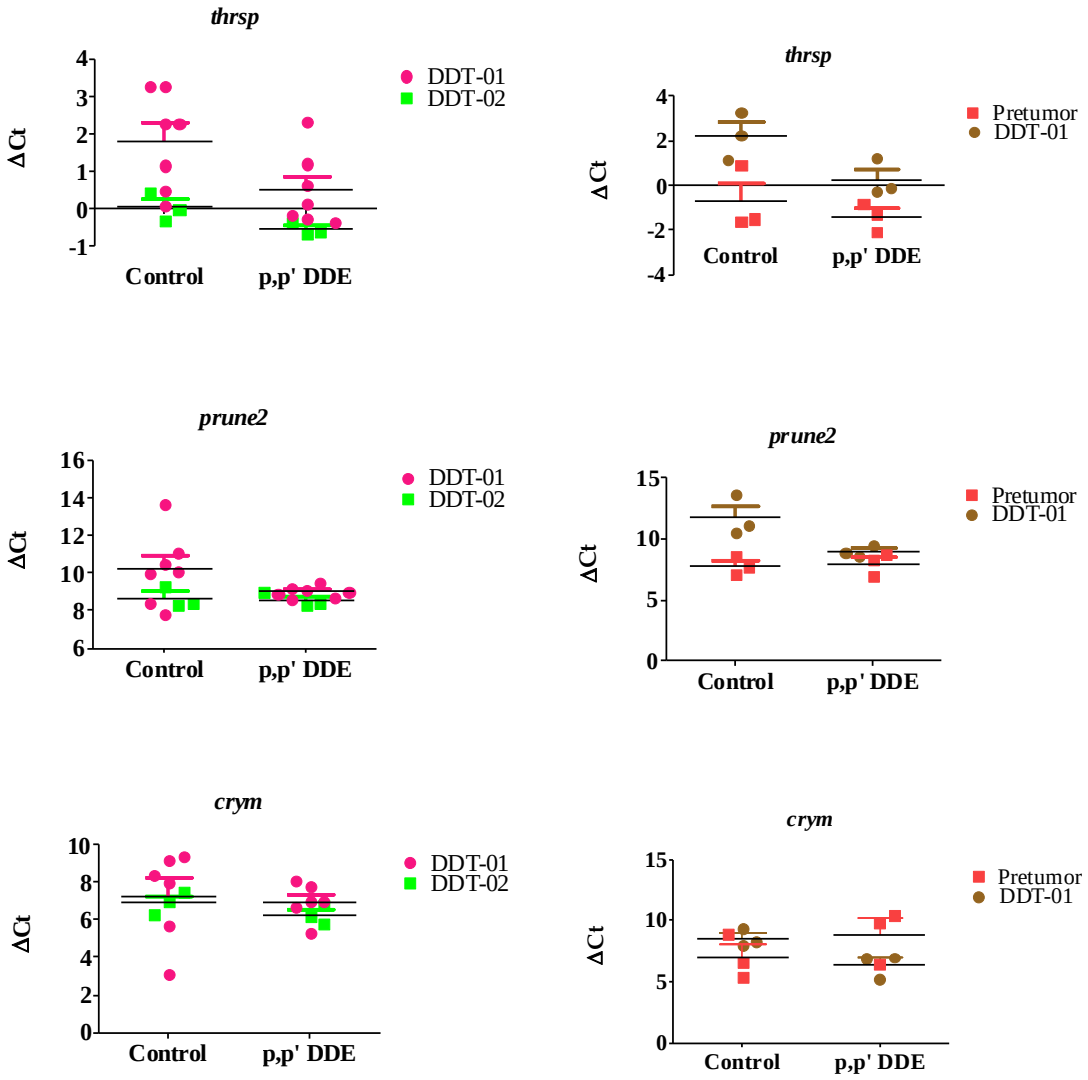


Figure 31. Expression variability between DDT-01 and DDT-02 was reduced in comparison to DDT-01 and Pretumor studies. When the seven candidate genes had their expression measured by real-time RT-PCR in the DDT studies, the ΔCt values of DDT-01 and DDT-02 studies displayed less variation than those in comparison to the DDT-01 and Pretumor studies. A higher ΔCt value is indicative of the transcript being expressed at lower levels, therefore requiring more amplification cycles before it can be detected. A Mann-Whitney test was used for statistical analysis. $p>0.05$. n=3 (Pretumor vs. DDT-01); DDT-01 vs. DDT-02 (n=6 and 3, respectively).

Due to the observed decrease in variability in the Ct levels for the seven candidate genes observed between DDT-01 and DDT-02 study, samples from both studies were combined

for the control and p,p´ DDE groups. Analyses on gene expression for the seven candidate genes were performed with the six animals from the DDT-01 study and three mice from the DDT-02 study (n=9). Only *csn1s2a* and *ifi44* were significantly up-regulated by p,p´ DDE treatment (p= 0.003 and 0.004, respectively, Mann Whitney test; Fig. 32).

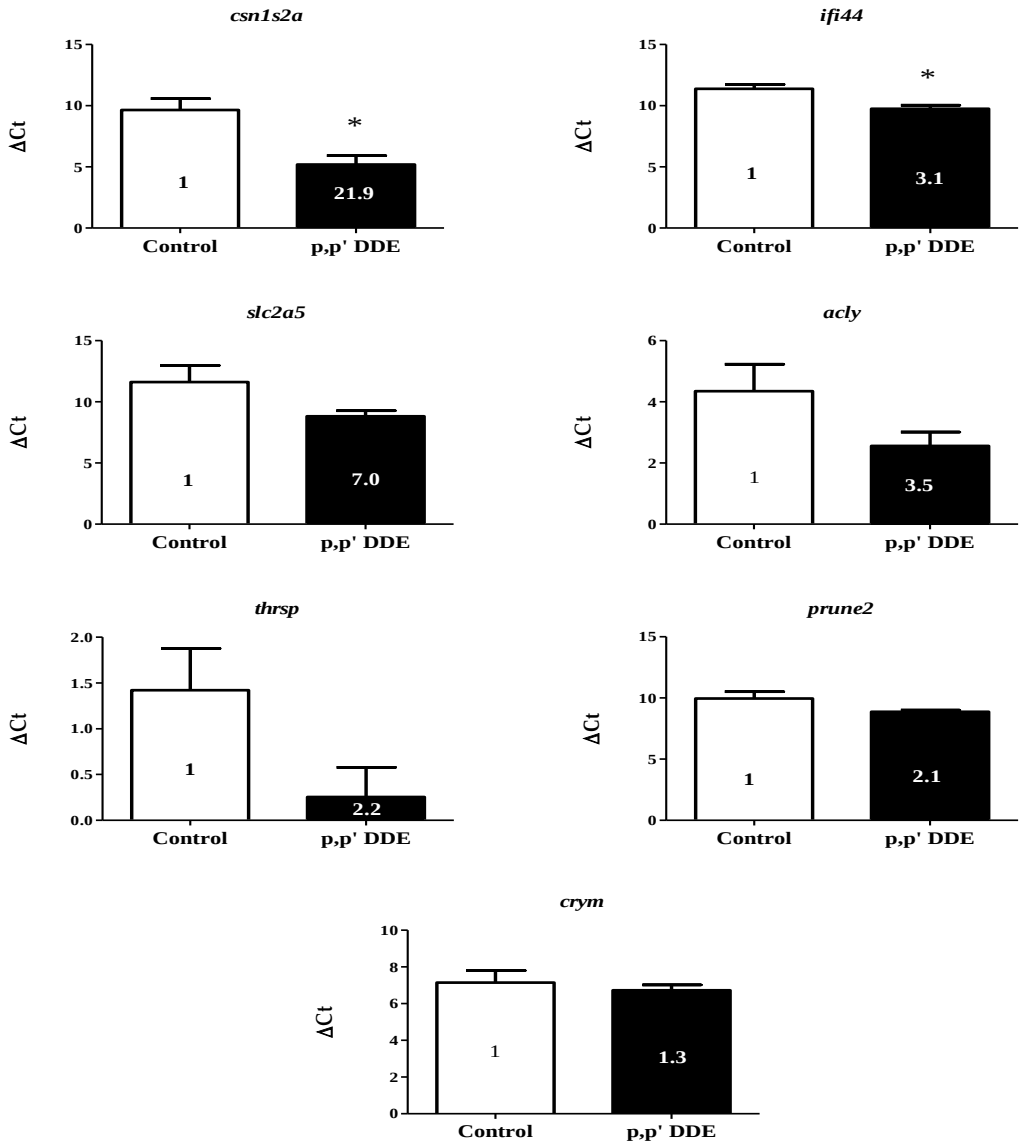


Figure 32. p,p´ DDE significantly stimulated candidate genes *csn1s2a* and *ifi44* in mammary tissue compared to control. Identification of candidate genes by RT-PCR revealed *csn1s2a* (p=0.003) and *ifi44* (p=0.004) were significantly up-regulated by p,p´ DDE when DDT-01 and DDT-02 studies were combined. *Thrsp* approached significance with a p value of 0.07, while all other genes also demonstrated a non-significant up-regulation in expression. Genes that are highly expressed have a lower ΔCt as a result of not requiring many amplification cycles prior to detection. The numbers within each bar represent the fold change compared to control using the 2- ΔΔCt method. A Mann-Whitney test was used for statistical analysis (*p<0.05). n=9.

Next, to identify additional genes differentially regulated by p,p´ DDE, a second set of genes were chosen from the microarray based on the predetermined criteria that were previously tested in the individual mice from DDT-01 study used in the second microarray (Table 18). The next genes analyzed were *egr2*, *sfrp5*, *aacs*, *sprr1a*, *insig1*, *thbs1*, *id4*, *rps12* and *csn2*. Expression was measured in lymph node-negative mammary tissue samples from a combination of DDT-01 and DDT-02 studies by real-time RT-PCR (Fig. 33). However, p,p´ DDE treatment did not result in a significant difference in gene expression for the second set of candidate genes compared to control mice.

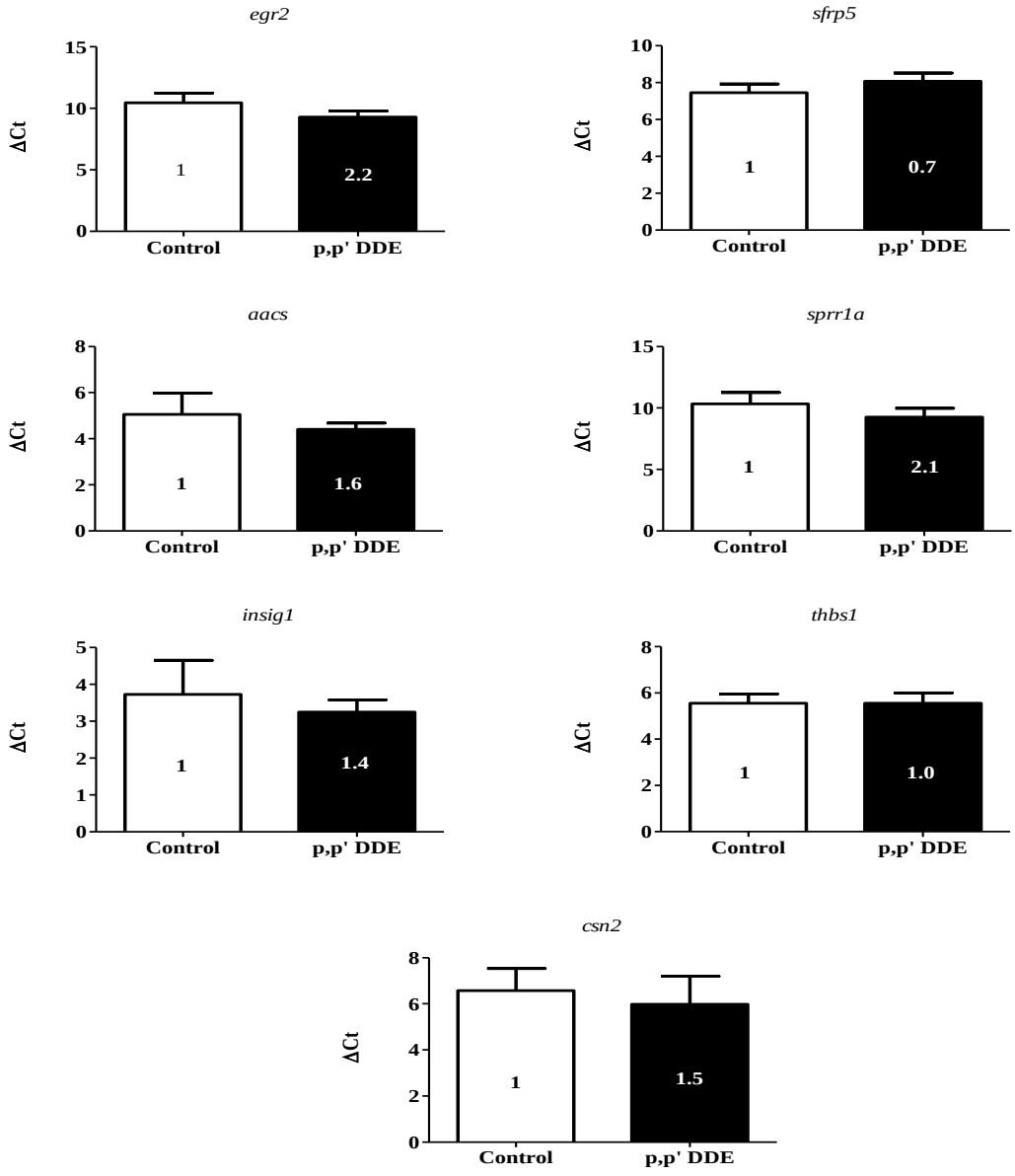


Figure 33. Second set of candidate genes were not significantly altered by p,p´ DDE treatment in 3-month-old MMTV-*neu* mice. When the second set of selected genes had their expression measured in mammary tissue by real-time RT-PCR, p,p´ DDE did not significantly alter gene expression compared to control. A lower ΔCt is indicative of a higher amount of transcript, therefore, less amplification cycles are needed before it is detected. The numbers within each bar represent the fold change compared to control using the $2^{-\Delta\Delta Ct}$ method. A Mann-Whitney test was used for statistical analysis ($p>0.05$). n=7.

Due to the inability to identify additional p,p´ DDE -induced markers from the second set of candidate genes, keratin 18 (*krt18*) expression was examined by real-time RT-PCR, to ensure the amount of mammary epithelial tissue in both studies was consistent. As shown in Figure 34, the amount of epithelial tissue was similar between the samples from the DDT-01 and DDT-02 studies in each group (Fig. 34A). However, krt18 was also significantly up-regulated by p,p´ DDE compared to control mice (Fig. 34B). Consequently, in total, three genes (*csn1s2a*, krt18, and *ifi44*) were identified to be significantly up-regulated by p,p´ DDE exposure (Fig. 35) compared to control animals in the mammary tissue from mice from the combined DDT-01 and DD-02 studies. These genes will be tested in the subsequent studies for Aim 3 (Chapter 5).

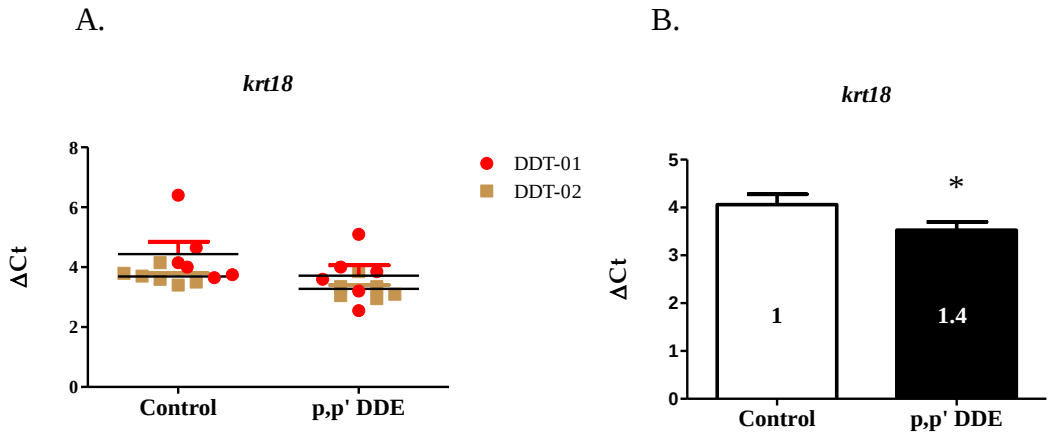


Figure 34. Epithelial marker *krt18* is significantly modified by p,p´ DDE. A) *Krt18* displayed uniform expression between DDT-01 and -02 studies when measured by real-time RT-PCR. B) Gene expression of *krt18* by real-time RT-PCR also revealed significant (*p=0.01) regulation by p,p´ DDE when study DDT-01 and -02 were combined. ΔCt is the normalized level of *krt18* to *ppia* (cyclophilin A). A lower ΔCt is indicative of a high abundance of message, which requires less amplification before it crosses the cycle threshold (Ct). The numbers within each bar represent fold change compared to control. A two-way ANOVA (A) and Mann-Whitney test (B) were performed for statistical analysis ($p<0.05$). n=6.

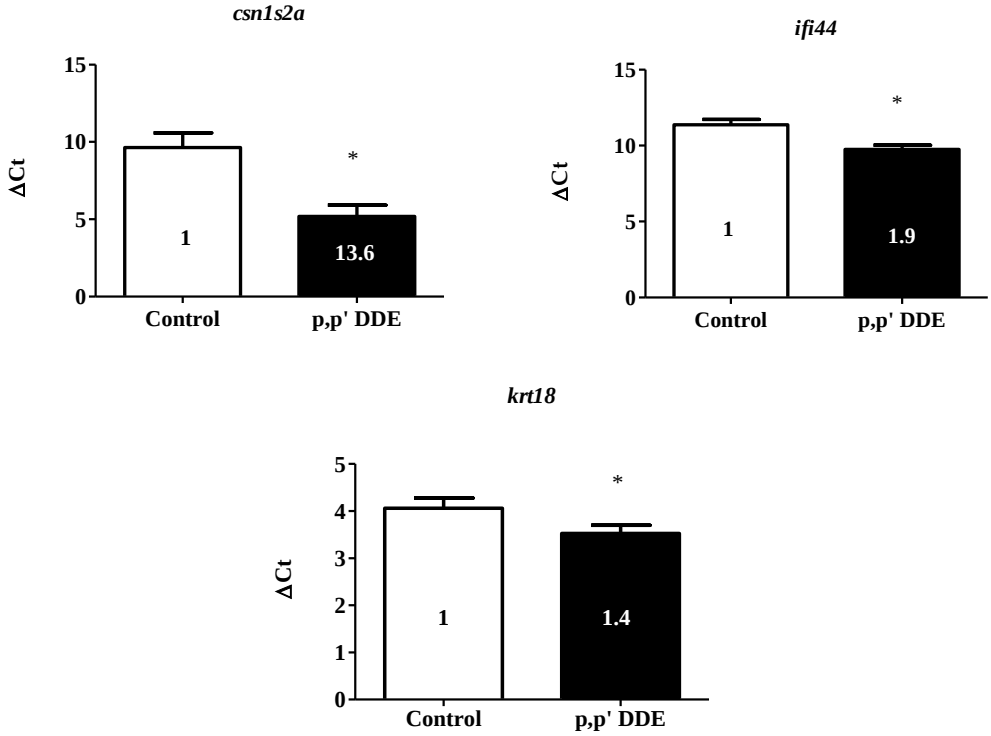


Figure 35. p,p´ DDE increased expression of the three identified markers in mammary tissue. *csn1s2a*, *ifi44* and *krt18* were identified as the final characterized genes of p,p´ DDE action by real-time RT-PCR. Differentiation marker *csn1s2a* was greatly modified by p,p´ DDE exposure, having a p value of 0.002 and an up-regulation in expression by a 13.6 fold change. Immune-related gene *ifi44* was also significantly (p=0.02) stimulated by p,p´ DDE, with an 1.9 fold change in expression. *krt18*, also a marker of differentiation was also significantly (p=0.01) up-regulated by p,p´ DDE, with a 1.4 fold change in expression by the $2^{-\Delta\Delta Ct}$ method. ΔCt values are the normalized level of gene expression compared to *ppia* (cyclophilin A). A high ΔCt means there is a low abundance of message and therefore requires more amplification before crossing the threshold cycle (Ct) of detection. Numbers within each bar represent the fold change compared to control ($p<0.05$). n=13.

DISCUSSION

Before Davis and colleagues, the direct, local effects of DDT metabolites such as p,p´ DDE had not been tested in the mammary gland by a controlled drug delivery system (ELVAX 40P). In this study, interferon-induced protein 44 (*ifi44*), casein gamma (*csn1s2a*) and keratin 18 (*krt18*) genes were chosen to investigate possible p,p´ DDE action. These genes were significantly regulated by p,p´ DDE and predetermined to be associated with cancer (*csn1s2a, krt18*), immune (*ifi44*) or hormone regulation.

Interferons are a family of proteins that are made and secreted by many different cell types of the immune system. They are made in order to modulate the response of the immune system to invading foreign substances like cancer. *Ifi44* was first discovered in association with microtubular structures in a hepatitis C virus-infected chimpanzee and, more than a decade later, nothing more is known about its possible function (Hallen et al., 2007). However, it has been considered a predictor of breast cancer outcomes and has been linked to aggregate patterns of gene expression that associate with lymph node status and recurrence (Huang et al., 2003). Here, it has been shown to be significantly up-regulated in the mammary gland by 5 μg/pellet p,p´ DDE . An increase in expression of this interferon-induced gene is suggestive of the need for additional immune response, perhaps due to early cancer causing events taking place. At approximately 3 months of age, this stimulation as well as many others may attempt to ward off tumor formation. However, in time, with longer exposure and higher accumulation, the response of this gene may not be adequate and/or may become down-regulated. Immune dysfunction

plays a key role in cancer development and although mechanisms are not fully understood, potential immune dysfunction in cancer does include defects in cytokines like interferons (Wang et al., 2011). In fact, interferon-stimulated gene *ifi44* has been found to be down-regulated in lymphocytes from breast cancer patients compared to controls (Critchley-Thorne et al., 2009), supporting the theory of decrease expression with time.

Cytokeratins (keratins) have been recognized for >20 years as structural markers specific to epithelial cells (Woelfle et al., 2004). They are regulated at the transcriptional level in normal mammary gland epithelia and alterations observed in breast cancer are characterized by partial loss of the normal regulation of cytokeratin expression (Su et al., 1996). Their expression also varies with differentiation of the cell (Clarke et al., 2004). A cytokeratin, *krt18*, was another gene significantly up-regulated by p,p´ DDE treatment in the mammary gland. It is well known that increased differentiation is associated with protection of the gland and this stimulation is not consistent with the tumor study outcome induced by p,p´ DDE . However, although this luminal epithelial marker (Abdel-Fatah et al., 2008) has been associated with being down-regulated in breast carcinomas as compared to normal breast tissue or fibroadenoma (Linder et al., 1996; Woelfle et al., 2004), use of the individual cytokeratin as a diagnostic marker for breast cancer might lead to false-negative findings due to the observed down-regulation of this protein (Woelfle et al., 2004). The disparity between p,p´ DDE-induced expression of *krt18* and the tumor study findings may also be due to an approximate exposure time of two months not being long enough. The regulation of this gene may eventually change

over time and the expression will become repressed and concur with the previous findings of down regulation (Franzen et al., 1996; Woelfle et al., 2004) or possibly that of Willipinski-Stapelfeldt and colleagues (2005) who propose loss of cytokeratin 18 is indicative of an epithelial to mesenchymal transition as seen in micrometastatic cancer cell lines. Moreover, the increased expression of this gene could infer an attempt of the gland to remain morphologically normal, as *krt18* is also important for normal breast epithelium and their proliferative epithelial processes (Böcker et al., 2009) as may be seen in a yet still growing gland. Another possible reason for the increased expression of *krt18* by p,p´ DDE could be due to the differential expression of HER-2/*neu* being associated with specific cytokeratin subtype distribution (Steinman et al., 2007). Considering *neu* was not significantly regulated by p,p´ DDE at approximately 3 months of age could imply *krt18* expression may be different at a later time. Conversely, stimulation of *krt18* could be occurring due to tumor cells already present in the mammary gland. Trask and colleagues (1990) have shown that normal mammary epithelial cells produce keratins (K) K5, K6, K7, K14 and K17, whereas tumor cells produce mainly K8 and K18. They also demonstrate in immortalized cells, which are preneoplastic or partially transformed, that *krt18* is increased. This finding is in better alignment with the tumor study and may, in fact, be what is occurring with p,p´ DDE exposure.

The third gene discovered to be significantly up-regulated by p,p´ DDE was casein gamma. Casein genes encode the major milk proteins and are associated with mammary ductal differentiation. Although mainly activated with pregnancy and lactogenic stimuli,

residual amounts of caseins have been located on the apical surface of epithelial cells surrounding the ductal lumen in virgin mammary gland sections (Barash et al., 1995). As mentioned previously with *krt18*, also a marker of ductal differentiation, increased differentiation within the mammary gland is generally deemed as being protective. Stimulation of casein gamma would not correspond to the p,p´ DDE-induced tumor formation observed in the tumor study. However, casein gamma, as well as all other caseins, have been found to be expressed in preneoplastic lesions, which are often developed prior to breast cancer and possess the attributes of hyperplastic normal tissue (Smith et al., 1984). Furthermore, when milk protein synthesis was examined in virgin mouse mammary tissue, which had been neoplastically transformed by a viral carcinogen (MMTV) and serially transplanted in gland-free fat pads of virgin mice, expression of casein genes accompanied the transformation (Smith et al., 1984). This finding also corresponds with the findings of Medina and colleagues (1987) who found tumors to have very high levels of casein expression. Still another possible reason casein gamma was up-regulated could be due to continued development of the gland. Postnatal development of the gland begins at puberty and is only completed upon giving birth. However, differentiation is initiated in virgin mice in a limited number of cells with each estrus cycle (Robinson et al., 1995). All animals in the study were sacrificed approximately at the same age in estrus to ensure the same level of hormonal influence. Therefore, the stimulation of both differentiation markers could also be attributed to organogenesis of the gland, thereby affecting development.

At this time, the exact roles of *ifi44*, *krt18* and casein gamma remain unclear in regards to their involvement in breast tumorigenesis. Though immune effects are more definitively associated with cancer formation, the role of mammary ductal differentiation is not as clear. It is possible that the expression of these genes are more of a reflection of earlier events that have already taken place, thus making their regulation more of a consequence of the exposure rather than an initial response to the exposure.

Chapter 5

Local Actions of p,p´ DDE in Mammary Tissue

INTRODUCTION

Two of the genes induced by p,p´ DDE which were identified in the previous chapter, casein gamma (*csn1s2a*) and cytokeratin 18 (*krt18*), are associated with mammary epithelial growth and differentiation. Keratin 18 and casein gamma are markers of luminal mammary epithelial cells (Steinman et al., 2007; Bocker et al., 2009; Barash et al., 1995). The third gene induced by p,p´ DDE action was interferon-induced protein 44 (*ifi44*). Immune dysfunction plays a key role in cancer development and although mechanisms are not fully understood, potential immune dysfunction in cancer does include defects in cytokines like interferons (Wang et al., 2011). As mammary gland development, growth, and differentiation are regulated by androgens, estrogens, and progesterone (Chambo-Dilho et al., 2005; Liao et al., 2002; Conneely et al., 2001; Jaga et al., 2000), the expression of the genes for these hormone receptors were studied in mammary tissue RNA from control and p,p´ DDE-treated MMTV-*neu* mice. In addition, to visualize the ductal tree of the mammary glands from the DDT-01 study mice for effects on mammary gland morphology that could correlate to the increased gene expression by p,p´ DDE , whole mounts were qualitatively examined for evidence of enhanced epithelial tissue and/or differentiation.

In the Tumor study, Davis and colleagues demonstrated that a dose of 5 µg/pellet of p,p´ DDE induces an earlier latency in MMTV-*neu* female mice (Fig. 2). However, how p,p´

DDE influenced the mammary gland is unclear and requires further investigation. To investigate the potential actions of p,p′ DDE in the mammary glands in Central Questions 2-5, DDT-01 and DDT-02 studies were conducted to generate tissues for analyzing Aim 3. For example, the anti-androgenic action of p,p′ DDE (Kelce et al., 1995) was investigated by comparing it to the well-known anti-androgen, hydroxyflutamide (OH-flut). If gene expression similarities are observed for the identified p,p′ DDE -induced genes from Chapter 4, these potential findings could imply p,p′ DDE has anti-androgenic properties in the mouse mammary gland.

The dose of 5 µg/pellet of p,p′ DDE accelerated mammary tumor onset; however, when a reduced dose of p,p′ DDE (3.3 µg/pellet) was co-administered in a 2:1 ratio with o,p′ DDE (1.7 µg /pellet; 5 µg/pellet total), the earlier latency did not occur as it did with 5 µg/pellet p,p′ DDE alone (Fig. 2). The comparable latency between the 2:1 ratio and control groups implied that the reduced dose of p,p′ DDE and/or the ratio of p,p′ DDE with its estrogenic isomer may be the reason for the inability of the 2:1 ratio group to accelerate tumor development. To investigate dose effects on gene expression, additional groups treated with varying doses of p,p′ DDE were generated; expression of the genes identified in Chapter 4 were tested in mammary tissue RNA from these groups. To examine whether o,p′ DDE affected p,p′ DDE actions in the 2:1 ratio, groups treated with 2:1, 5:1, and 10:1 ratios of p,p′ DDE:o,p′ DDE, with varying levels of o,p′ DDE and the 5 µg/pellet p,p′ DDE dose held constant, were generated to remove the potential dose effect of p,p′ DDE .

High levels of p,p´ DDT in young women were associated with increased breast cancer risk before age 50 (Cohn et al., 2007). These results suggest similar effects as observed with p,p´ DDE inducing early-onset mammary cancer in MMTV-*neu* mice; these potential similarities may be detected by examining expression of the p,p´ DDE -induced genes. Therefore, additional animals were treated and tested for gene expression changes between the p,p´ DDE, p,p´ DDT, and control groups. In addition, since p,p´ DDT is co-detected with p,p´ DDE in the serum/plasma and adipose tissue in women and o,p´ DDE ratio resulted in unexpected effects, a combination of p,p´ DDE and p,p´ DDT (1:1 ratio, 2.5 μg each) was also generated for examining expression of the three identified markers. Accordingly, additional groups of MMTV-*neu* mice were treated for investigating gene expression in order to address Central Questions 2, 3, 4, and 5 for local effects in the mammary gland outlined in Aim 3.

METHODS

Whole Mount Fixation and Staining

Mammary gland whole mounts allow for visualization of the entire gland structure, which can then be qualitatively or quantitatively assessed. Carmine alum stain was prepared by first adding 1 g of Carmine powder and 2.5 g of aluminum potassium sulfate to 250 mL of dH_2O in an Erlenmeyer flask. The combining of these reagents was followed by the addition of another 250 mL of dH_2O. The mixture was allowed to boil on a hotplate for 20 minutes and then the final volume adjusted to 500 mL. After the volume was adjusted, the solution was filtered twice using a mild vacuum and stored at 4°C with a pinch of thymol used as a preservative.

After mammary glands that were fixed overnight in 4% p-formaldehyde and stored in 70% ethanol were carefully trimmed away from the skin they were placed flat in a round tissue capsule (labeled with pencil) in order to maintain integrity and shape. On the first day of staining, mammary tissues were washed in a large beaker with a stir bar containing 70% EtOH for 15 minutes, followed by a 5 minute rinse in dH_2O. Tissue capsules were then placed in the Carmine Alum stain overnight at 4° C. On the second day, tissues were removed from the stain and washed in a large beaker of rapidly stirring 70% EtOH for 15 minutes. Next, tissues were washed in 95% EtOH followed by a 100% EtOH wash, both for 15 minutes. The last wash was prolonged if needed until all stain had been removed. Once all stain was removed, tissue capsules were transferred into toluene and spun for 30-40 minutes in order to remove the fat. Once the tissues appeared translucent, they were removed from the toluene and the glands transferred into scintillation vials filled with methyl salicylate for storage. Whole mounts were viewed using a Nikon stereoscopic microscope with an Olympus DP70 microscope digital camera. Whole mount digital images were captured using DP controller software using a blue filter background for optimal viewing quality.

Chapter 2 includes the methods on total RNA isolation, cDNA reverse transcriptase reactions, real time RT-PCR, relative gene expression and statistical analysis that apply to the studies within this chapter.

RESULTS

Hormone Responsiveness in the Mammary Gland with Localized p,p´ DDE Exposure

To examine whether local exposure to the anti-androgen p,p´ DDE had any effects on the hormone receptors that regulate hormone responsiveness in the mammary gland, expression of the genes for estrogen receptor alpha (*esr1*), G protein-coupled receptor 30 (*gpr30*), androgen receptor (*ar*), aryl hydrocarbon receptor (*ahr*) and progesterone receptor (*pgr),* were analyzed by real-time RT-PCR in animals from the DDT-01 study. In addition, the subtypes of the progesterone receptor (PR) were examined using primer sets that differentiate between the two receptor subtypes coded for the *prg* (progesterone receptor) gene, progesterone receptor-A (PRA) and progesterone receptor-B (PRB). Furthermore, to investigate whether a gene involved in mammary proliferation and cancer development and which is also influenced by *neu* (Taneja et al., 2009) was affected as a function of p,p´ DDE action, cyclin D1 (*ccnd1*) expression was also evaluated (Fig. 36). The 5 µg /pellet dose of p,p´ DDE did not significantly modulate the expression of any of the tested markers. However, the relative expression differences with p,p´ DDE treatment were slightly higher than the 1-fold for the control mice, 1.6-fold for *esr1,* 1.3-fold for *gpr30,* 1.2-fold for *ar,* 1.6-fold for *ahr,* 1.7-fold for *prg (includes both subtypes, PRA and PRB)* , 2.5-fold for the message for PRA, 2.5-fold for the message for PRB, and 1.7-fold for *ccnd1*.

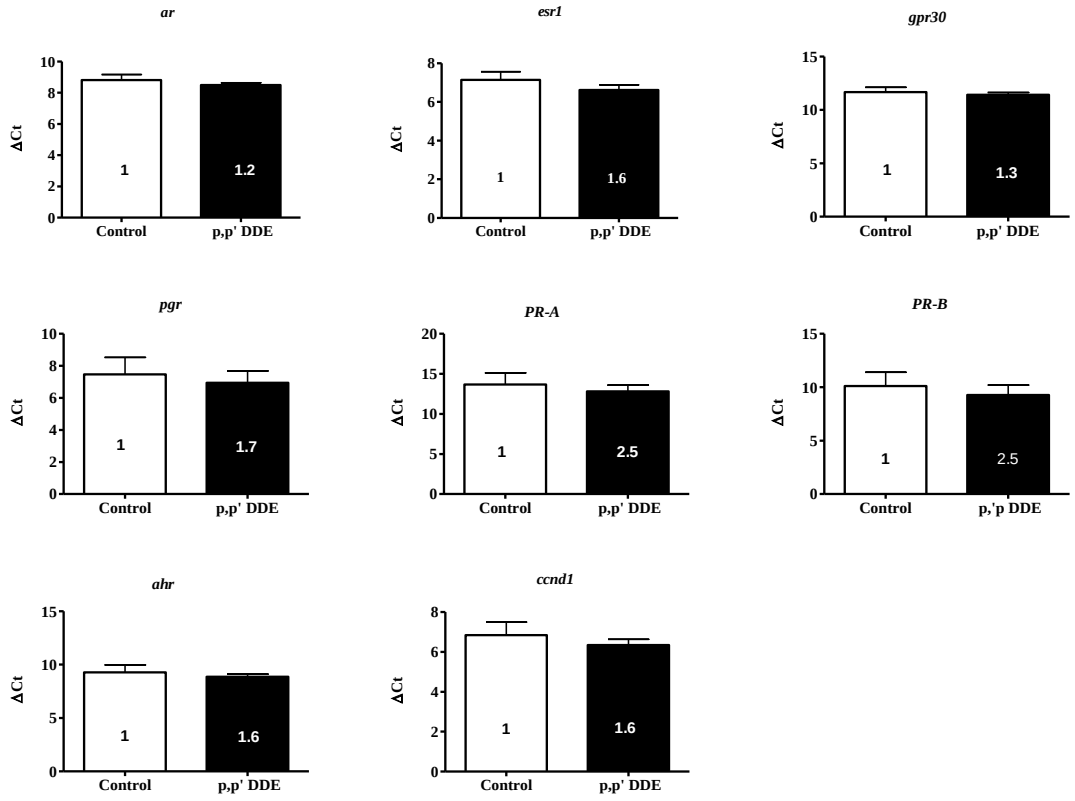


Figure 36. p,p´ DDE does not induce significant changes in hormone receptor gene expression in mammary tissue. Expression of hormone markers *esr1, ar, gpr30, ahr, pgr (all PR messages), subtypes PR-A and PR-B,* as well as a gene involved in mammary proliferation, *ccnd1*, were measured by real-time RT-PCR with SYBR-Green detection. No significant difference was observed among any marker with 5 μg/pellet treatment of p,p´ DDE, however fold-changes of all genes are slightly higher that the control group. ΔCt is the normalized level of the gene of interest compared to *ppia* (cyclophilin A). A higher ΔCt is indicative of the gene being expressed at lower level. The numbers within each bar represent the fold change compared to control using the $2^{-\Delta\Delta Ct}$ method. The Mann-Whitney test was used for statistical analysis, all genes had p values >0.05. n=6.

Treatment Effects on Mammary Gland Morphology

Since two of the p,p´ DDE-regulated genes (*csn1s2a* and *krt18*) are markers of ductal differentiation, digital images of mammary gland whole mounts were examined (Fig. 37). These images allowed the observation of potential morphological changes that may have occurred within the gland with treatment. Whole mounts from the nine treatment groups in the DDT-01 study were prepared and stained to allow visualization of the ductal tree

within the inguinal mammary glands. Possible effects of steroid hormone treatment on mammary gland morphology may include changes in mammary gland arborization, terminal end bud number, length and alveolar density (Fielden et al., 2002). Development of the mammary gland occurs in two major phases, hormone-independent and hormone-dependent. The latter occurs at puberty and results in ductal elongation and side branching, as a consequence of estrogen and progesterone, respectively (Brisken and O'Malley (2010). Therefore, anti-androgen influence by p,p´ DDE in the gland has the potential to also impact glandular development. Qualitatively, within each group, variations were observed such as the amount of fat pad occupied by the gland, secondary and tertiary branching and differences in growth patterns in relation to the ELVAX 40P pellet. For example, arrest of ductal growth beyond the pellet was observed with the highest concentration of p,p´ DDE, leaving the remainder of the mammary fat pad empty (Fig. 37E). However, this effect also occurred in some animals in other groups and may be due to the surgical procedure (such as scarring) or other unknown effects. Additionally, it was observed that some glands grew over the pellet (Fig. 37G), while others appeared to avoid it (Fig. 37I). Possible surgery effects could only potentially damage the adipose tissue and not the ducts themselves since pellet implantation occurred within the mammary fatpad prior to glandular development. Despite these observations, no consistent changes were apparent in the ductal structures among the groups to report them as a treatment effects; thus, quantitative analysis was not performed. Considering some of these genes were significantly stimulated by p,p´ DDE, the lack of noticeable morphological differences between the groups may suggest that the age of almost 3 months, when the animal is a mature adult and mammary gland development is

essentially complete, was too late to detect gross changes in gland morphology. These data suggest that further investigations into the impact of p,p´ DDE on mammary gland morphology is warranted except that glands should be examined at earlier time points during ductal development.

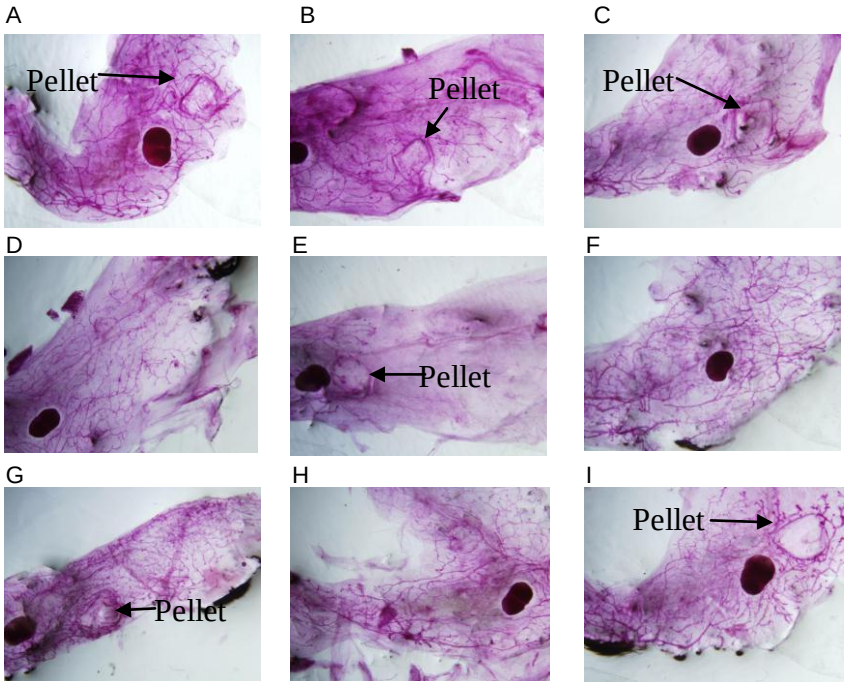


Figure 37. No apparent morphological changes were observed in the mammary glands. Qualitatively, no structural changes were observed in any treatment group; A) Control; B) 5 µg p,p´ DDE; C) 0.5 µg p,p´ DDE ; D) 2.0 µg p,p´ DDE; E) 10 µg p,p´ DDE; F) 2.5 o,p´ DDE ; G) 2:1 ratio; H) 5:1 ratio; I) 10:1 ratio. However, at the highest concentration, 10 µg p,p´ DDE, the ducts did not grow beyond the pellet (E). Whole mounts were viewed using a Nikon stereoscopic microscope with an Olympus DP70 microscope digital camera. Digital images were captured using DP controller software using a blue filter background for optimal viewing quality. Pictures above are representative images of all pictures in the group. Control n=4; 5 µg p,p´ DDE n=4; 0.5 µg p,p´ DDE n=5; 2.0 µg p,p´ DDE n=2; 10 µg p,p´ DDE n=2; 2.5 µg o,p´ DDE n=6; 10:1 n=5; 5:1 n=7; 2:1 n=6.

DDT-01 and -02 Studies Treatment Groups in MMTV-neu Mice

To test the actions of p,p´ DDE, DDT-01 study was conducted to investigate the effects caused by p,p´ DDE alone, at varying doses, and in combination with its estrogenic isomer, o,p´ DDE on the identified markers in Chapter 4. New treatment groups were created (Table 19) in addition to p,p´ DDE and o,p´ DDE alone at 5 µg/pellet, varying ratios of p,p´ DDE :o,p´ DDE (2:1, 5:1, 10:1) were tested. However, in these ratios, p,p´ DDE was held constant at 5 µg/pellet and only the o,p´ DDE concentration was modified. Gene expressions of the identified markers were also evaluated in varying doses of p,p´ DDE in order to gain a better understanding of its action.

Table 19. DDT-01 Study Treatment Groups

DDT-01 Study (Ratios)

Treatment n=10	Control	p,p´ DDE	o,p´ DDE	p,p´ DDE + o,p´ DDE (2:1)	p,p´ DDE + o,p´ DDE (5:1)	p,p´ DDE + o,p´ DDE (10:1)
p,p´ DDE o,p´ DDE (Doses per pellet)	0	5 µg	0 µg + 2.5 µg	5 µg + 2.5 µg	5 µg + 1.0 µg	5 µg + 0.5 µg

DDT-01 Study (Doses)

Treatment n=10	p,p´ DDE	p,p´ DDE	p,p´ DDE	OH-flut	OH-flut
Dose (per pellet)	0.5 µg	2.0 µg	10 µg	5 µg	2.5 µg

The DDT-02 study was conducted in order to gain a better understanding of the similarities and/or differences between p,p´ DDE and p,p´ DDT action. Identical treatment methods to deliver the treatment, time of exposure, age, and animal model were used for study DDT-02 treatment groups (Table 20). However, due to the information gained from the previous two studies (Pretumor and DDT-01), the lymph node was visually identified at necropsy and the mammary gland sectioned according to Figure 24.

Table 20. DDT-02 Study Treatment Groups

DDT-02 Study

Treatment (Doses per pellet) n=12	Control	p,p´ DDE	p,p´ DDE	p,p´ DDT	p,p´ DDT	p,p´ DDE + p,p´ DDT	OH-flut	OH-flut
p,p´ DDE	0	5 µg	2.5 µg			2.5 µg +		
p,p´ DDT				5 µg	2.5 µg	2.5 µg		
OH-flut							5 µg	2.5 µg

Gene Expression in Mammary Tissue of DDT-01 and -02 Studies

Using mammary tissue obtained from the two studies, real-time RT-PCR analysis of gene expression was performed on the three identified markers (*krt18, ifi44 and csn1s2a*) in Chapter 4. The three genes were chosen to be significantly modified by p,p´ DDE exposure using animals in the control and p,p´ DDE groups from both DDT-01 and -02 studies. Therefore, these genes can be tested in the different treatment groups in both studies to address all aspects of Aim 3. First, to examine the potential anti-androgenic

actions of p,p´ DDE in mammary tissue *in vivo* after chronic exposure (2 months), the 5 µg/pellet dose of p,p´ DDE was compared to the same dose of the known androgen antagonist, hydroxyflutamide (OH-flut). Next, to test the impact of additional doses of p,p´ DDE besides 5 µg/pellet, gene expression induced by a tenth (0.5), half (2.5), and double (10) the 5 µg dose were also tested. To examine the potential effects of p,p´ DDE when co-administered with its estrogenic isomer, o,p´DDE , ratios of p,p´ DDE with o,p´ DDE were investigated, and lastly, comparisons of p,p´ DDE to p,p´ DDT were also studied in order to identify similarities and differences in action between the two isomers. These studies will address Central Questions 2-5 for local effects of p,p´ DDE actions in the mammary gland, including as an androgen antagonist, dose effects, similarities and differences to p,p´ DDT, and effects of mixtures with the two DDT congeners, o,p´ DDE and p,p´ DDT.

Anti-androgenic Action of p,p´ DDE

The role of androgens within the breast has not been clearly defined, however, the consensus among most reports show androgens to be antiproliferative and to counteract estradiol stimulation of the mammary epithelial cells (Zhou et al., 2000; Leung et al., 2002; Suzuki et al., 2010). Additionally, 70-90% of primary breast tumors express the androgen receptor. Based on the results in the MMTV-*neu* mice, it has been verified that p,p´ DDE influences mammary carcinogenesis, but whether these effects are related to its reported anti-androgenic properties is unknown and there are very few studies that have investigated the modulation of gene expression by androgens in mouse mammary tissue (Aboghe et al., 2008). Androgen receptor antagonists compete with endogenous

androgens acting on the androgen receptor to prevent down-stream activation of androgen-dependent gene expression (Wilson et al., 2008). However, without a set of known genes regulated by androgens or anti-androgens in the mammary gland, the potential anti-androgenic actions of p,p´ DDE in mammary tissue must be tested in other ways. Therefore, in this study, the expression of the identified p,p´ DDE-induced genes was compared to OH-flut to determine if both treatments result in similar effects.

In order to investigate the anti-androgenic actions of p,p´ DDE, the modulation of the three candidate genes (*csn1s2a, ifi44, krt18*) by p,p´ DDE was compared to mice treated with the androgen receptor antagonist, OH-flut. It is hypothesized that if p,p´ DDE is behaving as an anti-androgen in the mammary gland, then the effect of 5 µg/pellet p,p´ DDE on *csn1s2a, ifi44,* and *krt18* should mimic those of 5 µg/pellet OH-flut. Even though significance was not reached, the pattern of regulation between OH-flut and p,p´ DDE was similar (Fig. 38). For *csn1s2a*, both p,p´ DDE and OH-flut increased expression, having fold changes of 13.6 and 5.0, respectively. However, among both groups compared to control, only p,p´ DDE treatment was significant ($p < 0.003$, one-way ANOVA). Epithelial marker *krt18* resulted in a non-significant stimulation by 5µg/pellet doses of p,p´ DDE and OH-flut, having similar fold changes of 1.4 and 1.3, respectively. However, p,p´ DDE expression was significant when compared to control ($p < 0.02$, Mann Whitney test), OH-flut approached significance ($p=0.065$, Mann Whitney test), and the two anti-androgens were not significantly different when compared to each other. Lastly, immune-related marker *ifi44* was not significantly modulated by p,p´ DDE or OH-flut (Fig. 38), but both induced an increase in expression, with fold changes of 1.9

and 1.4, respectively. Taken together, the common direction in expression of p,p´ DDE and OH-flut suggest both have a similar effect on the genes, possibly as anti-androgens, though the OH-flut effect was not as strong as p,p´ DDE.

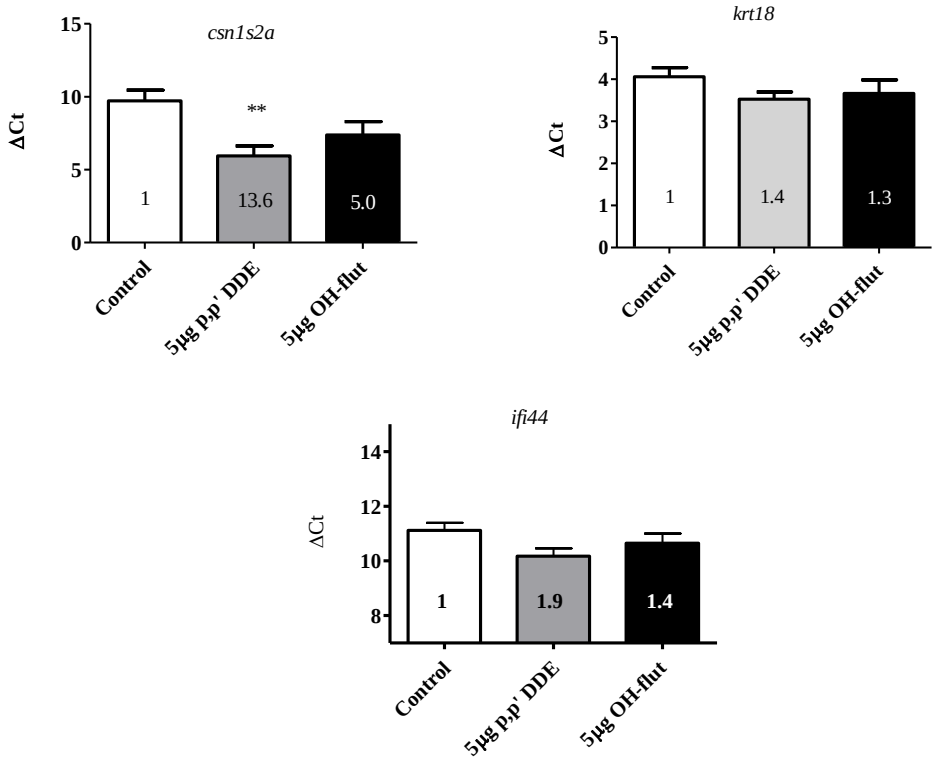


Figure 38. p,p´ DDE treatment showed similar trends in expression in *csn1s2a*, *krt18*, and *ifi44* when compared to OH-flut at an equivalent dose. Real-time RT-PCR demonstrated both anti-androgens evoked a common response in gene expression at the same concentration per pellet. *csn1s2a* expression was significant ($p < 0.003$, one-way ANOVA) and retained its significance for p,p´ DDE (** by Tukey's test), but not for hydroxyflutamide (OH-flut) compared to control. The p,p´ DDE and OH-flut groups were not significantly different than each other by Tukey's test, suggesting they share a similar increase in expression . The gene *krt18* was also stimulated, though not significantly by either p,p´ DDE or OH-flut ($p > 0.05$, 1 way-ANOVA; no groups significant by Tukey's test). Expression trends were similar in immune related *ifi44* as both anti-androgen treatments induced non-significant increases in expression compared to control ($p > 0.05$, one- way ANOVA; no groups significant by Tukey's test). The numbers within each bar represent fold change compared to control. ΔCt is the normalized expression of the target gene compared to the housekeeper gene, *ppia* (cyclophilin A). A lower ΔCt signifies the gene is expressed at a higher level. One-way ANOVA was used for statistical analysis ($p<0.05$), with Tukey's post analysis. Control and p,p´ DDE groups, n=13; OH-flut group, n=6. ** indicates level of significance by Tukey's test ($p<0.01$).

Dose Effects of p,p´ DDE

Previously, it was shown that p,p´ DDE treatment (5 µg/pellet, 4 pellets/mouse, 20 µg total dose) decreased tumor latency. In this investigation, the effects of p,p´ DDE dose were examined on *csn1s2a*, *ifi44,* and *krt18* gene expression. To test dose affects, varying doses of p,p´ DDE (0.5, 2.5, 5.0, and 10 µg/pellet) were delivered to the mammary glands as previously described. The results from this study will help to determine which doses of p,p´ DDE maximally and minimally modulate the three identified genes within the mammary gland.

To evaluate the dose effects of p,p´ DDE, the results of varying dosages of p,p´ DDE on gene expression of *csn1s2a*, *krt18,* and *ifi44* were measured in the mammary tissue by real-time RT-PCR. In Figure 39, the 5 µg/pellet dose of p,p´ DDE elicited the strongest stimulation of all three genes in comparison to both the higher (10 µg) and lower (0.5 µg, 2.5 µg) doses. Unexpectedly, the lower doses of p,p´ DDE either significantly down-regulated gene expression (*krt18*) or was without effect (*csn1s2a and ifi44)* (Fig. 39). At the highest dose tested, p,p´ DDE was without effect on *csn1s2a*, *krt18*, and *ifi44* gene expression when compared to control, but was significantly attenuated when compared to the gene expression induced by 5 µg/pellet p,p´ DDE (*krt18 and ifi44*). Results also demonstrate the 2.5 µg concentration of p,p´ DDE was significantly different than the 5 µg concentration for both *krt18* and *ifi44*. Collectively, these data show that the dose of p,p´ DDE differentially modulated the expression of *csn1s2a*, *ifi44,* and krt18 with the 5 µg/pellet p,p´ DDE being most influential, which is the dose that accelerated tumor onset.

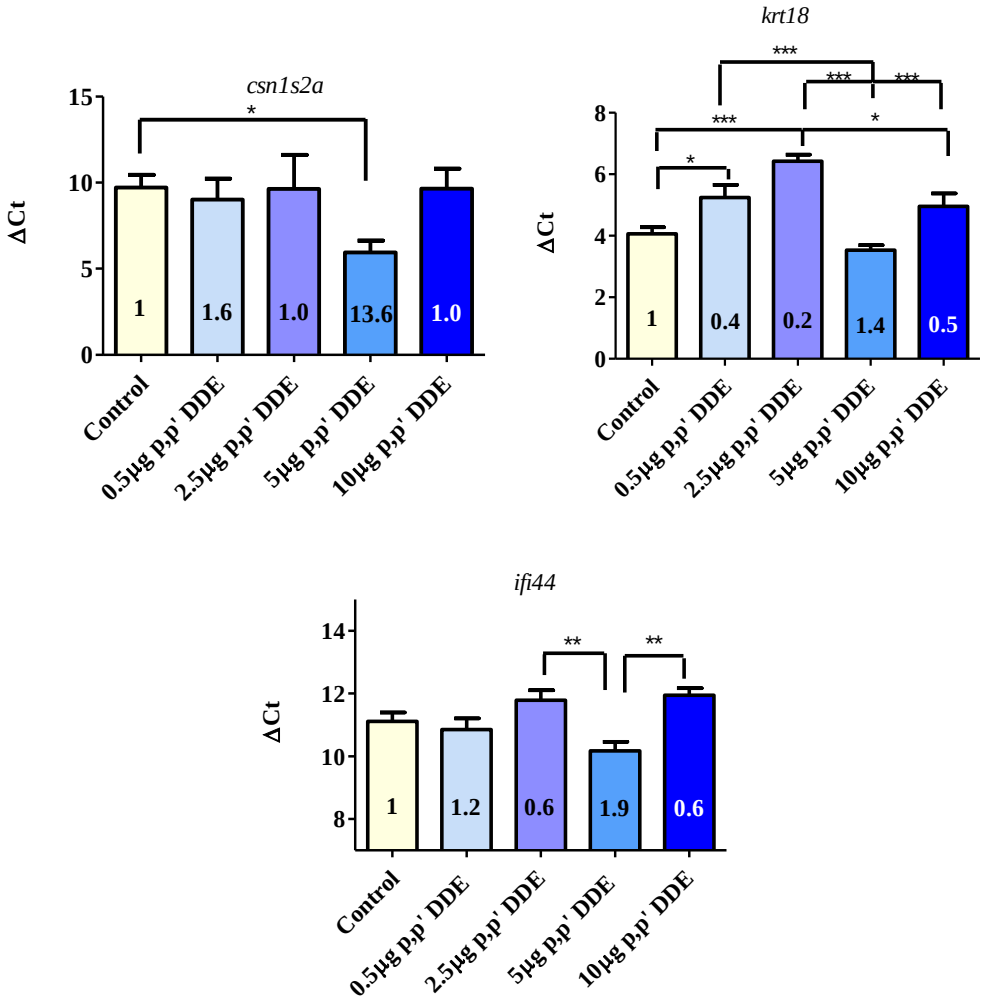


Figure 39. The 5 µg/pellet dose of p,p´ DDE evoked the strongest stimulation in expression for all the genes. Investigation of the potential doses of p,p´ DDE that modulated expression of marker genes by real-time RT-PCR revealed 5 µg/pellet increased expression of *csn1s2a, krt18, and ifi44* (markers of p,p´ DDE action). Among all treatment groups only the 5 µg/pellet dose of p,p´ DDE induced a significant change ($p<0.02$, one-way ANOVA) in expression in *csn1s2a*. Doses below 5 µg/pellet in *krt18* were significantly repressed compared to the control and the 5 µg/pellet group, while the repression induced by the highest dose was significantly different from both the repression of 2.5 µg p,p´ DDE and the stimulated expression of 5 µg/pellet p,p´ DDE. Significance $p<0.0001$, one-way ANOVA, with significance between the groups noted with the connecting lines by Tukey's test. Additionally, significant expression changes for *ifi44* ($p=0.002$, one-way ANOVA) occurred. Specifically, the 2.5 and 10µg/pellet

doses compared to the stimulated 5 µg/pellet dose. The lowest dose of p,p´ DDE in *ifi44* also induced non-significant stimulation; similar to what occurred in *csn1s2a*. ΔCt is the normalized gene expression compared to the housekeeper gene *ppia* (cyclophilin A). A higher ΔCt means the transcript is in low abundance. Numbers within each bar represent the fold change compared to control. One-way ANOVA used for statistical analysis (*p<0.05), with Tukey's post analysis. **p<0.01 and ***p<0.001 = level of significance as determined by Tukey's test, n=6-13. Significant comparisons are only between the closet hatch mark and the next when in a continuous horizontal line.

p,p´ DDE and o,p´ DDE Mixtures

The dose of p,p´ DDE that resulted in earlier tumor development was 5 µg. The 2:1 ratio of p,p´ DDE:o,p´ DDE did not induce the early tumor latency; however, in the Tumor study, the dose of p,p´ DDE in the mixture was 3.3 µg in each pellet and not 5 µg. Therefore, it is unknown whether the lower dose of p,p´ DDE and/or the inclusion of o,p´ DDE were responsible for the lack of effect on tumor latency for the 2:1 ratio, unlike p,p´ DDE (5 µg/pellet) alone. The dose was previously tested (Fig. 39), so the goal of these experiments were to test the ratios of p,p´ DDE with o,p´ DDE. To test whether or not mixtures of p,p´ DDE and o,p´ DDE influenced gene expression by removing the potential dose effects of p,p´ DDE, varying doses of o,p´ DDE were tested in the ratios with the concentration of p,p´ DDE held constant at the 5 µg/pellet dose that accelerated tumor onset. Specifically, three different ratios of p,p´ DDE:o,p´ DDE (2:1, 5:1, 10:1) were tested; the 2:1 ratio included 2.5 µg o,p´ DDE for at total of 7.5 µg , the 5:1 ratio included 1.0 µg o,p´ DDE for at total of 6.0 µg, and the 10:1 ratio included 0.5 µg o,p´ DDE for at total of 5.5 µg in each pellet. If o,p´ DDE modifies p,p´ DDE action, then the expression of *csn1s2a*, *ifi44*, and *krt18* genes would not resemble the 5 µg/pellet dose of p,p´ DDE alone in groups with higher doses of o,p´ DDE. If the dose of p,p´

DDE was the most important, it could be hypothesized little variation in expression of these genes would be evident for all three ratios compared to the 5 µg p,p´ DDE group.

As shown in Figure 40, the 2:1 ratio resulted in similar stimulated expression levels for all three genes compared to p,p´ DDE alone (5 µg); but, expression was reduced by o,p´ DDE alone again in all three genes compared to control, although only significantly for *krt18*. For *csn1s2a*, the 5 µg/pellet concentration of p,p´ DDE induce expression that was significantly different than its estrogenic isomer, o,p´ DDE. This trend was also demonstrated in *krt18,* where the 2:1 ratio was also significantly different than its individual components, as was the case for *csn1s2a* as well. The gene *ifi44* was not significantly affected. These results suggest that the dose and not the combination with o,p´ DDE affect expression of these three genes in mammary tissue. However, the 5:1 and 10:1 ratios do not result in similar expression patterns compared to the p,p´ DDE group alone. Since there are no groups tested with the lower doses of o,p´ DDE alone (0.5 and 1µg), these findings are difficult to interpret, but may suggest some influence of the estrogenic o,p´ DDE isomer at the lower doses.

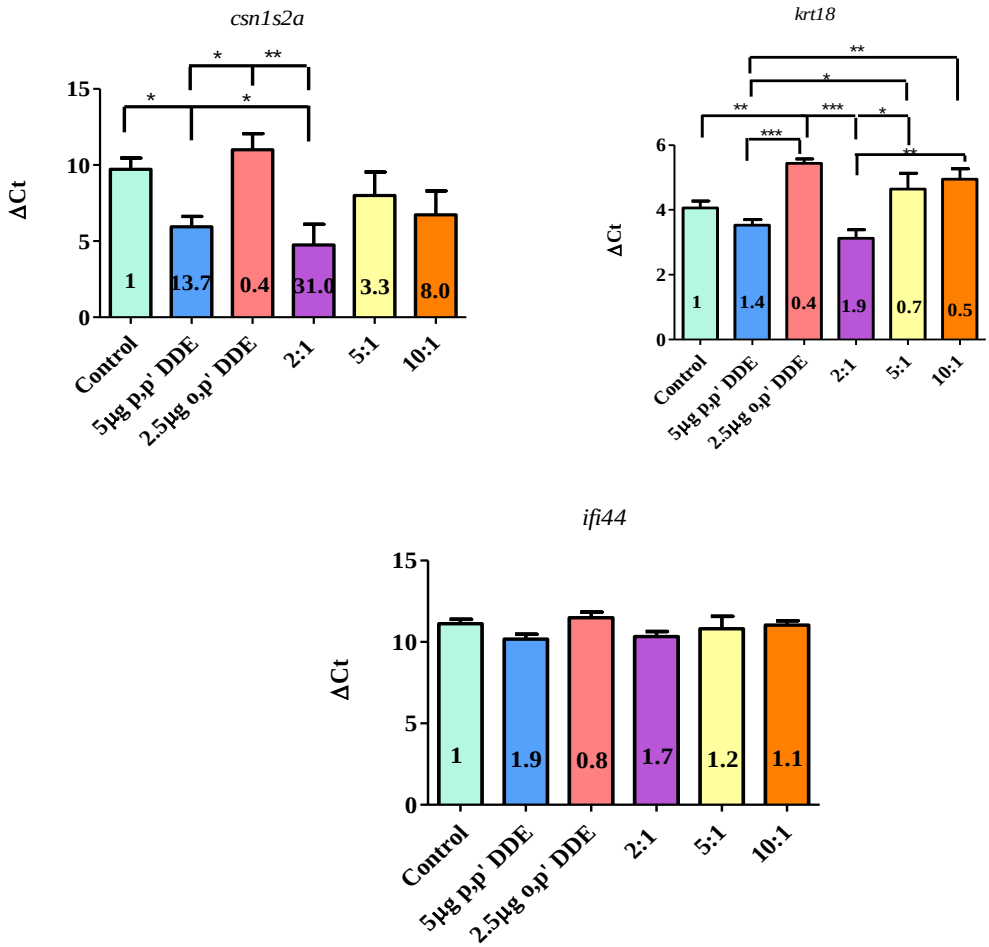


Figure 40. Ratios of p,p´ DDE to o,p´ DDE influence expression of *csn1s2a* and *krt18* in the mammary gland of MMTV-*neu* mice. Investigation into varying ratios of p,p´ DDE to o,p´ DDE reveal by real-time RT-PCR that both *csn1s2a* and *krt18* expression was significantly altered. For *csn1s2a*, the groups for the individual isomers and the ratios are significant (p=0.0013, one-way ANOVA). The 5 µg p,p´ DDE group was significantly stimulated compared to control and gene expression induced by the 2:1 ratio was significantly different than either of its components (Tukey's test). Similarly, *krt18*, repression caused by the o,p´DDE group was significantly different from the 2:1 ratio, the 5 µg group and control. Additionally, the 5:1 and 10:1 ratios were significantly different from both the 2:1 ratio and control group (Tukey's test). Overall significance for krt18 is p<0.0001,one-way ANOVA . Expression of *ifi44* was unaffected (p>0.05,one-way ANOVA). ΔCt is the normalized expression of gene compared to *ppia* (cyclophilin A). Numbers within each bar represent fold change compared to control. One-way ANOVA and Tukey's post analysis used for statistical analysis (p<0.05). *p<0.05, **p<0.01, ***p<0.001 = level of significance by Tukey's test, n=6-13. Significant comparisons are only between the closet hatch mark and the next when in a continuous horizontal line.

p,p´ DDT, Alone and Combined with p,p´ DDE

Davis and colleagues have previously shown p,p´ DDE is capable of causing early mammary tumor formation. Cohn and colleagues (2007) reported more women exposed to higher levels of p,p´ DDT before age 14 developed breast cancer before age 50. Their study was the first to investigate DDT and breast cancer risk in terms of the significance of age at exposure. In addition, p,p´ DDT is reported to have anti-androgenic activity, though weaker than p,p´ DDE (Kelce et al., 1995). Furthermore, p,p´ DDT is the primary constituent of commercial grade DDT (Cohn et al., 2007); therefore, it is likely to also be present in human tissue along with p,p´ DDE.

To investigate whether or not p,p´ DDE and p,p´ DDT similarly modulate *csn1s2a*, *ifi44*, and *krt18*, animals were exposed to two doses of either p,p´ DDE (2.5 µg and 5 µg) or p,p´ DDT (2.5 µg or 5 µg) or to a combination (1:1 ratio) of the two (2.5 µg p,p´ DDT:2.5 µg p,p´ DDE, total 5 µg in each pellet) (Fig. 41). The 5 µg dose of p,p´ DDT was included to match the dose of p,p´ DDE demonstrated to cause early tumor formation in the mammary gland by Davis and colleagues. In addition, every animal study has some variation and the goal was to compare expression of the identified markers to this dose of p,p´ DDE, this treatment as well as the control (vehicle pellets) were included in both studies. A 1:1 ratio of p,p´ DDE and p,p´ DDT was also tested since neither of these congeners exist alone in the environment or *in vivo* and, therefore, may influence each other. Since both congeners have previously been linked to breast cancer (Wolff et al. 1993; Charlier 2003; Cohn et al., 2007) and p,p´ DDE accelerated mammary cancer in MMTV-*neu* mice, comparison of these congeners alone and together on expression of the

p,p´ DDE-induced markers may suggest if existing mixtures act similarly or not in women exposed to DDT.

Expression of the *csn1s2a* gene induced by 5 and 2.5 µg of p,p´ DDE and p,p´ DDT or a 1:1 ratio (2.5 µg p,p´ DDE: 2.5 µg p,p´ DDT) was minimally affected across most groups; with only 5µg p,p´ DDE being significantly different than 2.5 µg of p,p´ DDT ($p=0.0031$, one-way ANOVA). However, when p,p´ DDE (5 µg) and p,p´ DDT (5 µg) were compared to each other individually , their difference in expression approached significance ($p= 0.05$, Mann-Whitney test). The 2.5 µg/pellet concentration of p,p´ DDT was significantly different than the 1:1 ratio of p,p´ DDE : p,p´ DDT ($p =0.0043$, Mann-Whitney test) , containing the same concentration of both congeners (2.5 µg/pellet), producing fold changes of 0.1 and 4.1, respectively.

Interestingly, gene expression of *krt18* among the different p,p´ DDE and p,p´ DDT treatment groups was very similar, despite the 5 µg/pellet concentrations of p,p´ DDE and p,p´ DDT approaching significance ($p= 0.065$, Mann-Whitney test) and having fold changes of 1.4 and 1.0, respectively. They were also both significantly different from their half (2.5 µg) concentrations. At the half concentration (2.5 µg/pellet), p,p´ DDE and p,p´ DDT expression was identical. Not only were both significantly different from control, but they were also not significantly different from one another and had identical fold changes of 0.2. Furthermore, 2.5 µg/pellet of p,p´ DDE and p,p´ DDT were also both significantly different from 5 µg/pellet of p,p´ DDE and p,p´ DDT. Also, both 2.5 µg/pellet concentrations of p,p´ DDE and p,p´ DDT were significantly different from the

1:1 ratio, although it comprised the individual doses. When p,p′ DDE and p,p′ DDT were placed in a 1:1 combination (2.5 µg each), they no longer elicited expression as they did alone, as demonstrated by the 0.2-fold change of the individual isomers compared to the co-administration concentration 1.2-fold change.

The expression of *ifi44* was similar to *krt18* with the exception of the 5 µg/pellet concentration of p,p′ DDE, which was significantly different than the 5 µg concentration of p,p′ DDT, having fold changes of 1.9 and 0.6, respectively. Additionally, 5 µg p,p′ DDE-induced expression was significantly different from both 2.5 µg concentrations. Furthermore, also comparable to *krt18*, the 2.5 µg/pellet concentration of p,p′ DDT was significantly different from the 1:1 ratio although it contained the same concentration of p,p′ DDT.

Overall, these trends suggest p,p′ DDE and p,p′ DDT have both similar and dissimilar actions amongst themselves and between each other. Furthermore, when they are combined, they induce an opposite response to what is induced by each individually. These results revealed that the presence of one isomer influences the other.

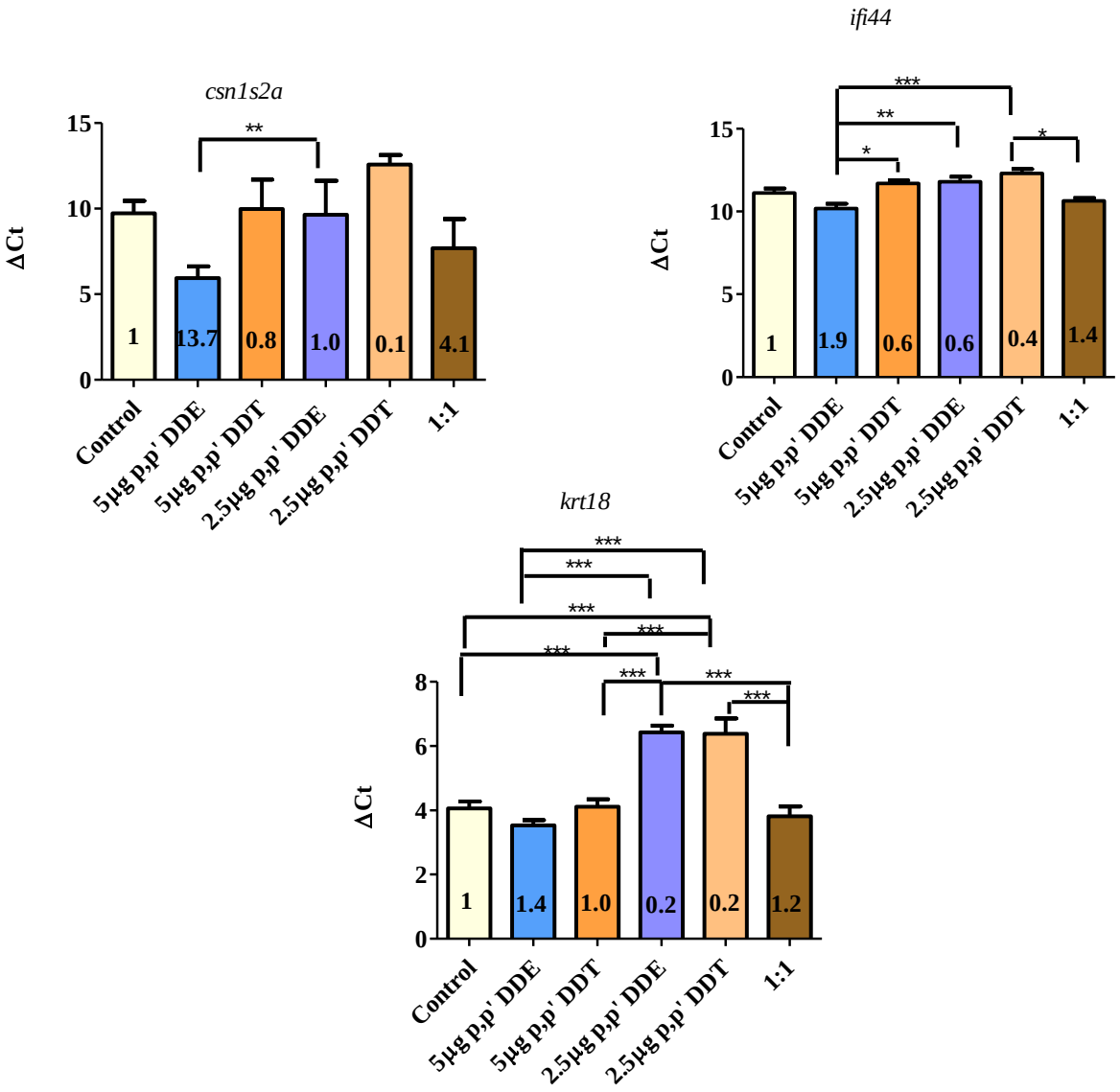


Figure 41. p,p´ DDE and p,p´ DDT induce similar and dissimilar expression in genes regulated by p,p´ DDE. Using specific primers for *csn1s2a*, *ifi44* and *krt18*, induced expression by p,p´ DDE, p,p´ DDT and a 1:1 combination of both was measured by real-time RT-PCR. For *csn1s2a*, among all groups (p=0.0031, one-way ANOVA), only the 2.5 µg/pellet of p,p´ DDT was significantly different from the 5 µg/pellet dose of p,p´ DDE by Tukey's test, however, this distinction was not significant for the comparisons being investigated . *Ifi44* expression was also significantly modified (p<0.0001, 1-way ANOVA). p,p´ DDE and p,p´ DDT were significantly different at the 5 µg/pellet dose (Tukey's test). Additionally, both half doses of 2.5 µg/pellet were also

significantly repressed compared to the stimulation induced by 5 µg/pellet dose of p,p′ DDE (Tukey's test). Lastly, *ifi44* expression at the 2.5 µg concentration of p,p′ DDT was significantly different from the 1:1 ratio group consisting of the same concentration test. expression of k*rt18* was also significantly modulated by the treatment groups ($p<0.0001$, one-way ANOVA). Both 5 µg/pellet doses of p,p′ DDE and p,p′ DDT were significantly different from their half concentrations (2.5 µg), which were also significantly repressed compared to control (Tukey's test). Additionally, the 2.5 µg/pellet concentrations were both repressed by p,p′ DDE and p,p′ DDT but induced significant stimulation when co-delivered using the same concentrations (Tukey's test) . ΔCt reflects how strongly or weakly the gene is expressed. A low ΔCt indicates the gene is highly expressed and is the normalize level of the gene compared to the housekeeper gene *ppia* (cyclophilin A). The numbers within each bar represent the fold change compared to control. One-way ANOVA and Tukey's tests were used for statistical analysis ($p<0.05$). n=13 (control and 5 µg p,p′ DDE); n=6 (5 µg p,p′ DDT, 2.5 µg p,p′ DDE and p,p′ DDT and 1:1 ratio).*$p<0.05$, **$p<0.01$, ***$p<0.001$ = level of significance by Tukey's test. Significant comparisons are only between the closet hatch mark and the next when in a continuous horizontal line.

DISCUSSION

An effect of p,p´ DDE, besides its reported androgen receptor binding and inhibition of androgen-regulated genes, may be to modify hormone responsiveness of the mammary gland. One way to modify its responsiveness would be to alter expression levels of the hormone receptors involved in modulating responses important in proliferation, differentiation, and neoplastic formation. Therefore, gene expression for the androgen receptor (*ar),* estrogen receptors (*esr1* and *gpr30),* progesterone receptor (*pgr)* and its subtypes PRA and PRB, cyclin D1 (*ccnd1*) and the aryl hydrocarbon receptor (*ahr)* were investigated. The gene for e*sr2* (estrogen receptor beta) was not tested because it was not detected in other studies. Previous studies show p,p´ DDE binds to the androgen receptor, binds poorly to the estrogen receptor (Kelce et al., 1995), has relatively low binding affinity for GPR30 (Thomas and Dong 2006), acts as a progesterone receptor antagonist (Li et al., 2008), and binds to the aryl hydrocarbon receptor (Long et al., 2006). In this study, p,p´ DDE did not significantly modify the expression of any of these genes.

The lack of changes in expression of these genes by p,p´ DDE does not mean these receptor levels are or are not modified. Since investigations in this study were on their transcript levels, the measured expression of these genes may not reflect their protein levels or activity. Therefore, no obvious changes in receptor gene expression suggest hormone levels are unchanged. The presence of these receptors in mammary tissue suggests the receptors are available for binding to p,p´ DDE or to induce responses from other hormones. But, if the mRNA levels reflect the receptor levels, the gland would not

be more or less responsive to these hormones or DDT congeners. However, other p,p´ DDE-induced changes could occur to affect hormone responsiveness in the mammary gland, such as by modifying androgen, estrogen, and progesterone synthesis or metabolism.

Although DDT has been shown to increase cyclin D1 protein synthesis in MCF-7 breast cancer cells (Dees et al., 1997), p,p´ DDE treatment in <3-month-old female MMTV-*neu* mice did not significantly alter cyclin D1 (*ccnd1*) gene expression. These results are in agreement with Silva and colleagues (2010) who found that p,p´ DDE is a fairly weak transcriptional inducer of CCND1 in breast cancer cells.

DDT has been reported to be a morphogen, increasing cell proliferation and promoting the maturation of the undifferentiated terminal end buds to more differentiated lobular terminal ductal structures (Brown and Lamartinier 1995). The gene expression effects in this study suggest this effect may have occurred in the MMTV-*neu* mice. However, gross observation of the glands did not reveal any morphological changes despite both differentiation genes, *csn1s2a* and *krt18*, being significantly up-regulated by p,p´ DDE. The lack of consistent change in morphology is likely due to variations within groups that obscure potential treatment effects; therefore, additional studies will be required to determine if mammary gland development and differentiation are modified by p,p´ DDE.

Anti-androgenic Effects of p,p´ DDE

In the mammary tissue, OH-flut stimulated the expression of three genes similar to p,p´ DDE, but its expression was not significantly different from control or p,p´ DDE for *csn1s2a*, *krt18*, and *ifi44*. Although these data suggest some similar actions by these two treatments, the lack of significance does not confirm that p,p´ DDE is acting as an anti-androgen. As the genes tested in the mammary tissue have not been reported to be regulated by androgens in this tissue, it may be expected that OH-flut would not significantly influence their expression. Androgen-stimulated genes may need to be identified to accurately test whether p,p´ DDE (and OH-flut) have anti-androgenic action *in vivo* on gene expression in mammary tissue; then, the anti-androgenic actions can be confirmed by repressing its expression in the presence of endogenous androgens. Expression of *neu* via the MMTV promoter would be the closest test for this anti-androgen action. However, due to regulation of the MMTV promoter by progesterone which is present in high levels in the mammary glands and the very weak binding of p,p´ DDE to the progesterone receptor, p,p´ DDE may not be able to reduce its expression. The ability of OH-flut to reduce *neu* transgene expression suggests it may be a better progesterone receptor antagonist than p,p´ DDE.

Dose dependence

Dose effects of p,p´ DDE were evident in the three markers of p,p´ DDE action. Of the four p,p´ DDE doses, 0.5, 2.5, 5, and 10 µg/pellet, the 5 µg dose elicited the strongest stimulation for all three genes. There was also a noticeable biphasic trend involving the 2.5, 5, and 10 µg doses. In all three genes, fold changes were either identical to control or

suggestive of repression by 2.5 and 10 µg p,p′ DDE, while the 5 µg dose resulted in up-regulation, which was significant versus control mice (Fig. 39). Due to this particular pattern of response, the concept of hormesis may be applicable. Hormesis is defined as a dose-response relationship in which there is a biological activation at low doses, but an inhibition at high doses, or vice versa, resulting in a U, J or inverted U-shaped dose response (Calabrese 2002). Although no studies exist linking a hormetic effect of p,p′ DDE to gene regulation in the mammary gland, Fukushima and colleagues (2005) have explored non-genotoxic carcinogens, like DDT, to examine possible mechanisms of hormesis and have demonstrated a hormetic effect in the liver of rats. Hepatocarcinogenic hormesis-like effects of DDT have been witnessed previously (Sukata et al., 2002) and most recently in rat hypothalamus (Shutoh et al., 2009).

The results show the 5 µg dose stimulated the p,p′ DDE -induced genes. For *krt18*, the three other doses were significantly different than the 5 µg dose (Fig. 39). For *ifi44*, the 2.5 and 10 µg doses were significantly different that the 5 µg dose that accelerated mammary tumor development. Collectively, these results demonstrate that expression of p,p′ DDE-induced genes *csn1s2a*, *ifi44*, and *krt18* depends on the dose of p,p′ DDE in the ELVAX pellets. The lower dose of p,p′ DDE (2.5 µg) resulted in no effect for *csn1s2a* (1.0-fold) or significantly lowered expression in *krt18* and *ifi44* compared to 5 µg p,p′ DDE. These results suggest that if a similar effect to what occurred with these genes also affected genes or responses involved in mammary tumor development, the reason for the lack of effect on tumor latency by the 2:1 ratio in the Tumor study may be related to the lower dose of p,p′ DDE (3.3 µg in the 2:1 ratio). Future studies will be

required to determine if these three genes tested in the mammary tissue in this study are related to the tumor outcomes.

Mixtures

p,p´ DDE in Combination with o,p´ DDE

Commercial grade DDT exists as a mixture of p,p´ DDT and o,p´ DDT. None of these congeners exist alone in nature and the entire population of the world presents with detectable residues of more than one organochlorine pesticide (OC) (Valerón et al., 2009), such as DDT. Furthermore, a DDT metabolite mixture consisting of p,p´ DDE , p,p´ DDD, and o,p´ DDD was found to be highly cytotoxic in human mammary epithelial cells (Valerón et al., 2009). Aubé and colleagues (2011) found the proliferative effects of an OC mixture containing 15 compounds, with p,p´ DDE as a major compound, on CAMA-1 cells appeared mainly due to the anti-androgenic effect of p,p´ DDE. Additionally, Sprague-Dawely rat neonates exposed to a mixture of p,p´ DDE, p,p´ DDT, and polychlorinated biphenyls (PCB) stimulated the development of MNU-induced mammary tumors (Desaulniers et al., 2001). Finally, technical grade DDT (a mixture) has been shown to inhibit the functional activation of murine macrophages in response to infection (Nuñez et al., 2002). These findings imply that mixtures of DDT and its metabolites are, indeed, capable of exerting effects on many systems including affecting human mammary epithelial and breast cancer cell lines and the murine immune system to promote tumor formation, proliferation, and other cancer-related events.

In the Tumor study conducted by Davis and colleagues, the 2:1 ratio group, consisting of 3.3 μg p,p′ DDE and 1.7 μg o,p′ DDE (5 μg total), resembled control. In this combination, since the 3.3 μg of p,p′ DDE did not cause the early latency it induced alone (5 μg), one possible explanation could be the influence of o,p′ DDE. Another possible reason is the lower dose, which was tested above (Fig. 39). It is also possible that both the dose of p,p′ DDE and the inclusion of its estrogenic isomer have a role in the different latency effects between p,p′ DDE and the combination with o,p′ DDE(2:1 ratio). In the current study, the influence of o,p′ DDE on p,p′ DDE was investigated by varying the concentrations of o,p′ DDE while holding the concentration of p,p′ DDE constant at the 5 μg dose shown to cause an earlier tumor onset and differentially induce expression of the three identified genes. The results revealed that both *csn1s2a* and *krt18* were significantly up-regulated by the 2:1 ratio group compared to control and had a similar effect on p,p′ DDE (Fig. 40). The effect on these genes with the revised 2:1 ratio (5 μg + 2.5 μg ; 7.5 μg total) mimicked the 5 μg p,p′ DDE alone, unlike the 2:1 ratio in the Tumor study (3.3 μg + 1.7 μg; 5 μg total) which mimicked the control group and not p,p′ DDE on tumor latency. Suppression of *csn1s2a* and *krt18* by o,p′ DDE was not observed with the 2:1 ratio. A similar effect was observed for *ifi44*, although significant differences did not occur. Since this ratio correlated with p,p′ DDE and not o,p′ DDE, these findings suggest that, for this combination, p,p′ DDE is the dominant isomer influencing the gene expression which obscures any potential actions of o,p′ DDE.

In contrast to the 2:1 ratio, the gene levels for the 5:1 and 10:1 ratios did not follow the pattern of expression of the 5 μg p,p′ DDE . However it is difficult to determine the true impact of these two ratios without knowing the effect of 1 μg and 0.5 μg of o,p′ DDE

alone. Therefore, further work will be required to understand the effects of these ratios with lower o,p´ DDE concentrations.

p,p´ DDE in Combination with p,p´ DDT

The p,p´ DDT congener has also most recently been associated with a 5-fold increased risk of breast cancer (Cohn et al., 2007). Their study was the first to examine the significance of age at exposure and breast cancer risk. With higher serum levels of p,p´ DDT, the women in this study (average age of 26) developed breast cancer before the age of 50, which suggests accelerated tumor formation similar to what was observed in the Tumor study conducted by Davis and colleagues. Cohn and colleagues hypothesize that it is the initial exposure to p,p´ DDT early in life which is more important for breast cancer development rather than chronic exposure to its metabolite, p,p´ DDE. The preclinical studies conducted by Davis and colleagues as well as the current study mimic important aspects of the human study conducted by Cohn and colleagues. First, all the animals were exposed initially to p,p´ DDE prepubertally, whereby the pellets were implanted in the mice at weaning (3 weeks of age). Second, the mice were exposed prior to puberty, when the gland is most vulnerable (Macmahon et al., 1970). Third, this study also investigated the effect of p,p´ DDT to compare its effects to p,p´ DDE due its similar effects on early-onset breast cancer (Cohn et al., 2007) compared with the early-onset mammary cancer with p,p´ DDE in the Tumor study in MMTV-*neu* mice.

The 1:1 ratio of p,p´ DDE to p,p´ DDT up-regulated expression of *csn1s2a* (4.1-fold), *ifi44* (1.4-fold), and krt18 (1.2-fold). The components of the ratio, 2.5 µg of p,p´ DDE

and p,p´ DDT, did not correlate with the 1:1 ratio for these same genes. However, the expression of the genes in the 1:1 ratio group was most similar to the 5 µg p,p´ DDE treatment which also increased expression of *csn1s2a* (13.6-fold), *ifi44* (1.9-fold), and *krt18* (1.4-fold). Moreover, 5 µg p,p´ DDE and p,p´ DDT were not similar as 5 µg p,p´ DDT was close to control or repressed for the three genes. Therefore, the 1:1 ratio appeared to have influences from both p,p´ DDT and p,p´ DDE since the lower doses had the opposite response. However, the more dominant effects appears to come from p,p´ DDE, but at the 5 µg dose; this result suggests that the two congeners add up to act like the 5 µg of p,p´ DDE and not 5 µg p,p´ DDT, even though the 2 isomers do not have the same effect on the genes.

The divergent expression between the individual p,p´ DDT and p,p´ DDE at the 5 µg dose may be related to their differences in metabolism. Both congeners are highly lipophilic and p,p´ DDE is more stable in the breast tissue for long durations of time; whereas, p,p´ DDT is more readily metabolized to p,p´ DDE (Tebourbi et al., 2006). Humans and animals perform this conversion particularly during periods of active exposure (Morgan and Roan 1975). Because the ELVAX 40P pellets were actively releasing p,p´ DDT over a 2 month period, the conversion of p,p´ DDT to p,p´ DDE during this time is likely. Thus, the 5 µg p,p´ DDT mice may contain a reduced level of p,p´ DDT as well as measurable levels p,p´ DDE in the mammary gland. This potential metabolism of p,p´ DDT may explain why the 1:1 ratio resembles the 5 µg p,p´ DDE treatment as the p,p´ DDT may augment its metabolite's levels. To determine how the gene expression effects correlate to the levels of p,p´ DDT and p,p´ DDE in the animals in the 5 µg, 2.5 µg , and

1:1 ratio treated groups, the levels of both congeners need to be measured to determine if the effects on the genes are related to p,p´ DDT concentrations and/or to its metabolism.

Chapter 6

Effects of Systemic p,p´ DDE Exposure

INTRODUCTION

The studies of Davis and colleagues (Pretumor and Tumor studies) as well DDT-01 and DDT-02 studies are the first to investigate p,p´ DDE action in the mammary gland. Moreover, detectable levels of both DDE isomers have been measured in serum (Table 10), even though the delivery was localized to mammary glands. Therefore, systemic effects in addition to local effects tested in the previous chapters may also influence mammary gland responses and tumor development. Thus, two known actions of DDT congeners were examined, relating to potential influences on the immune system and on the hormonally-responsive uterus.

DDT exposure has been reported to affect the immune system, such as decreasing human natural killer cell lytic function by as much as 55% (Udoji et al., 2010) and modulating other humoral and cellular responses in rats (Banerjee et al., 1996). Furthermore, p,p´ DDE is known to modulate immunoglobulin G and antinuclear antibodies in humans (Cooper et al., 2004) and it decreased cell viability and proliferation while also increasing apoptosis of leukocytes in Chinook salmon, leading to immune suppression (Misumi et al., 2005). The basic structure and functional characteristics of fish and mammalian immune systems are quite similar (Misumi et al., 2005). Since immune dysfunction can influence cancer development, including defects in cytokines (Critchley-Thorne et al., 2009), pro- and anti-tumor cytokines were measured in the circulating cells of the

immune system collected from the spleen as a concentrated source of these leukocytes. Four cytokine genes were investigated in leukocytes of MMTV mice treated for 2 months in the DDT-02 study, interleukin 10 (*il10*), interleukin 12 (*il12*), interleukin 18 (*il18*), and transforming growth factor β1 (*tgfb1*).

As p,p´ DDE has anti-androgenic actions *in vitro* and *in vivo* in male rats (Kelce et al., 1995), the presence of circulating levels may influence hormonally-responsive tissues outside the mammary gland, such as the uterus. If the serum levels of p,p´ DDE stimulate or repress uterine wet, these data would suggest that exposure to this endocrine disruptor may modify the systemic hormone environment. The uterine wet weight was investigated in all three animal studies, Pretumor, DDT-01, and DDT-02 studies after two months of exposure.

These endpoints were examined in mice treated with hydroxyflutamide (OH-flut) in comparison to p,p´ DDE to see if this isomer mimics the actions of the pharmaceutical anti-androgen. In addition, as p,p´ DDT has anti-androgenic actions (Kelce et al., 1995) and has been associated with increased breast cancer risk in an epidemiology study (Cohn et al., 2007), its effects alone and in combination with p,p´ DDE were also investigated. Effects on uterine wet weight from varying doses of p,p´ DDE and in ratios with the estrogenic isomer, o,p´ DDE, were also examined in animals from DDT-01 studies. These studies will address Aim 4 and Central Questions 3 and 5.

METHODS

Chapter 2 includes the methods on total RNA isolation, cDNA reverse transcriptase reactions, real time RT-PCR, relative gene expression and statistical analysis that apply to the studies within this chapter.

RESULTS

Cytokine Expression in Splenic Leukocytes

To test whether local administration of p,p´ DDE to the mammary gland has systemic effects on immune functions, gene expression of anti- and pro-tumor cytokines interleukin 10 (*il10*), interleukin 12 (*il12*), interleukin 18 (*il18*), and transforming growth factor β1 (*tgfb1*) were measured in isolated splenic leukocytes by real-time RT-PCR (Fig. 42). The expressions of *il12* (anti-tumor) and *il18* (pro-inflammatory) genes were minimally altered by p,p´ DDE as their levels were similar to the control group (Fig. 42). However, the *il10* gene, which codes for the cytokine that inhibits cell functions such as T-helper cell activity (Kundu et al., 1996), was significantly decreased by treatment with 5 µg/pellet p,p´ DDE ($p=0.008$, Mann Whitney). The gene for *tgfb1*, which codes for the growth factor that is considered to have both pro-and anti-tumor activities (Cox et al., 2007), was significantly up-regulated by 5 µg/pellet p,p´ DDE treatment ($p=0.01$, Mann Whitney). These data demonstrate that local administration of p,p´ DDE to the mammary gland produced systemic effects within the leukocytes and modulated genes involved in immune function.

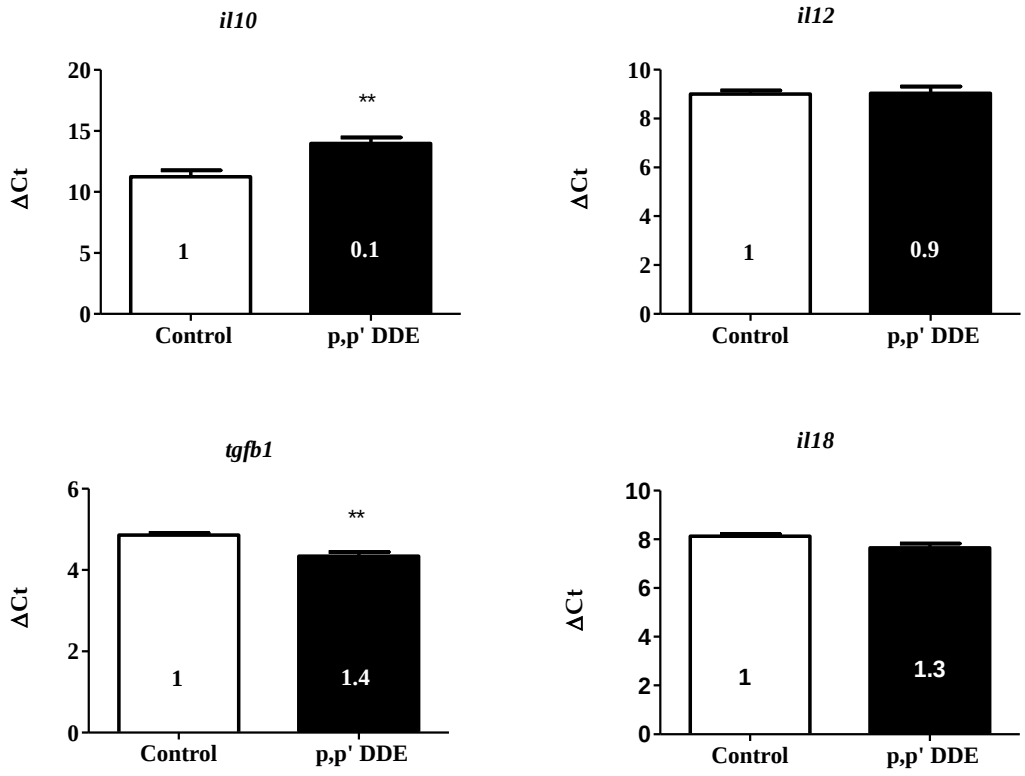


Figure 42. Expression of cytokines *il10* and *tgfb1* were significantly modified by p,p´ DDE in splenic leukocytes. Local exposure of p,p´ DDE in the mammary gland of MMTV-*neu* females treated for 2 months elevated significant changes in expression of genes for the cytokines *il10* ($p<0.009$) and *tgfb1* ($p<0.013$) in splenic leukocytes. Genes for the cytokines *il12* and *il18* were not significantly altered. ΔCt is the normalized level of gene expression compared to the housekeeper *ppia* (cyclophilin A). A low ΔCt is indicative of a higher abundance of message. Numbers within each bar represent fold change compared to control. The Mann-Whitney test was used for statistical analysis ($p^{**}<0.01$). n=6.

Cytokine Expression in Lymph Node-positive Mammary Gland

As the expression of the cytokines in leukocytes could influence mammary tissue as these cells circulate throughout the body, their expressions in lymph node-positive mammary glands were examined. The lymph node would be expected to contain high levels of leukocytes, but these immune cells may also be affected by the higher p,p´ DDE levels in the mammary gland versus serum. Therefore, the same four cytokines were also examined by real-time RT-PCR in lymph node-positive mammary glands of six animals (Fig. 43). The p,p´ DDE treatment did not induce significant changes in expression of the genes for cytokines *il10*, *il12*, *il18* or *tgfb1* in the mammary gland; however, *tgfb1* expression approached significance ($p = 0.065$). These data suggest that this cytokine may be modulated by p,p´ DDE in the mammary gland.

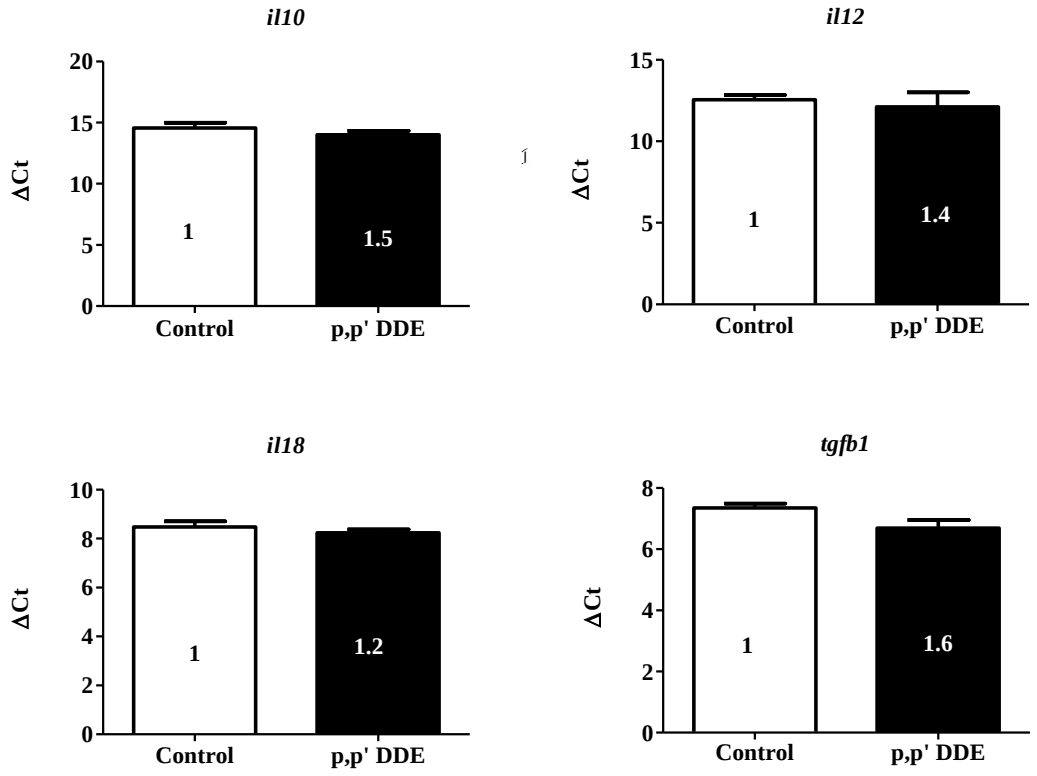
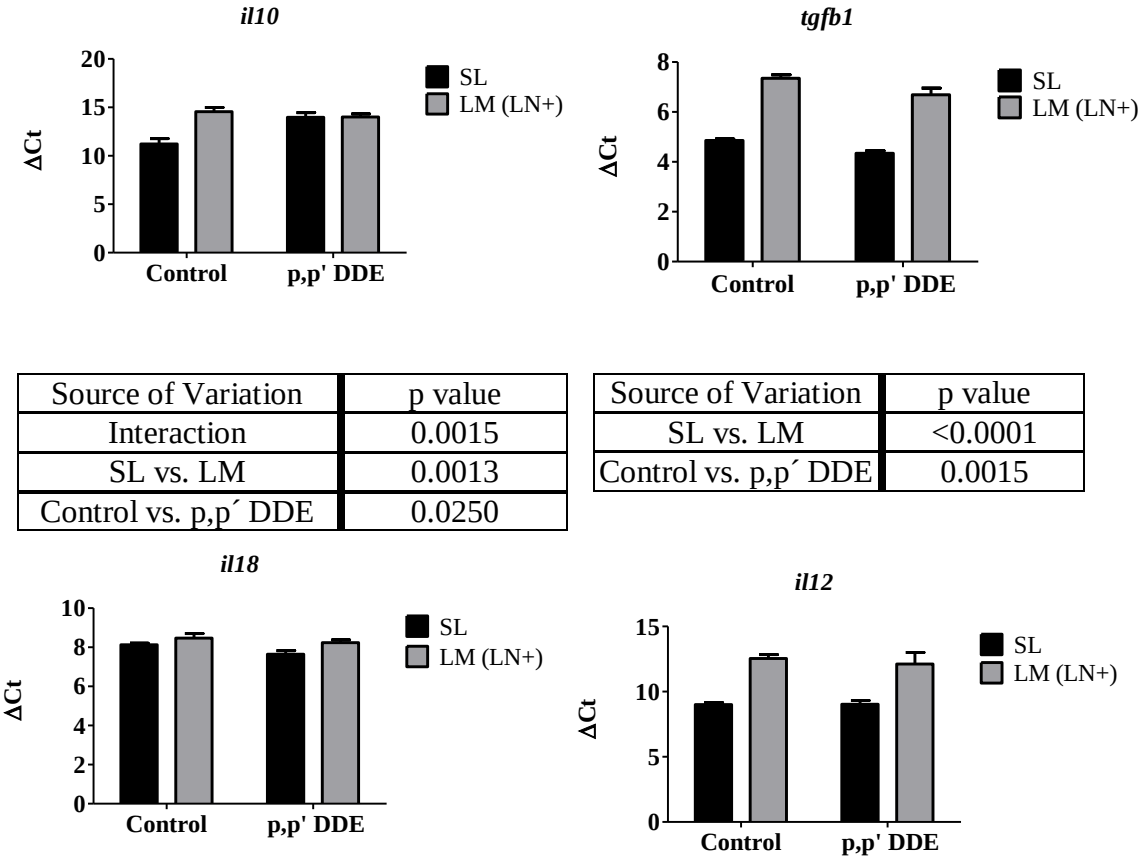


Figure 43. p,p´ DDE does not elicit significant changes in expression of genes for cytokines *il10, il12, il18* or *tgfb1* in lymph node-positive mammary gland of MMTV-*neu* mice. Using specific primers for *il10*, *il12*, *il18* and *tgfb1*, expression was measured by real-time RT-PCR with SYBR-Green detection in lymph node-positive mammary glands. None of the cytokines were significantly altered by p,p´ DDE treatment, although fold changes were indicative of slight increases in expression in all genes. Additionally, *tgfb1* expression was nearly significant, having a p value of 0.065. ΔCt is the normalized level of the target gene compared to *ppia* (cyclophilin A). A higher ΔCt value means the gene is expressed at a lower level. The numbers within each bar represent the fold change compared to control using the $2^{-\Delta\Delta Ct}$ method. A Mann-Whitney test was used for statistical analysis ($p>0.05$). n=6.

To compare RNA expression levels in the two tissues (splenic leukocytes and lymph node-positive mammary glands), tissue and treatment effects were examined by a two-way ANOVA. All four immune-related genes examined showed significantly higher expression in splenic leukocytes to lymph node-positive mammary glands (Fig. 44). This effect is likely due to the multiple cell and tissue types in the mammary gland

containing the lymph node which would contribute to the RNA in these samples. Thus, the amount of RNA from the leukocytes alone (and not blood or lymphatic vessels, mammary tissue, fat, etc.) would be reduced. Additionally, p,p´ DDE treatment effects were significant for all genes. These data indicate that with the exception of *il12*, there were both significant treatment and tissue effects induced by p,p´ DDE in theses cytokines locally and systemically.



Source of Variation	p value
Interaction	0.0015
SL vs. LM	0.0013
Control vs. p,p´ DDE	0.0250

Source of Variation	p value
SL vs. LM	<0.0001
Control vs. p,p´ DDE	0.0015

Source of Variation	p value
SL vs. LM	0.0126
Control vs. p,p´ DDE	0.0475

Source of Variation	p value
SL vs. LM	<0.0001

Figure 44. Tissue and treatment effects observed in expression of cytokine genes *il10, il12, il18* and *tgfb1* in splenic leukocytes and lymph node-positive mammary glands. Isolated splenic leukocytes (SL) and lymph node-positive mammary glands [LM (LN+)] from 6 MMTV-*neu* animals from the DDT-02 study were prepared into RNA for analysis of the cytokine genes by real-time RT-PCR. *il10* expression demonstrated an overall significant interaction (p=0.0015), with tissue and treatment effects also being significant, having p values of 0.0013 and 0.0250, respectively. Extremely significant tissue effects were see with *il12* expression (p<0.0001) and *il18* and *tgfb1* had significant tissue and treatment effects without significant interaction. *IL18* expression in tissue and between treatments had p values of 0.0126 and 0.0475, respectively. *tgfb1* also had marked differences in expression when comparing tissue and treatments, have p values of

<0.0001 and 0.0015, respectively. ΔCt is the normalized level of target gene expression when compared to a housekeep such as *ppia* (cyclophilin A). A low ΔCt means the gene is highly expressed. Two-way ANOVA with Bonferroni post test used for statistical analysis ($p<0.05$). n=6. SL=splenic leukocytes; LM (LN+) = lower mammary, lymph node positive. Expression levels in relative to the control animals (fold difference) are noted in Fig. 42 and 43.

Cytokine Expression in Leukocytes from Other Treatment Groups

Cytokine expression in splenic leukocytes was also measured by real-time RT-PCR in the additional groups of the DDT-02 study (5 µg/pellet p,p´ DDT and OH-flut, 2.5 µg/pellet p,p´ DDE and p,p´ DDT, and 1:1 ratio) in order to obtain a better understanding of their levels in relation to p,p´ DDE (Fig. 45). Also, cytokine RNA levels were assessed in p,p´ DDT-exposed animals to determine if similar patterns of gene expression occurred compared to p,p´ DDE. Anti-androgenic influences on systemic immune effects were also tested using 5 µg/pellet of OH-flut. Similar trends in gene expression for p,p´ DDE compared to OH-flut may suggest an anti-androgenic effect by circulating levels of this DDT metabolite. Dose effects of p,p´ DDE and the influence of o,p´ DDE on p,p´ DDE's action on immune-related genes in splenic leukocytes were also addressed. Cytokine *il18* was not pursued further than the initial tests in control and 5 µg/pellet p,p´ DDE in splenic leukocytes and lymph node positive mammary glands because it was shown to not be significantly regulated by p,p´ DDE (Fig. 42 & 43). Although p,p´ DDE did not significantly modulate the expression of *il12* in leukocytes, *il12* was explored further because of this cytokine's role as the inverse counterpart to IL10; that is, the ratio of these two cytokines (protein) may play a role in mammary tumorigenesis as well as many other cancers (Jacobs et al., 1998;Takeuchi et al., 2002; Lopez et al., 2005).

tgfb1 expression was significantly up-regulated in mice exposed to 5 µg/pellet p,p´ DDE compared not only to the control group, but all other groups as well (p=0.0004, one-way ANOVA) (Fig. 45). Additionally, all of the other treatment groups were similar to the control group in expression for this cytokine. Therefore, p,p´ DDE had a unique response that was unlike its lower dose (2.5 µg/pellet); the anti-androgen, OH-flut; the p,p´ DDT congener; or the 1:1 ratio of p,p´ DDE:p,p´ DDT.

il10 expression in splenic leukocytes was significantly regulated by all treatments ($p<0.0001$, one-way ANOVA) (Fig. 45) and gene expression by 5 µg/pellet p,p´ DDE was similar to the expression induced by 5 µg/pellet OH-flut. This similarity, may be suggestive of a common systemic hormonal influence. For example, both anti-androgens (5 µg) significantly downregulated *il10* expression compared to control, having fold changes of 0.2 and 0.1, respectively. Furthermore, expression induced by p,p´ DDE and OH-flut was similarly significantly different from 2.5 µg/pellet p,p´ DDE and 5 µg/pellet p,p´ DDT, and their expression was not different from one another. However, il10 expression by OH-flut was significantly different from two treatment groups, the 1:1 ratio and 2.5 µg/pellet p,p´ DDT, both of which were not significantly different from 5 µg/pellet p,p´ DDE. The differential effects on *il10* expression of p,p´ DDE and p,p´ DDT are suggestive that these congeners may have different modes of action in leukocytes on this cytokine gene and it also demonstrates that dose is an important factor as well.

Unlike the other two cytokines, expression of *il12* in isolated splenic leukocytes was not substantially altered in any treatment group compared to control mice. However, because IL12 serves as an inverse counterpart to IL10 (Jacobs et al., 1998; Takeuchi et al., 2002; Lopez et al., 2005), p,p′ DDE- and p,p′ DDT-induced changes in il10 mRNA levels without changes in il12 mRNA levels could still change the ratio of IL10:IL12 cyotkine protein levels, thus affecting tumorigenesis.

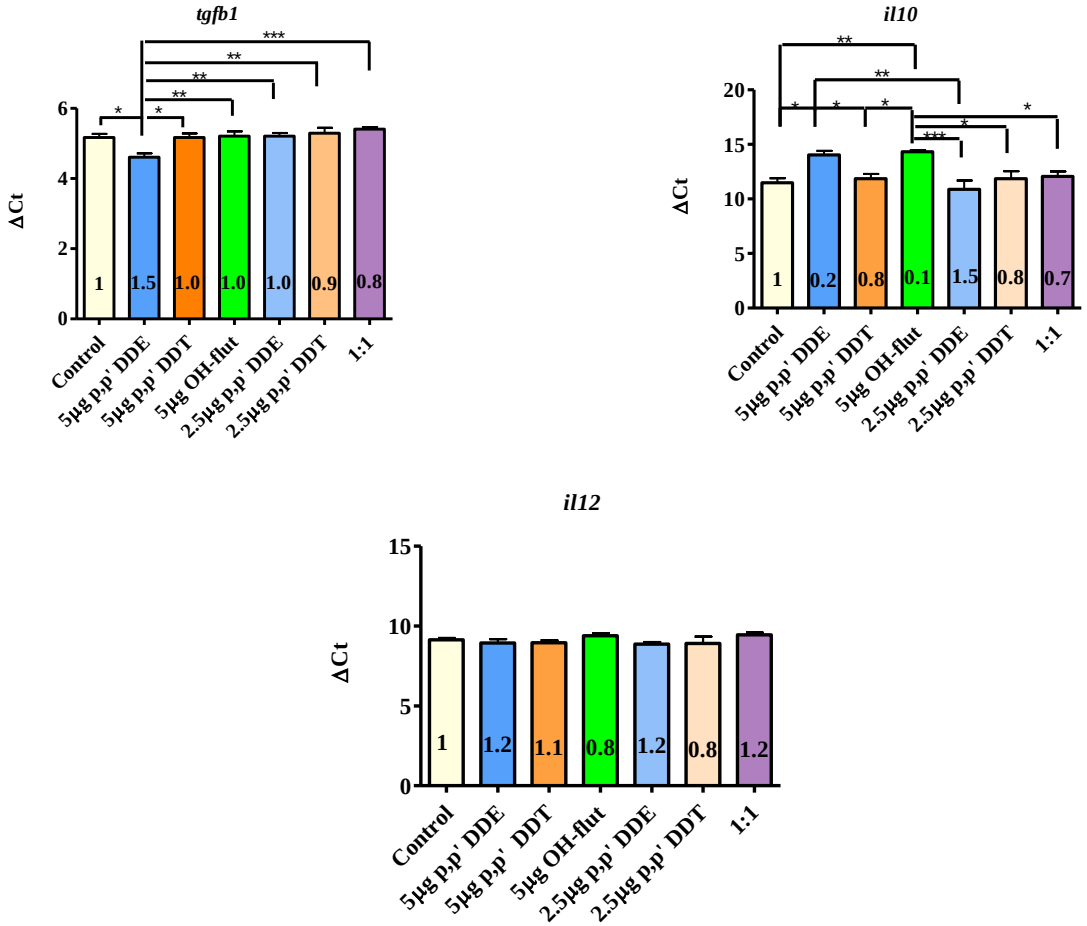


Figure 45. Expression of the *il10* gene is repressed by both p,p´ DDE and OH-flut in isolated splenic leukocytes. Real-time RT-PCR with SYBR-Green detection in study DDT-02 study animals: *Tgfb1* expression was significantly altered by 5 µg/pellet p,p´ DDE, which was also significantly different from all other treatments, demonstrating its unique action. p,p´ DDE and OH-flut evoked significant repression in *il10*, having fold-changes of 0.2 and 0.1, respectively. Their expression was also similarly and significantly different from expression induced by 2.5 µg p,p´ DDE and 5 µg p,p´ DDT. Additionally, 5 µg/pellet p,p´ DDT was significantly different from OH-flut, possibly suggestive of distinct hormonal differences. *il12* was not substantially altered in any treatment group. ΔCt is the normalized level of the target gene compared to the housekeeper, *ppia* (cyclophilin A). A lower ΔCt is indicative of a higher abundance of message. A one-way ANOVA with Tukey's post test was used for statistical analysis ($p<0.05$).).*$p<0.05$, **$p<0.01$, ***$p<0.001$ = level of significance by Tukey's test. (n=4 for 2.5 µg p,p´ DDT); (n=6 for control, 5 µg p,p´ DDE, 5 µg p,p´ DDT, 5 µg OH-flut , 2.5 µg p,p´ DDE and 1:1). Significant comparisons are only between the closet hatch mark and the next when in a continuous horizontal line.

Effects of p,p´ DDE Exposure on Uterine Wet Weight

As the uterus is highly responsive to hormone effects, uterine wet weights (normalized to body weight) were measured in all three animal studies for mice in estrus at necropsy (Fig. 46). There were no significant differences in uterine weight with any treatment compared to the control group within each study for the DDT-01, DDT-02 or Pretumor studies. However, in the Pretumor study, the OH-flut treatment group was significantly different than the 17β-estradiol (E_2) group for uterine weight, demonstrating their unique effects on this tissue during the estrus phase of the cycle. As no estrogenic treatments were included in the DDT-02 study, no significant effects were observed for OH-flut in that study.

To ensure that the body weights were similar for the normalized uterine weights and for examining treatment effects on the mice, the animal weight was measured at necropsy 2 months after implant surgery. The significant difference between the 17β-estradiol and OH-flut groups in the Pretumor study observed for uterine weight was also evident in body weight. Other than OH-flut, little variation in body weight was evident between the control group and the treatments within each study. These results suggest the animals in all studies were healthy and did not have any major adverse effects with DDT congener administration that would result in reduced body weights.

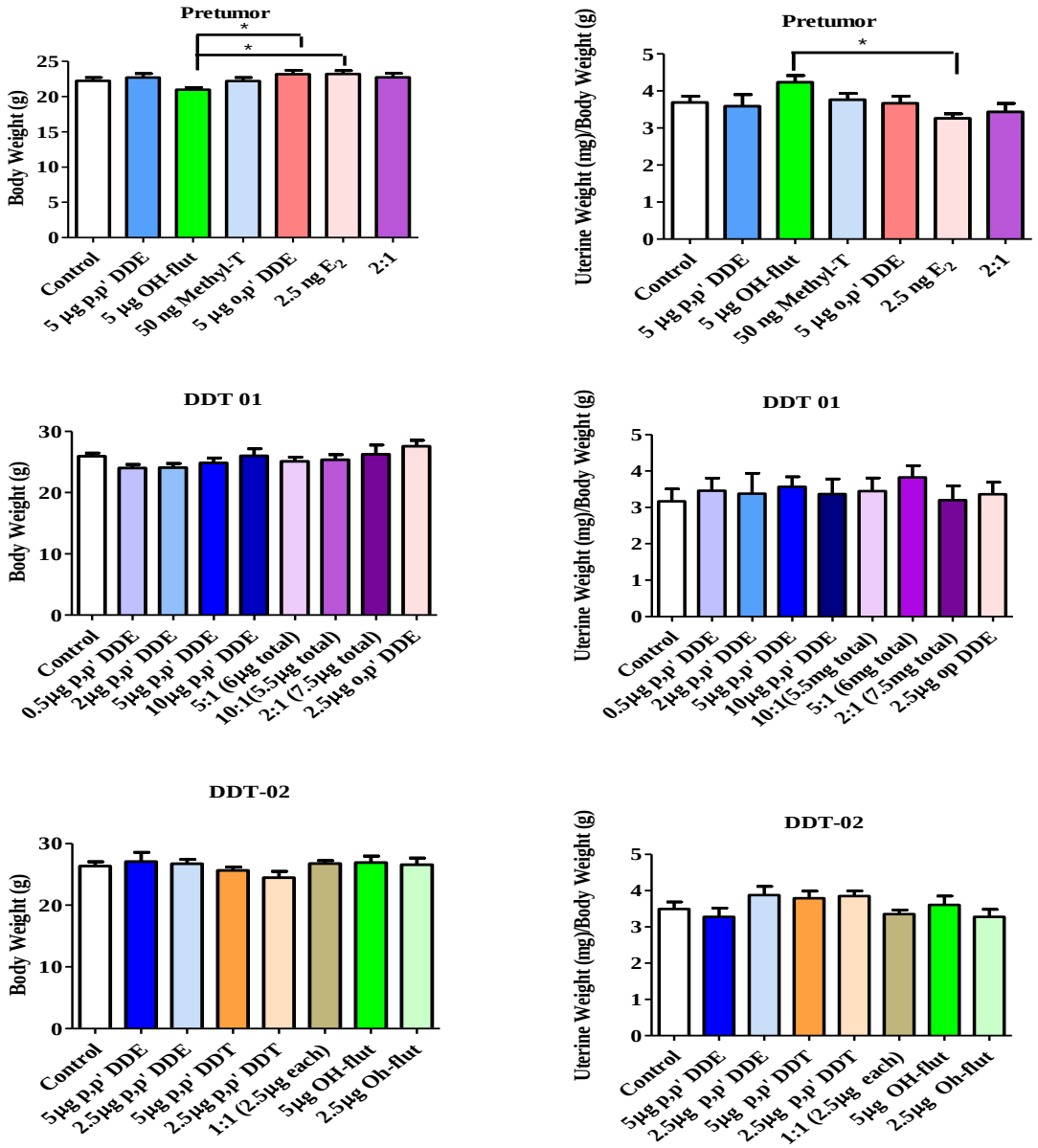


Figure 46. Body weights and uterine weights of < 3-month-old MMTV-*neu* mice in Pretumor, DDT-01 and DDT-02 studies were unaffected by treatment. At termination of the study, body weights and uterine wet weights were taken at necropsy. Uterine weights, only for mice in estrus at necropsy, are shown in the right panels. No significant difference in either weight was observed compared to control, in all studies; suggesting there were no adverse treatment effects and the animals remained healthy. However, in pretumor animals, Tukey's test showed body weights in the OH-flut group were significantly different from both the estrogenic treatments of 5 µg/pellet o,p´ DDE and 2.5 ng E_2. Similarly, uterine weights in the pretumor animals of the OH-flut group were also significantly different from the 2.5 ng E_2 group. Uterine weight p value

equaled 0.04. One-way ANOVA was used for statistical analysis ($p>0.05$). n=19-20 (Pretumor); n=4-13 (DDT-01 and DDT-02). $E_{2=}$ (Estradiol).

DISCUSSION

Immune System

Decreased immune surveillance and overall immune dysfunction play a vital role in cancer development, promotion and progression and may also have a role in the accelerated mammary tumor onset by p,p´ DDE. DDT and its metabolites have been reported to modulate immune responses in humans (Cooper et al., 2004), by negatively affecting human natural killer cells (Udoji et al., 2010), and producing modest increases in white blood cells, lymphocytes and immunoglobulin A levels, indicative of DDT having the ability to evoke immune response (Vine et al., 2001). Furthermore, Dutta and colleagues (2008) have reported that DDT is able to activate the complement system, induce the significant production of tumor necrosis factor-α and nitric oxide in macrophages and contribute to inflammatory reactions, cytokine imbalance and immune dysregulation. Due to the ability of DDT to influence the immune system, isolated leukocytes from the spleen provided an ideal tissue to examine host defense mechanisms in response to p,p´ DDE. Splenic leukocytes have also been used previously to study immune system responses to p,p´ DDE in Chinook salmon (Misumi et al., 2005). Basic structure and functional characters of fish and mammalian immune systems are quite similar and their findings also revealed immune suppression, by direct action of DDE on leukocytes. Message levels of the cytokines with both pro- and anti-tumor properties were also examined in lymph node-positive mammary glands for local immune responses in the gland. The following cytokines were investigated: interleukin 10 (*il10*), interleukin 12 (*il12*), interleukin 18 (*il18)*, and transforming growth factor β1 (*tgfb1*),

whose functions are to regulate differentiation, growth, and activity of different immune cells, thereby influencing the immune response, both locally and systemically.

Interleukin 10 is a potent anti-inflammatory cytokine, capable of down regulating antigen-specific immune responses by inhibiting cytokine production (Heckel et al., 2011) and T-cell response (Woo et al., 2007). TGFβ1 has been described as both a tumor suppressor and a tumor promoter (Cox et al., 2007), with conflicting results regarding its association with breast cancer. Its signaling has been reported to elicit a preventative or tumor suppressive effect during earlier stages of tumorigenesis, but later in tumor development, when carcinoma cells become refractory to TGFβ-mediated growth inhibition, response to TGFβ*1* signaling elicits predominately tumor progressing effects (Connolly and Akhurst 2011). It has also been shown to prevent T-cell proliferation and is associated with increased invasiveness and metastasis (Heckel et al., 2011). Interleukin 12 is a major inducer of Th1 T-cell differentiation and cytokine production, including the induction of interferon gamma production by T-cell response (Heckel et al., 2011). Lastly, interleukin 18 is a pro-inflammatory, systemic cytokine produced by activated macrophages, epithelial cells and cancer cells and has well known anti-tumor activity (Park et al., 2009). It also promotes the differentiation of T-cells and enhances the cytotoxic activities of natural killer cells. (Khalili-Azad et al., 2009). (See Chapter 4 for more background on the functions of these cytokines.)

The significant changes in expression of *il10* and *tgfb1* by p,p´ DDE suggest that these cytokines may have a role in the early onset of mammary cancer in the MMTV-*neu* mice.

However, it is unknown if the protein levels of these cytokines correlate with their messages and, accordingly, their function.

Evidence of p,p´ DDE anti-androgenic action was observed with *il10* expression in splenic leukocytes. The 5 µg dose of p,p´ DDE significantly decreased expression of this gene (0.2-fold) as did the equivalent dose of OH-flut (0.1-fold). An attenuation of *il10* expression could potentially mean there is a need for increased immune involvement following p,p´ DDE and/or anti-androgenic exposure. Additionally, both these treatments were significantly different from the 2.5 µg dose of p,p´ DDE and the 5 µg dose of p,p´ DDT. This similarity in expression supports that p,p´ DDE is acting as an anti-androgen. The exact roles and mechanisms of androgens in mammary carcinogenesis remain undefined (Liao et al., 2002; Suzuki et al., 2010); however, one well accepted notion is that androgens counteract or inhibit estrogen-induced mammary epithelial and breast cell proliferation (Zhou et al., 2000; Dimitrakakis et al., 2003; Hofling et al., 2007). With this activity, it is conceivable that blocking androgen action with an anti-androgen, such as p,p´ DDE and OH-flut, could result in increased proliferation and, thus, tumorigenesis by interfering with hormonal actions that influence mammary tumor development (Desaulniers et al., 2001). Therefore, the anti-androgenic effects of p,p´ DDE are one means for its cancer-promoting activity.

In the mammary gland, p,p´ DDE did not significantly modify the expression of any of the cytokines. Also, p,p´ DDT alone and in a 1:1 ratio with p,p´ DDE in the node-positive mammary glands did not significantly alter the expression of *tgfb1*, *Il10*, or *il12*, although

organochlorine pesticides, including p,p´ DDT, have been shown to affect interleukin secretion from lymphocytes (Beach and Whalen 2006). The lack of significant expression in the mammary gland could be due to several effects. First, it may be suggestive of a homeostatic immune environment. Expression similar to control may have been caused by the immunologic balance being properly maintained within the microenvironment of the gland. Another reason could be that there may have been no significant changes in immune expression with the chosen markers due to hormonal action within the gland, as hormones influence immune function. The differing levels of p,p´ DDE in the mammary gland versus the serum may result in different effects on the leukocytes within the lymph node in the mammary glands compared to those observed in the leukocytes. Also, in the mammary gland, other tissues may express some cytokines, like TGFβ1, to contribute to the levels detected from the leukocytes. Additionally, a different percentage of some types of leukocytes may be present in the lymph node compared to the spleen, and some subtypes may express the cytokines at different levels.

A significant increase in *tgfb1* expression was detected in isolated splenic leukocytes. The *tgfb1* gene codes for a cytokine that is involved in many different processes, including cell differentiation and proliferation, inflammation, wound healing, cellular adhesion, and host immunity (reviewed in Teicher 2001). It has been described as both a tumor suppressor and a tumor promoter, with conflicting results regarding its association with breast cancer (Cox et al., 2007). The results demonstrate that 5 μg/pellet p,p´ DDE significantly increased expression of *tgfb1* in splenic leukocytes. These increased transcript levels could influence p,p´ DDE mammary tumor formation, considering the

findings of Nicolini and colleagues (2006) who state that cytokines such as TGFβ1 stimulate breast cancer proliferation and/or invasion. However, there is also the possibility TGFβ1 may be acting as a tumor suppressor in response to p,p´ DDE, thereby regulating events, such as immune suppression and angiogenesis and, therefore, inducing effects that are more protective in the gland. The significant 1.4 fold increase in expression is possibly indicative of an induced response to suppress tumor formation as a result of p,p´ DDE exposure.

Conversely, *il10* expression was significantly repressed in leukocytes isolated from the spleen. However, similar to *tgfb1*, this cytokine also has both immunosuppressive (potentially cancer promoting) and anti-angiogenic (potentially cancer inhibiting) properties (Howell et al., 2006). With this in mind, it is difficult to determine exactly what role *il10* has with p,p´ DDE exposure. Considering the fatty environment of the mammary gland, it is possible the function of this cytokine is being modulated by the stromal environment, as adipose derived stem cells of breast cancer patients has been reported to influence both *il10* and *tgfb1* expression (Razmkhah et al., 2011). A decrease in *il10* expression, as seen in this study with p,p´ DDE exposure, could be interpreted as a needed anti-tumor event, causing a reduction in the inhibition of cytokine production. Woo and colleagues (2007) also found an overexpression of *il10* to be correlated with a down-regulation in T cell response. These findings are suggestive of *il10* repression possibly being associated with increased T cell response and tumor formation protection. In contrast, *il10* has been found to have anti-tumor activity and be a potent anti-metastatic

agent in a murine mouse model of breast cancer (Kundu et al., 1996); these findings are suggestive of p,p´ DDE acting to reverse these effects.

Additionally, expression of both anti-tumor cytokines *il12* and *il18* were unaffected by p,p´ DDE treatment in splenic leukocytes. The specific immunomodulatory activity taking place is uncertain, despite the significant increase and decrease in expression observed with *tgfb1* and *il10*, respectively. No changes in expression imply these two cytokines are not influenced by exposure to p,p´ DDE. However, the levels of the cytokines need to be measured to determine how the transcript concentrations compare to the translated proteins and secreted cytokines.

The results show p,p´ DDE is capable of inducing systemic effects despite its localized exposure to the mammary gland. The lack of significant changes in expression in the mammary gland compared to the isolated splenic leukocytes may be the result of concentration differences in the tissues. Since the leukocytes were isolated from the spleen, cytokine expression is presumably more robust because of the dense population of leukocytes, whereas expression in the mammary gland was diluted by the multiple tissue and cell types in the gland. Although the mammary gland tissue was lymph node-positive, the leukocytes were not isolated and, therefore, dispersed amongst all the other tissue types of the gland, decreasing their concentration and weakening their expression, which is observed by the higher ΔCt values in the RNAs from the mammary gland versus splenic leukocytes. Therefore, treatment effects may be obscured by lower transcript levels for the cytokines compared to the increased message levels for the housekeeping

gene (present in all cell types in the mammary gland) used for normalizing the expression.

Body and Uterine Weights

For the uterine weight, no changes were detected between the control group and any of the treatment groups in three animal studies in the MMTV-*neu* mice. If there was higher estrogen stimulation as result of one treatment in the intact mice, the uterus might be found to have an increased weight (uterotropic effect). However, as progesterone reduces estrogen stimulation in the uterus, a lower uterine weight might be detected for animals that enhance its activity or response. The significant differences in uterine weight in the Pretumor study between the anti-androgen, OH-flut, and the estrogenic treatments, 5 μg o,p′ DDE and 17β-estradiol, suggests that anti-androgens and estrogens would have opposing effects on uterine weight. No significant differences between p,p′ DDE and OH-flut were detected, so it cannot be determined if p,p′ DDE has anti-androgenic effects in the uterus. Therefore, the lack of difference in uterine weight compared to the control groups suggests the DDE isomers and p,p′ DDT do not sufficiently modify the hormonal environment in intact mice with endogenous estrogens, progesterone, and androgens. However, p,p′ DDT concentrations were not measured in serum, therefore it cannot be determined if this isomer has a systemic presence after local mammary gland administration. The absence of a systemic effect by p,p′ DDE on uterine weight may be expected considering p,p′ DDE levels in serum were approximately eight times lower than in mammary adipose tissue. The lack of effect by the DDT congeners is in agreement with Desaulniers and colleagues (2005), who also reported a lack of

uterotropic effects in Sprague-Dawley rats after exposure to p,p´ DDT, p,p´ DDE, and PCBs.

Another systemic exposure endpoint examined was body weight. There has been some speculation about the role of endocrine disruptors, which interfere with the body's adipose tissue biology, endocrine hormone systems, or central hypothalamic-pituitary-adrenal axis, thereby altering homeostatic mechanisms important to weight control (Grün et al., 2009). In the present study, no change in body weights in both DDT-01 and DDT-02 studies occurred in response to treatment. Dirinick and colleagues (2010) found no association between p,p´ DDE and body weight index. A uniform body weight among animals in all treatment groups in both studies also signifies that p,p´ DDE did not have any adverse effects and that the animals were relatively healthy.

Collectively, these results demonstrate that local exposure of 2 months to p,p´ DDE has systemic effects, by modifying cytokine expression in leukocytes isolated from the spleen. Therefore, gene expression and tumor development in the mammary gland may be influenced by systemic effects on tissues outside the mammary gland that can impact responses within the gland. In addition, the animals remain relatively healthy with no apparent influences on body weights and on the hormonal environment, as observed with no significant changes in uterine weight.

This document has not been reviewed or approved by Dr. Vicki Davis, who oversaw the research project.

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APPENDIX

Real-time PCR results were analyzed using the ΔΔCt method as described by Livak and Schmittgen (2001). The cycle threshold (Ct) value is the cycle at which the fluorescence crosses the threshold, which is set at 100 relative fluorescence units (RFU). The ΔCt for each sample is calculate by subtracting the average Ct value of the housekeeping gene, cyclophilin A (*ppia*), from the average Ct value of the gene of interest/target gene. The ΔCt values for each individual sample are then averaged per group. Next, the ΔΔCt is calculated by subtracting the average ΔCt for the treatment group from the average ΔCt of the control group. That number is then used to calculate the relative fold change in expression ($2^{-\Delta\Delta Ct}$) among treatment groups compared to control.

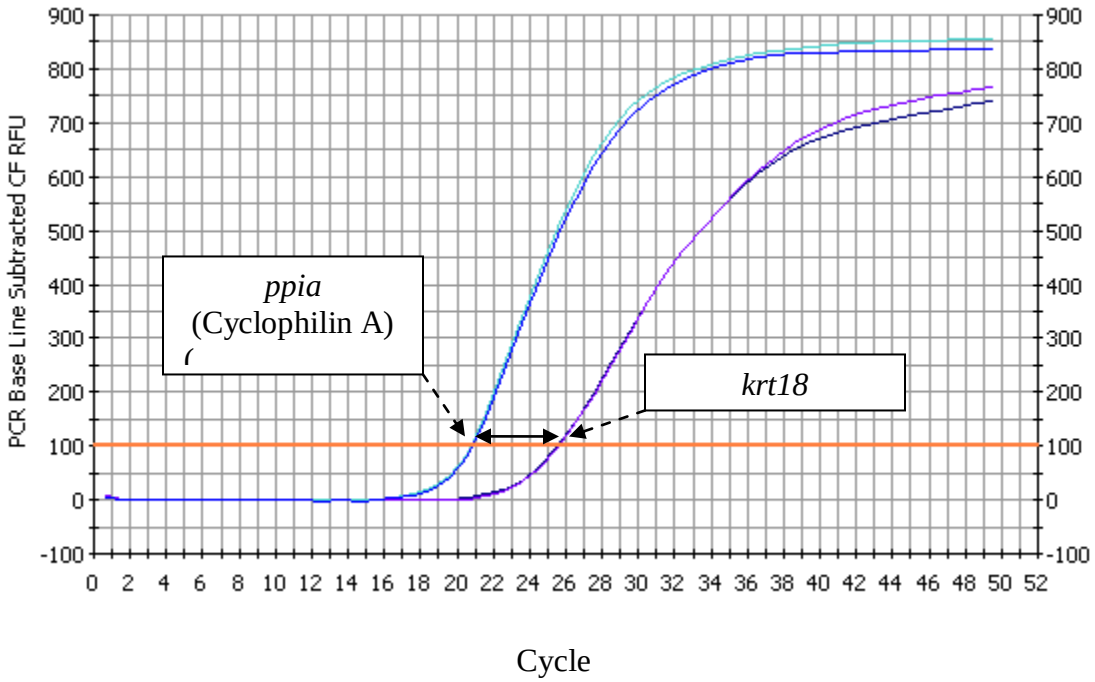


Figure 47. Example of a small ΔCt. Real-time PCR data for *krt18* analysis in mammary tissue. Dotted arrows are indicative of cycle threshold (Ct) values for *ppia* and *krt18* gene expression. The ΔCt is indicated by the double-headed arrow. The ΔCt value for this example is 4.9.

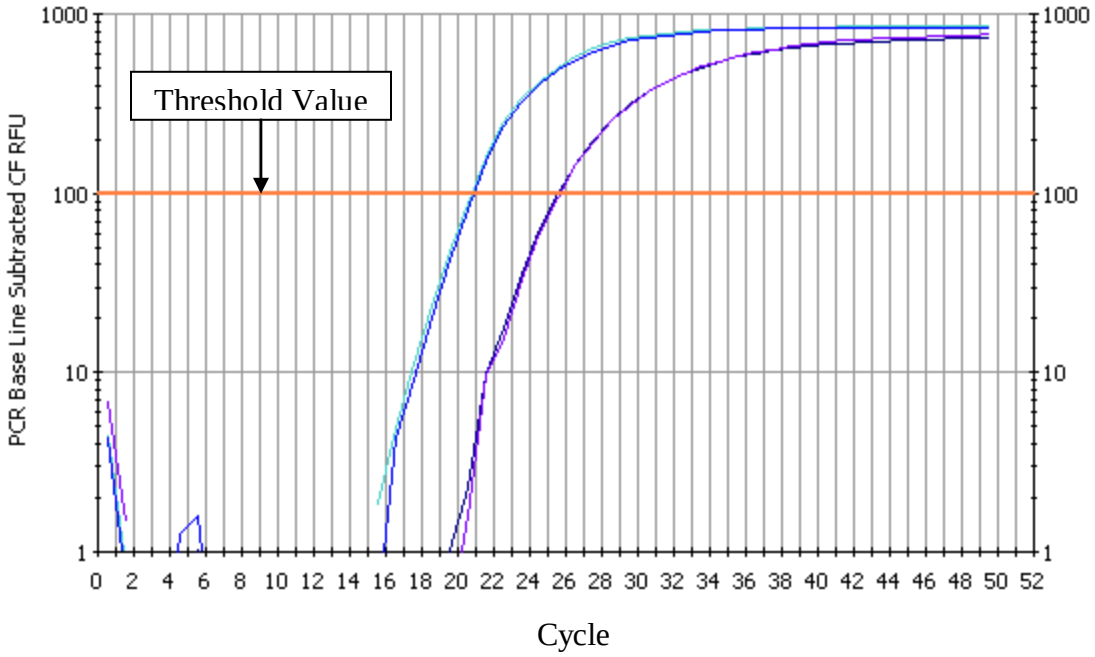


Figure 48. Example of the logarithmic graph view of real-time PCR data from Figure 47. The fluorescence threshold value was set to 100 RFU for all experiments. The threshold is consistently above background fluorescence and within the linear phase of the real-time PCR reaction.

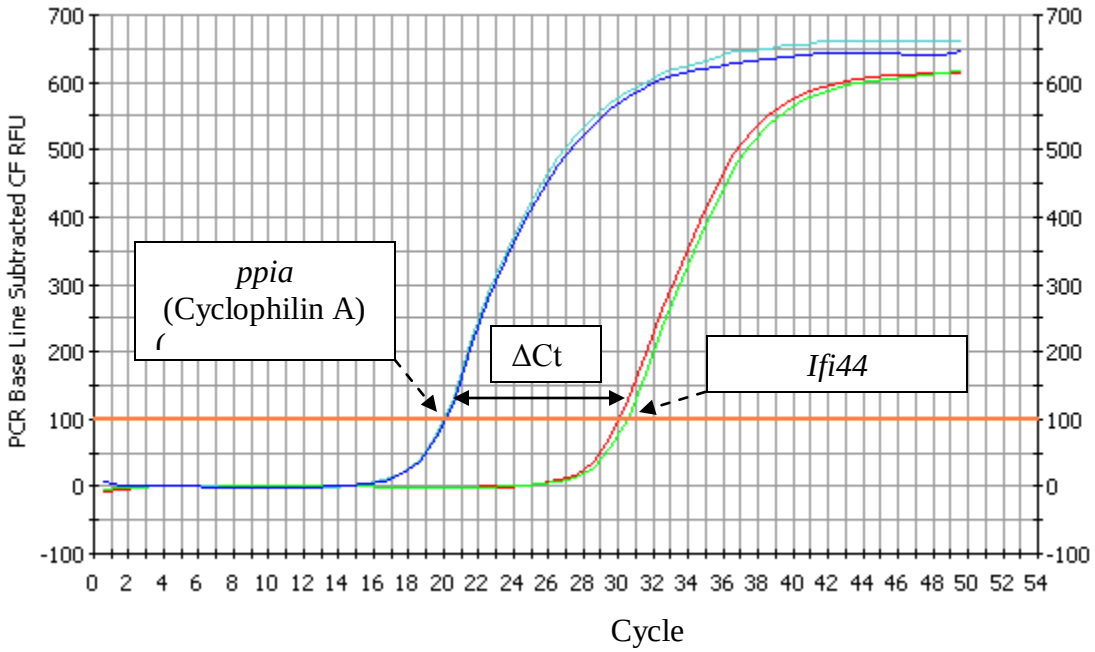


Figure 49. Example of a large ΔCt. Real-time PCR data for Ifi44 analysis in mammary tissue. Dotted arrows are indicative of cycle threshold (Ct) values for *ppia* and *ifi44* gene expression. The ΔCt is indicated by the double-headed arrow. The ΔCt value for this example is 10.15.

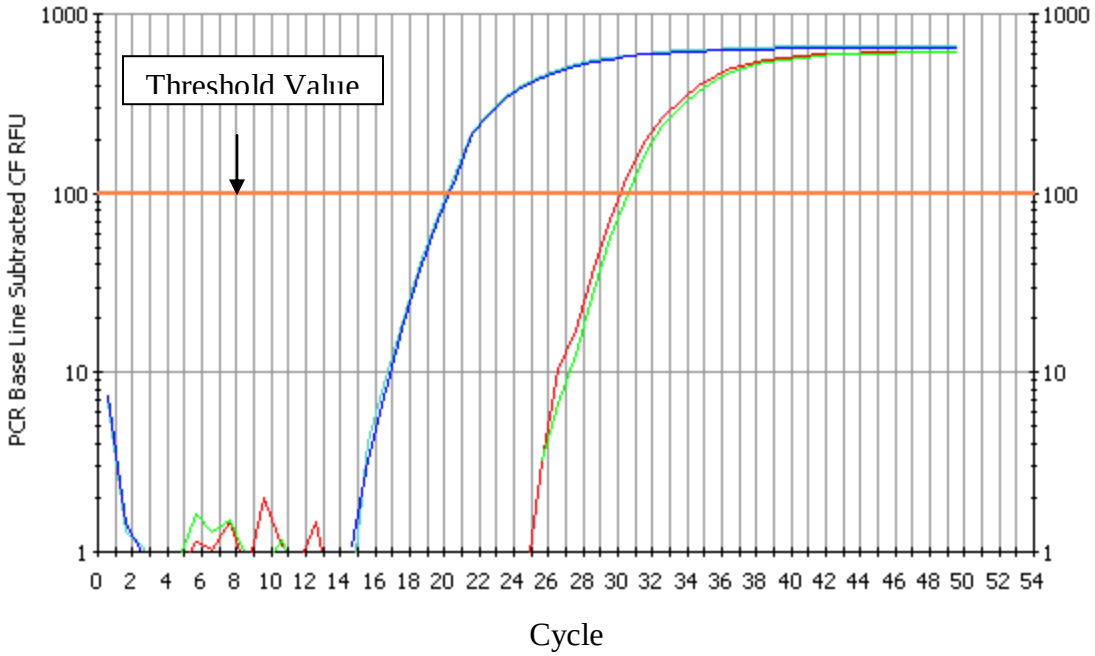


Figure 50. Example of the logarithmic graph view of real-time PCR data from Figure 49. The fluorescence threshold value was set to 100 RFU for all experiments. The threshold is consistently above background fluorescence and within the linear phase of the real-time PCR reaction.

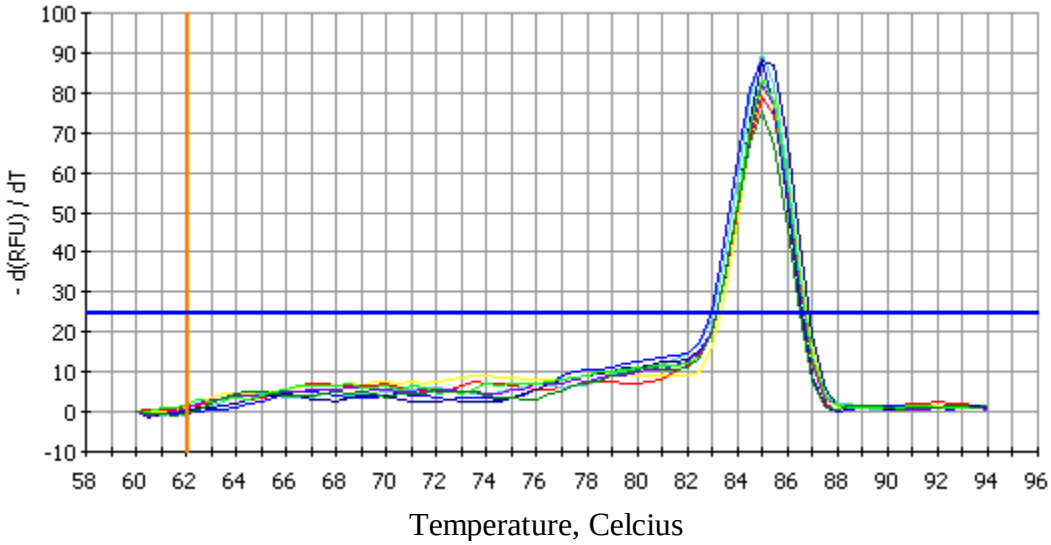
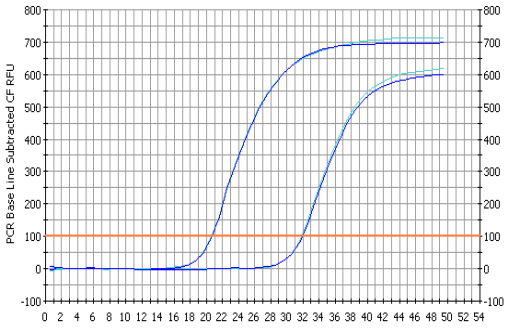
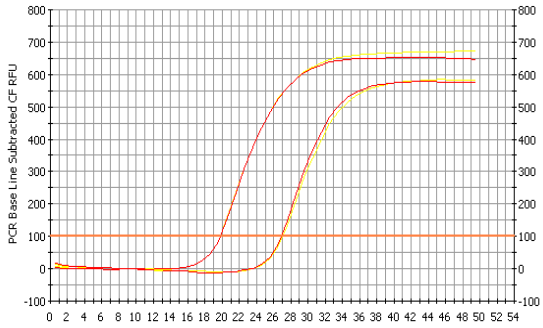


Figure 51. Example of real-time PCR melt curve analysis. Melt curve for krt18 reactions illustrated in figures 47 and 48 . The presence of one peak at 85°C is indicative of the purity of the in each reaction well.

Control



p,p´ DDE



Control			p,p´ DDE		
Gene	**Csn1s2a**	**Cph2**	**Gene**	**Csn1s2a**	**Cph2**
Raw Ct value	31.8	20.6	**Raw Ct value**	27.0	19.8
Raw Ct value	31.9	20.6	**Raw Ct value**	26.9	19.8
Average Ct	31.85	20.6	**Average Ct**	26.95	19.8

ΔCt = Avg. Ct (gene of interest) - Avg. Ct (housekeeping gene)

Control ΔCt = 31.85 - 20.6 = 11.65
p,p´ DDE ΔCt = 26.95 – 19.8 = 7.15

ΔΔCt = ΔCt (of treated group) – ΔCt (of control group)

ΔΔCt = 7.15 – 11.65 = - 4.5

$2^{-\Delta\Delta Ct}$ = Relative fold change

$2^{-\Delta\Delta Ct} = 2^{-(-4.5)} = 22.6$

Figure 52. Example calculation using the $2^{-\Delta\Delta Ct}$ method. Example of csn1s2a expression induced by p,p´ DDE treatment. The two raw Ct values are averaged by both genes and the housekeeping gene average is subtracted from it. For the ΔΔCt, the ΔCt of the control group is subtracted from the treatment group. Lastly, in order to get the relative gene expression, $2^{-\Delta\Delta Ct}$ is used.

Real-Time RT-PCR Primer Sequences

Housekeeper Gene

Gene	Abbreviation	Sequence
Cyclophilin	*ppia F1*	AGGTGAAAGAAGGCATGAAC
	ppia R1	ACAGTCGGAAATGGTGATCT

MMTV Promoter Investigation

Gene	Abbreviation	Sequence
Mouse neu	*neu F1*	CATTCACCCTACCACCCTCT
	neu R1	GCTTCCTTAGCTCTGTCTCC
Rat neu	*neu F1*	TGGATGTACCTGTATGAGACG
	neu R1	GGATTCAAGCAGCAAGGAAAG

Microarray #1

Gene	Abbreviation	Sequence
Aldo-keto reductase fam1 memC18	*akr1c18 F1*	GTCACTCCATTCCTGTCCTG
	akr1c18 R1	GCCTGGCCTATCTCTTCTTC
Bcl2-like 11	*bcl2l11 F1*	GTCAACACAAACCCCAAGTC
	bcl2l11 R1	GTTTCGTTGAACTCGTCTCC
Brain expressed gene 1	*bex1 F1*	TGGAGTCCAAAGATCAAGGCGT
	bex1 R1	TGGCTCCCTTCTGATGGTATCT
Breast Cancer 1	*brca1 F1*	CAAGATAAAGCCGGAATTGA
	brca1 R1	TATCCACTTTCCTCCTGCAA
Casein beta	*csn2 F1*	AAGGTGAATCTCATGGGACA
	csn2 R1	ATCTGTTTGTGCTTGGGAAG
CD52 antigen	*cd52 F1*	AACAAAAACAGCACCTCCAC
	cd52 R1	GCTGAGGTAGAAGAGGCACA
CD81 antigen	*cd81 F1*	GCCAGTCCTTCAGAAAGTGA
	cd81 R1	ATATCACAGGCGAAGTCCAA
Chemokine (C-C motif) ligand 19	*ccl19 F1*	AGCCTTCCGCTACCTTCTTA
	ccl19 R1	CTTCAGTCTTCGGATGATGC
Chemokine (C-C motif) ligand 21b	*ccl21b F1*	GACTCTGAGCCTCCTTAGCC
	ccl21b R1	AGGGTTTGCACATAGCTCAG
Chemokine (C-C motif) ligand 5	*ccl5 F1*	GCCCACGTCAAGGAGTATTT
	ccl5 R1	TGAACCCACTTCTTCTCTGG
Chemokine (C-C motif) receptor 7	*ccr7 F1*	GATTTCTACAGCCCCCAGAG
	ccr7 R1	CCACGAAGCAGATGACAGAA
Glycosylation adhesion molecule	*glycam1 F1*	AATGAAGACTCAGCCCACAG
	glycam1 R1	CTCTTGATTCTCTGGCTTGG
Histone deacetylase 3	*hdac3 F1*	GATCGATTAGGCTGCTTCAA
	hdac3 R1	GGATGGAGTGTGAAATCTGG
IL2-inducible T-cell kinase	*itk F1*	TCCTGTATGTGTTTGCTCCA
	itk R1	CATTCTTGGATGGGTCGTAG
Inhibitor of DNA binding 1	*id1 F1*	GACATGAACGGCTGCTACTC
	id1 R1	CTTCAGACTCCGAGTTCAGC

Microarray #1 (continued)

Gene	Abbreviation	Sequence
Interleukin 18	*il18 F1*	GCGTCAACTTCAAGGAAATG
	Il18 R1	AGTCTGGTCTGGGGTTCACT
Interleukin 2 receptor, gamma	*il2rg F1*	CGGGCTCCAGAAAATCTAAC
	il2rg R1	TACCGTTTCAGCTCATCCAC
Interleukin 7 receptor	*il7r F1*	TTGGTCATCTTAGCCCATGT
	il7r R1	TCGGGATTGAAACTCACATT
Keratin 18	*krt18 F1*	TTGCGAATTCTGTGGACAAT
	krt18 R1	TTCCACAGTCAATCCAGAGC
Lymphocyte protein tyrosine kinase	*lck F1*	TCTAGTCCGCCATTACACCA
	lck R1	AGTACCCCATCCACACTTCC
Pou domain, class 2, associating factor 1	*pou2af1 F1*	TACCAGGGTGTTCGAGTCAA
	pou2af1 R1	GGAAGCAGAAACCTCCATGT
Schlafen 2	*slfn2 F1*	ACTGCTGTGGGTGTCTCAAG
	Slfn2 R1	CGGTGATGTTCATTTTCCAG
Selectin	*sell F1*	CTGGACACTGCTCT TTGTG
	Sell R1	ACCCCAGTTCTCTGCTTCTT
Serum Amyloid A1	*saa1 F1*	CATTTGTTCACGAGGCTTTC
	saa1 R1	CAATGGTGTCCTCATGTCCT
Serum Amyloid A2	*saa2 F1*	TCATTTATTGGGGAGGCTTT
	saa2 R1	AAGGCCTCTCTTCCATCACT
Solute carrier organic anion transporter family, member 3a1	*slco3a1 F1*	GTGATGTGTGTGTGCAGCTT
	slco3a1 R1	GTGCTCCAGAACAGACAGGT
T-cell receptor alpha chain	*tcra F1*	TGGAGTCAGTGTTCTGTGGGA
	tcra R1	ACTTTGTCTCACCTGCTGCT
Telomerase reverse transcriptase	*tert F1*	TGACTTCTTCCTGCACTTCC
	tert R1	TTCACCACTGTCTTCTGCAA
Thioredoxin 2	*txn2 F1*	TGTTGTGGACTTTCATGCAC
	txn2 R1	AATGGCAAGGTCTGTGTGAT
Tropomyosin-2 F1	*tpm2 F1*	AGAGGACGAGGTGGAAAAGT
	tpm2 R1	ATCTCCTGCAGCTCCATCTT
Tumor necrosis factor receptor superfamily member 13c	*tnfrsf13c F1*	GACATACAAGCAGCCTGGAG
	tnfrsf13c R1	CTCTTGCTGGACTCCTTCTG
Tumor necrosis factor super family, member 11	*rankl F1*	CTATGATGGAAGGCTCATGG
	rankl R1	ACCCTTAGTTTTCCGTTGCT

Microarray #2

Gene	Abbreviation	Sequence
Acetoacetyl-CoA synthetase	*aacs*	TCCTTGGTCCTGTATGATGG
	aacs	TGTGGAGGTTGTGAGTTTCC
ATP citrate lyase	*Acly F1*	GCACAGAAACTCACATGACG
	Acly R1	TCGGGACTCAGAAAAAGATG
Casein gamma	*Csn1s2a F1*	CAGAGCAGTGTGAACCAGTG
	Csn1s2a R2	GGTATCTGGGGATGAAGAGC
Early growth response 2	*Egr2 F1*	TTTGACCAGATGAACGGAGT
	Egr2 R1	TTGCCCATGTAAGTGAAGGT
Inhibitor of DNA binding 4	*id4 F1*	GTGAACAAGCAGGGTGACAG
	Id4 R1	GGAATGACAAGACGAGACGA
Insulin induced gene 1	*insig1F1*	GGCTGTTGTCGGTTTACTGT
	insig1 R1	CCAAAGAGAGGGCTGCTAA
Interferon-induced protein 44	*Ifi44 F1*	CCCTGCCATTTATTCTGTGT
	Ifi44 R1	GCAGTGGGTCATGGGTATAG
Keratin 18	*Krt18 F1*	TTGCGAATTCTGTGGACAAT
	Krt18 R1	TTCCACAGTCAATCCAGAGC
Prune homolog 2	*prune2 F1*	CTGGAGGACTACCTGCTTCA
	prume2 R1	GGCAAGGGTTCTGACTTTCT
Ribosomal protein S12	*rps12*	TGTATGTCAAGCTGGTGGAG
	rps12	TAACCACTACGCAACTGCAA
Secreted frizzled-related sequence protein 5	*sfrp5 F1*	ATGGCCTCATGGAACAGAT
	sfrp5 R1	GTCCCCGTTGTCTATCTTGA
Small proline-rich protein 1A	*sprr1a F1*	CATCACCATACCAGCAGA AG
	sprr1a R1	GATTGAAGGTCACAGCATTG
Solute carrier member2 fam5	*Slc2a5 F1*	TTTGGCTCATCCTTCCAATA
	Slc2a5 R1	TGCCCAGTTTATTCACCAAG
Thrombospondin 1	*Thbs1 F1*	AAGGAACCTCCCAAAATGAC
	Thbs1 R1	GACTGGTAGCCGAAAACAAA
Thyroid hormone responsive SPOT14 homologue	*Thrsp* F1	GGAGGAGCTGGACCTAGAAG
	Thrsp R1	TACAGAACCTGCCCTGTCAT

Hormone Responsiveness

Gene	Abbreviation	Sequence
Androgen Receptor	*ar F1*	AATTTCGGAGGAAAAATTGC
	ar R1	CAAAGGAATCTGGTTGGTTG
Arylhydrocarbon receptor	*ahr F1*	GCG GCG CCA ACATCA CCT AT
	ahr R1	AGC CGGTCT CTG TGT CGCTT
Cyclin D1	*ccnd1 F1*	CGTACCCTGACACCAATCTC
	ccnd1 R1	AAGACCTCCTCTTCGCACTT
Cyp19a1	*cyp19a1 F1*	GTTCCATGTCATGAAGCACA
	cyp19a1 R1	AACTTCCACCATTCGAACAA
Estrogen receptor 1 (alpha)	*esr1F1*	TATCCGGCACATGAGTAACA
	esr1 R1	CACACCAGCCACCACCTTCT
G protein-coupled receptor 30	*gpr30 F1*	AGATCAGGACACCCAACAGA
	gpr30 R1	TTAAGGGGAGCAGAGTCCTT
Progesterone Receptor	*pgr F1*	GGCAAATCCCACAGGAGTTTG
	pgr R1	AGACATCATTTCCGGAAAATTC
Progesterone Receptor (for PR-A)	*pgr F1*	CAGTGGTGGATTTCATCCATG
	pgr R1	CTTCCAGAGGGTAGGTG
Progesterone Receptor (for PR-B)	*pgr F1*	ATGACTGAGCTGCAGGCAAAG
	grb R1	CTTCTACCCCAGAGAAAGC

Cytokines

Gene	Abbreviation	Sequence
Interleukin 10	*il10 F1*	AGCCTTATCGGAAATGATCC
	il10 R1	ACTCTTCACCTGCTCCACTG
Interleukin 12	*il12 F1*	CCCTCCTAAACCACCTCAGT
	il12 R1	GTCTTCAGCAGTGCAGGAAT
Interleukin 18	*il18 F1*	GCGTCAACTTCAAGGAAATG
	il18 R1	AGTCTGGTCTGGGGTTCACT
Transforming Growth Factor β1	*tgfb1 F1*	GCCAGATCCTGTCCAAACTA
	tgfb1 R1	TTCCCGAATGTCTGACGTAT