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Radiation Induces Estrogen Receptor-Mediated Genomic Surveillance to Restrict Mammary Progenitor Cell Expansion

Aliza Majewski

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Radiation Induces Estrogen Receptor-Mediated Genomic Surveillance to Restrict Mammary Progenitor Cell Expansion

A Thesis Presented

by

ALIZA MAJEWSKI

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

February 2018

Animal Biotechnology and Biomedical Sciences, Veterinary and Animal Sciences Department
Radiation Induces Estrogen Receptor-Mediated Genomic Surveillance to Restrict Mammary Progenitor Cell Expansion

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To my patient and loving family.
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I am extremely thankful to my advisor, Dr. Karen A. Dunphy, for her constant support and belief in me as a person and a scientist. I also need to thank Dr. D. Joseph Jerry. Together they inspire my growing interest in science and discovery, and without them I would never have had this adventure. Working with Karen and Joe has allowed me to make strides towards a better understanding of science, and more so, who I am.

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ABSTRACT

RADIATION INDUCES ESTROGEN RECEPTOR-MEDIATED GENOMIC SURVEILLANCE TO RESTRICT MAMMARY PROGENITOR CELL EXPANSION

FEBRUARY 2018

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Breast cancer is the leading cause of women’s cancer death in the United States. Estrogen exposure and reproductive history are the greatest influencing factors towards developing breast cancer. The following experiments compare the impact of parity on genomic surveillance as evaluated by radiation sensitivity to treatment with estrogen (E2) and estrogen receptor agonists. Mice are treated with agonists for 14 days and harvested after 5 weeks of involution. Apoptotic response to radiation indicated background estrogen, confounding the results, and failure to elicit appropriate response from E2 treatment. Upon further analysis, the cellulose matrix vehicle was identified as providing biologically active estrogen. To further test the effect of treatment with estrogen receptor specific agonists, a new vehicle for hormone delivery was required.

We tested tocopherol stripped corn oil as an oral vehicle to deliver estrogen agonists; estrogenic background was no longer detected in control-treated animals. E2 treatment increased apoptosis, development, and gene expression in the mammary gland of mice treated for 4 days. Treatment with estrogen receptor agonists PPT and DPN was unable to alter apoptosis but did impact uterine wet weights and gene expression within the mammary glands. These results indicated that treatment, while not completely effective, may still be biologically absorbed via oral treatment.
Parity is also known to affect the population of mammary progenitor cells, decreasing Wnt4 expression and the proliferative capability of stem cells. Full-term pregnancy early in reproductive life reduces breast cancer risk, therefore mice were bred at 3 different ages: 5, 9, and 24 weeks. After involution, mammary glands were disassociated and plated as mammospheres to study the radiation sensitivity of progenitor cells. Early pregnancy, at 5 and 9 weeks, resulted in radiation sensitivity of mammary progenitor cells that was no longer present in the mammary epithelium of mice bred at 24 weeks. Treatment of nulliparous mammospheres with Wnt4 or Sfrp1 increased the number of mammospheres relative to control. Sfrp1, a Wnt agonist, enhanced the radiation sensitivity similar to the parous mammospheres. Parity-induced alterations to the mammary gland can be used to explain and further study the protective effect against the development of breast cancer.
# TABLE OF CONTENTS

| ACKNOWLEDGMENTS | ................................................................. | iv |
| ABSTRACT | ............................................................................. | v |
| LIST OF TABLES | ............................................................................. | ix |
| LIST OF FIGURES | ............................................................................. | x |

## CHAPTER

### 1. INTRODUCTION ........................................................................... 1

  - Significance ............................................................................. 1
  - Estrogen Receptors .................................................................. 2
  - Mammary Gland Development .................................................. 3
  - Mouse Mammary Stem Cells ..................................................... 3
  - Hypothesis and Objectives ....................................................... 5

### 2. COMPARE PERSISTENT RADIATION-INDUCED RESPONSE AND PROLIFERATION IN MICE EXPOSED TO ESTROGEN RECEPTOR AGONISTS .................................................. 7

  - Introduction/Rationale ........................................................... 7
  - Materials and Methods ............................................................ 8
  - Results ................................................................................... 10
    - Tissue harvest in relation to estrous cycling ........................... 10
    - Mammary gland development indicates uneven hormonal impact .... 12
    - Irradiation-induced apoptosis ................................................. 13
    - Investigating the abnormal outcome ...................................... 14
    - Discussion ........................................................................... 19

### 3. ACUTE ORAL TREATMENT WITH DIFFERENT AGONISTS TO INDUCE OPTIMAL RADIATION RESPONSE ........................................................................... 37

  - Introduction/Rationale ........................................................... 37
  - Material and Methods ............................................................. 38
  - Results ................................................................................... 40
    - Determine optimal E2 dosage ............................................... 40
    - Physical Changes of Ovariectomized Mice Caused by Hormone Treatment .... 40
    - Gene Expression from Hormone Treatment ........................... 44
    - Apoptosis as a Marker for Genomic Surveillance .................... 45
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1: Staging estrous to determine tissue harvest timing</td>
<td>25</td>
</tr>
<tr>
<td>2.2: Estrous cycle stage based on 14-day hormone treatment</td>
<td>26</td>
</tr>
<tr>
<td>3.1: Estradiol delivery in various mouse experiments</td>
<td>50</td>
</tr>
<tr>
<td>3.3: Estrous cycle stage in ovariectomized mice treated with agonists for 4 days</td>
<td>50</td>
</tr>
<tr>
<td>4.1: Secondary mammospheres from females bred at varying ages</td>
<td>73</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1: DBD and LBD homologies for ER(\alpha) and ER(\beta)</td>
<td>6</td>
</tr>
<tr>
<td>1.2: Mammary gland development</td>
<td>6</td>
</tr>
<tr>
<td>2.1: Endogenous estrogens and estrogen specific agonists</td>
<td>26</td>
</tr>
<tr>
<td>2.2: Endogenous E2 serum levels 6 weeks after hormone removal</td>
<td>27</td>
</tr>
<tr>
<td>2.3: Mammary gland whole mounts influenced by treatment type</td>
<td>28</td>
</tr>
<tr>
<td>2.4: Parous mice see significant increase in radiation-induced apoptosis</td>
<td>29</td>
</tr>
<tr>
<td>2.5: Quantitative analysis of serum levels influenced by pellet sterilization technique</td>
<td>30</td>
</tr>
<tr>
<td>2.6: Mammary gland ductal and alveolar development</td>
<td>31</td>
</tr>
<tr>
<td>2.7: E2-mediated gene expression is not impacted by sterilization of the 50(\mu)g E2 pellet</td>
<td>32</td>
</tr>
<tr>
<td>2.8: Serum E2 levels of mice treated with 50(\mu)g or 100(\mu)g E2 pellets</td>
<td>33</td>
</tr>
<tr>
<td>2.9: Relative gene expression of 50(\mu)g and 100(\mu)g over 14-day time course</td>
<td>34</td>
</tr>
<tr>
<td>2.10: Percent BrdU positive cells in mice treated with E2 pellets</td>
<td>35</td>
</tr>
<tr>
<td>2.11: E2 and biological activity found in control pellets</td>
<td>36</td>
</tr>
<tr>
<td>3.1: Previous work with acute hormone delivery in ovariectomized mice</td>
<td>51</td>
</tr>
<tr>
<td>3.2: Oral E2 treatment pre-test</td>
<td>52</td>
</tr>
<tr>
<td>3.3: 17-(\beta) estradiol serum concentrations</td>
<td>53</td>
</tr>
<tr>
<td>3.4: Uterotrophic response to orally dosed animals</td>
<td>54</td>
</tr>
<tr>
<td>3.5: Mammary gland development</td>
<td>55</td>
</tr>
<tr>
<td>3.6: Progesterone receptor relative gene expression</td>
<td>56</td>
</tr>
<tr>
<td>3.7: Ampheregulin relative gene expression</td>
<td>56</td>
</tr>
<tr>
<td>3.8: Wnt5a relative gene expression</td>
<td>57</td>
</tr>
</tbody>
</table>
3.9: Tgfβ2 relative gene expression ................................................................. 58
3.10: Cebpδ relative gene expression ............................................................... 58
3.11: Esr1 relative gene expression ................................................................. 59
3.12: Esr2 relative gene expression ................................................................. 60
3.13: TUNEL staining for apoptotic cells .......................................................... 61
4.1: Parity-induced changes in epithelial cell populations .................................. 73
4.2: Parous mammary stem/progenitor cells are susceptible to radiation .......... 74
4.3: Secondary mammospheres from female bred at varying ages .................... 75
4.4: Tertiary mammospheres from female mice put with a male at 9 weeks of age .... 76
4.5: Cells/mL in 2D culture after 3-day treatment ............................................ 76
4.6: Unsuccessful treated TM40A Mammospheres ........................................ 77
4.7: Wnt4 and Sfrp1 treatment of nulliparous mammospheres ......................... 78
4.8: Nulliparous mammospheres treated at primary and secondary levels ........ 79
4.9: Secondary Mammospheres ..................................................................... 80
A.1: Wholemount 3rd Mammary Gland from 4d oil Treatment ......................... 86
A.2: TUNEL 4th Mammary Gland from 4d oil Treatment .................................... 89
B.1: BrdU incorporation ..................................................................................... 101
B.2: Preparation of pellets ................................................................................ 101
B.3: TUNEL incorporation ................................................................................ 101
CHAPTER 1

INTRODUCTION

Significance

Each year in the United States, one in four deaths are caused by cancer, with breast cancer being the leading cause of cancer death among women (Siegel et al. 2012). Aside from age and genetics, reproductive history and estrogen exposure is the greatest risk factor for the development of breast cancer (Travis & Key 2003, Anderson 2014). Pregnancy causes a rise in hormone levels with increased growth of mammary epithelium and a short-term increase in breast cancer risk (Meier-Abt & Bentires-Alj 2014) in the years immediately following the pregnancy. While pregnancy is associated with this transient increase risk in breast cancer, it has been recognized for almost 200 years (Weinberg 2004) that there is also a long-term protective effect of parity against breast cancer.

This protective effect is also seen in parous mice, and the protective effect of pregnancy can be mimicked by 14-day treatment with exogenous estrogen in rodents. This protection of the mammary gland is sustained after removal of exogenous estrogen and is no longer dependent on consistent high doses of hormone (Medina 2005, Dunphy et al. 2008). The BALB/c mouse mammary tumor model demonstrates a decrease in carcinogen-induced mammary tumor incidence from 63% to 17% when treated with hormones. Prior exposure to estrogen and progesterone persistently increased the radiation-induced responsiveness of p53 and, consequently, genomic surveillance (Dunphy et al. 2008). This result is similar to the effects of parity, with both parous and hormone-treated mice retaining p53 responsiveness for weeks after the end of hormone exposure.
**Estrogen Receptors**

When mimicking pregnancy, mice are treated with high doses of 17β-estradiol (E2), which is the most commonly produced estrogen in the ovaries of pre-menopausal women (Gruber et al. 2002). E2 has an equal affinity for the two estrogen receptors (ER), ERα and ERβ. The DNA binding domains (DBD) of ERα and ERβ are 97% homologous, indicating that they are capable of binding to the same estrogen response elements (ERE) in the promoters of estrogen-responsive genes (figure 1.1) (Leitman et al. 2010). The two receptors are only 55% homologous in the ligand binding domain (LBD), implying that they can be individually studied using different agonists, due to differing binding affinities. The two receptors also differ in the two activating function (AF) domains, where the percent homology between the two receptors in AF1 is 17% and AF2 is 18%. These differences may account for why ERβ is generally a weaker transcriptional activator in comparison to ERα. Although ERβ may be a weak transcriptional activator, it has also been shown to repress ERα-mediated proliferation (Hodges-Gallagher et al. 2008).

High levels of ERα, as well as serum estrogen, have been found to relate to an increased risk of breast cancer (Clemons & Goss 2001). In the normal breast, ERα is expressed exclusively in the mammary epithelium (Brisken & O’Malley 2010). ERβ is also expressed in the mammary epithelium, as well as other cells in the breast tissue including myoepithelial cells, fibroblasts, adipose tissues, and endothelial cells. In normal breast tissue ERβ is found at high levels, but when breast cancer occurs levels of ERβ expression decline or disappear completely (Williams et al. 2008). However, when reintroduced to cancer cell lines ERβ is anti-proliferative, decreasing the activity of ERα and therefore regulating cell cycle control and proliferation.
**Mammary Gland Development**

Mammary gland development is unique, just barely beginning during fetal development and continuing at different stages postnatally (Hennighausen & Robinson 2005), figure 1.2. At puberty, when estrogen production begins, ducts form and evenly spread to the edge of the gland. Mice that remain nulliparous or that are not exposed to an estrogen receptor agonist treatment have minimal development of the mammary gland architecture, limited to bifurcation and minimal tertiary branching (Hughes & Watson 2012). The mammary gland is arrested at this stage unless exposed to pregnancy hormones which induce expansion of the epithelium to produce tertiary branching and alveolar buds, along with the ability to produce milk during lactation. After cessation of lactation, the mammary gland involutes, lobules and alveoli regress, and the mammary gland resembles the nulliparous gland, but can expand again upon subsequent pregnancies. It is expected that treatment with different estrogen receptor agonists would mimic pregnancy, including development of tertiary branching and lobular/alveolar development in the mammary gland (Dunphy et al. 2008).

**Mouse Mammary Stem Cells**

This ability of mammary glands to develop postnatally has lead scientists to believe that there is a form of stem or progenitor cell in the mammary gland. The first supported evidence for mammary gland stem cells came in the 1950s when small pieces of a mammary gland were transplanted into the cleared fat pad of a different mouse (DeOme et al. 1959). These expanded and differentiated, forming fully functional mammary glands from the small transplanted sections. Likewise, some hypothesize that many cancers originate from stem cells or tumor-initiating cells (Tiede & Kang 2011). Breast cancer is a
heterogeneous disease, occurring in multiple forms that could derive from mammary stem cells (MaSCs) or lineage restricted progenitor cells.

Restriction of MaSCs may also provide an alternative mechanism for the protective effect of pregnancy. Controversy over whether parity alters stem cells is prominent in this field because breast cancers are believed to start from different subpopulations within the mammary gland. Britt et al. (2009) found that there are no significant differences in stem cell populations within basal and luminal cells of parous versus nulliparous animals. Alternatively, Meier-Abt et al. (2013) showed that while the basal stem progenitors were not altered by parity, luminal progenitor cells were. In luminal epithelial cells, \( Wnt4 \) expression is decreased by early parity. In mammary stem and progenitor cells this results in a decrease in the Wnt/Notch signaling pathway ratio (Meier-Abt et al. 2013). Wnt proteins have a clear impact on MaSCs, and could cause uncontrolled self-renewal and growth (Many & Brown 2010). Thus, the decrease in Wnt associated with parity would result in increased control of cell division to restrict progenitor cells and the development of tumors.

Frizzled polypeptides with cysteine-rich domains function as receptors for Wnt molecules (Finch et al. 1997). Finch et al. (1997) found a secreted frizzled related protein (SFRP) that resembles frizzled receptor proteins but does not contain a transmembrane domain. Consequently, they determined that SFRP inhibits Wnt-dependent development in \textit{Xenopus}, and determined it to be a dominant-negative receptor in the Wnt system. In cells collected from SFRP1\(^{-/-}\) mouse mammary glands, there is a significant increase in mammospheres relative to those formed from SFRP1\(^{+/+}\) tissue (Gauger et al. 2012). This
indicates that SFRP1, likely by inhibiting Wnt signaling, plays a role in regulating progenitor cells and mammosphere initiating cells.

**Hypothesis and Objectives**

Discovering a means for protection against breast cancer is a lofty goal. Working towards a better understanding of our natural protective mechanisms, and how to mimic some of these factors is the first step. Therefore, we intend to treat animals with hormones and specific hormone receptor agonists to compare the proliferation and radiation-induced responses to that of parity. Based on acute responses with estrogen receptor agonists, I hypothesize that radiation-induced responses will be modified by ERβ. Furthermore, studying the radiation sensitivity of MaSCs will help to understand the mechanisms behind the differences in DNA damage response between parous and nulliparous individuals. I hypothesize that this response is regulated by the wnt pathway and will be induced by a decrease in wnt signaling.
**Figure 1.1: DBD and LBD homologies for ERα and ERβ** (Leitman et al. 2010)

Estrogen Receptor alpha (ERα) and Estrogen Receptor beta (ERβ). ERα and ERβ share a 97% homology and the DNA binding domain (C, DBD) but only a 55% homology at the ligand binding domain (E, LBD). Differences in the LBD impact the receptors ligand binding specificity.

**Figure 1.2 Mammary gland development** (Hennighausen & Robinson 2005)

Mammary gland development occurs postnatally in conjunction with hormone production. Duct expansion progresses at puberty (A), but then remains static until increased hormones due to pregnancy (C) and lactation (D) promote alveolar development. Post-weaning, alveoli regress and glandular structure resembles that of a mature nulliparous animal (B).
CHAPTER 2

COMPARE PERSISTENT RADIATION-INDUCED RESPONSE AND PROLIFERATION IN MICE EXPOSED TO ESTROGEN RECEPTOR AGONISTS

Introduction/Rationale

Pregnancy has long been associated with a short-term increase in breast cancer risk (Lyons et al. 2009) in the years immediately following a full-term pregnancy. However, for women who are under the age of 25 at the time of their first childbirth, this transient increased risk is modest and furthermore, it is followed by a 40% lower lifetime risk of developing postmenopausal breast cancer (Machon et al. 1970, Clemons & Goss 2001). This protective effect can also be seen in the decreased development of mammary tumors in parous rodents or rodents treated with estrogen to mimic serum levels during natural pregnancy (Medina 2005, Dunphy et al. 2008).

Naturally occurring estrogens are steroids that are derived from cholesterol (Gruber et al. 2002), of which 17β-estradiol is the most abundant in women (figure 2.1). Along with naturally occurring phytoestrogens and synthetically produced xenoestrogens, there are also synthetic estrogen receptor specific agonists that can be used to activate the two estrogen receptors independently. Propyl pyrazole triol (PPT) has 400-fold selectivity for ERα over ERβ (Frasor et al. 2003). The specific actions of ERβ can be stimulated using 7-Ethenyl-2-(3-fluoro-4-hydroxyphenyl)-benzoxazolol (ERB041), which has 200-fold selectivity to ERβ or Diarylpropionitrile (DPN), with 70-fold selectivity for ERβ over ERα (Leitman et al. 2010). When compared to nulliparous mice, the expression of ERα was seen to decrease in the mammary epithelium of parous mice (Meier-Abt et al. 2013). The
increased surveillance response in parous and E+P treated mice might be due to a more favorable ERα to ERβ ratio. I hypothesize that treatment with an ERβ agonist will induce persistent surveillance and mimic the protective effect of parity.

**Materials and Methods**

*Animals and animal experiments:* BALB/c mice were housed in a temperature-controlled rodent vivarium with 12hr alternating day/night light cycle. All procedures were in accordance with protocols approved by the Institutional Animal Care and Use Committee. All mice were maintained on LabDiet 5058 (PicoLab St. Louis, MO, USA) 20% protein diet and water ad libitum. At 8 weeks of age, treatment pellets were implanted subcutaneously at the base of the neck with one of five treatments: 50µg E2, 400µg PPT, 400µg ERB041, 400µg PPT+400µg ERB041 or cellulose control. There were 12 female mice per treatment group. After two weeks the pellets were removed from the animal and the mice were then allowed 5 weeks to go through involution and quiescence. On the day of tissue harvest, the mice were irradiated (5 Gy) at 6 hours and given BrdU (70µg/g of body weight) injections at 2 hours prior to euthanasia. As a positive control for a natural pregnancy, 12 female mice were housed with males. Four days after natural birth the pups were removed to force involution.

*Pellet preparation:* Pellets were made using silastic tubing (Fisher) sealed with silicone (DAP Inc) approximately 1.2 cm long. These were packed with one of five compounds: control (cellulose; Fischer, # AC382312500), E2 (50µg; Sigma Aldrich; Cat#E27858), PPT (400µg; R&D Systems, Cat#1426-50), ERB041 (400µg; R&D Systems; Cat#427-50), and ERB041+PPT (400µg each), each packed in cellulose. The proper ratio of cellulose to hormone was combined prior to packing the capsules and then measured in a
pre-tared capsule to 0.015g so each would contain the appropriate concentration of agonist. Pellets were sterilized with 5kGy gamma irradiation and primed in standard phenol red-free DMEM:F12 media (Sigma Aldrich; #D2906) for 24 hours prior to implantation.

**RT-qPCR:** RNA was isolated from frozen tissues using 1mL TRIzol reagent (Life Technologies) according to the manufacturers instruction. cDNA was then made using 1μg RNA and amplified using ProtoScript II first strand cDNA synthesis kit (New England Biolabs). Gene expression was quantified using SYBR (Kapa) as per manufacturer instruction. Pgr forward primer: 5’-GGTGGGCCTTCCTAACGAG-3’, Pgr reverse primer: 5’-GACCACATCAGGCTCAATGCT-3’; Areg forward primer: 5’-GGCTTGCAATGATTCAACT-3’, Areg reverse primer: 5’-AAGAAACCGGACTGTGCAT-3’.

**Hematoxylin and Eosin Stain:** Vaginal smears were taken and spread on slides immediately at time of tissue harvest. The smears were allowed to air dry for multiple days, fixed in 10% NBF, stained in hematoxylin for 4 minutes and eosin for 12 seconds.

**Whole Mount Preparation:** Whole 3rd thoracic mammary glands were fixed in Carnoy’s Fixative (6:3:1 ethanol:chloroform:acetic acid) and then stained in carmine alum solution (2g/L; 10mM aluminum potassium sulfate). Glands were dehydrated in graded ethanol, cleared in xylenes and mounted on slides with permount.

**Enzyme-Linked Immunosorbent Assay (ELISA):** Coagulated whole blood was centrifuged at 7500rpm for 5 minutes to separate serum. Serum estrogen was quantified using 17β-estradiol specific ELISA (Calbiotech) as per manufacturer instruction.
**Terminal Deoxynucleotide Transferase dUTP Nick-End Labeling (TUNEL):** Four-micron paraffin embedded sections were de-paraffinized and subjected to TUNEL assay (Millipore; cat# S7101) to observe apoptosis within the mammary epithelium. Protocol was altered slightly from manufacturers suggestion to best suit mouse mammary glands, see appendix B. A total of 1200 mammary epithelial cells were counted for each slide to determine the percent of cells that were undergoing apoptosis. Cells were counted at an increased magnification on ImageJ (http://rsb.info.nih.gov/ij/download.html). Percentages calculated as total positive out of 1200. Error bars indicate SEM.

**5-Bromodeoxyuridine (BrdU):** Four-micron paraffin embedded sections were de-paraffinized and subjected to BrdU assay (Invitrogen; Cat# 93-3943). The number of proliferating cells was quantified by counting the percent BrdU positive, counting 1200 cells per slide.

**Results**

**Tissue harvest in relation to estrous cycling**

Each cage contained 6 mice that were all treated with the same hormone and had been housed together since the start of the experiment. In order to best mimic conditions in which humans develop breast cancer, these mice were not ovariectomized. Vaginal smears were collected and stained with H&E to determine the best time to harvest tissues. Optimal tissue harvest would be when the mouse is in diestrus and has the lowest endogenous estrogen levels, 5-20 pg/mL serum E2 (Walmer et al. 1992). Housing the mice together meant there was a potential for them to be in sync and cycle through estrous together. To determine if this was the case, initially all six of the mice were assessed by vaginal smear. This first assessment demonstrated that all the mice in the cage appeared to
cycle together, so on consecutive days only two mice per cage were tested by random selection. By not staging all the mice daily, there would be less physical stimulation leading to a divergence from normal estrous cycle progression. At tissue harvest the mice in each cage were not all at the same stage, indicating that this plan did not work accordingly. Additionally, the majority of mice were not caught in diestrus (table 2.1).

The experience with the Group 1 mice helped to change the plan for Group 2 mice. While the original goal was to stage the mice, and catch them in diestrus at time of harvest, the results showed that staging may have increased the risk that the mice would be pushed into pseudo-pregnancy and prolonged estrus with a potential for higher levels of E2 in the serum. This is not entirely unexpected, as the rodent estrous cycle lasts only 4 to 5 days (Byers et al. 2012). While staging for estrus was not confirmed to have caused prolonged estrus in the first group of mice, to reduce the risk in group 2, vaginal smears were only taken at time of harvest.

Quantifying the results of the vaginal smear H&Es shows that most of the mice were in estrus (50%) at the time of tissue harvest (table 2.2). 25% of the mice were euthanized in diestrus, 15% at metestrus and 10% at proestrus. This is concerning, especially considering the fact that during estrous staging some mice, although repeatedly sampled, were only found in proestrus/estrus.

Enzyme-linked immunosorbent assay specific for E2 was conducted on a set of the animals to determine serum E2 concentration at tissue harvest and compared to estrous stage. The serum concentrations and estrous stage seem to be correlated, indicating that the mice had returned to normal cycling levels of serum E2 following treatment and 5 week clearing period (figure 2.2).
Mammary gland development indicates uneven hormonal impact

Mammary gland development is dependent on the quantity of hormone exposure in the animal. This experiment includes a parous control that has gone through mammary gland expansion for lactation, followed by involution, which would decrease the quantity of lobules/alveoli, although the tertiary branching will still be greater than that of a nulliparous animal. The five-week period between pellet removal and tissue harvest in hormone treated animals is designed to have the same involution impact. Control treated animals are expected to have the least mammary gland development. The E2-treated mice should have mammary glands that appear closer to that of a parous animal. The effect of the other agonists, PPT or ERB041, on mammary gland development was not certain. The results show, that in terms of mammary gland morphology, there was considerable unexpected variation within each of the treatment groups, such that some glands appear nulliparous with little branching (figure 2.3A) while others appear more parous with highly developed branching (figure 2.3B). The development of the parous mammary glands is consistent between animals within the parous group. The control treated animals were inconsistently branched, some being minimally branched properly mimicking nulliparity, but others demonstrate branching of the E2-treated animals. The E2-treated animals were generally further developed than control animals, but with less alveolar development than parous animals. PPT and ERB041 were similarly developed to E2-treated mice. As with the other treatments, animals varied within these groups. PPT animals varied towards less developed controls, and ERB041 varied towards that of parity with the highest developed animal being indistinguishable from the parous animals. There is no correlation between the varied stage of estrous and the developmental level of the mammary glands, suggesting
that each stage of estrous and consequent serum E2 levels are too brief to influence mammary gland morphology.

**Irradiation-induced apoptosis**

Each treatment group contains animals that were exposed to 5Gy γ-irradiation and non-irradiated mice to compare both radiation-induced and spontaneous apoptosis due to treatment. Parous mice were used as a control for induced genomic surveillance, those exposed to 5Gy γ-irradiation saw a significant increase in TUNEL positive cells (9.02%) relative to spontaneous apoptosis (3.04%) (figure 2.4), *p*=0.0018. This is consistent with historical data that has spontaneous apoptosis of parous animals around 3%, increasing to 8-12% TUNEL positive cells after irradiation. Conversely, the control mice had levels of apoptosis that were above historical values for both spontaneous and radiation-induced apoptosis. The non-irradiated control mice typically have 1-2% TUNEL positive, however, in this experiment the spontaneous apoptosis values are higher than expected – between 1.7% and 3.2%. Irradiated controls were also above typical values, with a mean >8% compared to the expected 3%, and with one as high as 18.08% TUNEL positive. The hormone treated animals: E2, PPT, and ERB041, had lower irradiation-induced apoptosis levels than that of parous animals or the abnormal controls. E2-treated animals had an average of 4.8% TUNEL positive in the irradiated group and 1.28% TUNEL positive in the spontaneous group. PPT irradiated animals presented 3.95% TUNEL positive compared to their non-irradiated PPT counterparts who exhibited 2.25% TUNEL positive. ERBO41 irradiation-induced apoptosis and spontaneous apoptosis were at 6.96% and 3.11% TUNEL positive respectively. Combined ERB041+PPT pellets had 6.22% TUNEL positive irradiation-induced apoptosis and 0.92% spontaneous TUNEL positive.
The abnormally high level of apoptosis within control-treated animals, as well as insignificant differences between irradiation-induced and spontaneous apoptosis within a treatment suggests that there may be an issue with hormone delivery by the pellets. The problematic controls need to be addressed in order to continue and repeat this experiment, and to have a better understanding of the impact of the receptor specific agonists.

**Investigating the abnormal outcome**

While PPT, ERB041, and the combined pellet did not provide the hypothesized outcome, the 50μg E2 pellets did not either. To confirm that sterilization of the pellets was not causing the decreased activity, mice were treated with E2 pellets for 14 days that were sterilized by 5kGy irradiation (IR), ethylene oxide (EO), or not sterilized. Serum E2 levels were quantified via ELISA. Sterilization by IR and EO resulted in equivalent levels of serum E2 on day 14 as non-sterilized pellets: IR: 70.45+/−19.66, EO: 68.45+/−22/17, and no sterilization: 71.02+/−21.28 pg/mL (figure 2.5; p<0.05 relative to controls). Sterilization technique did not have an impact on the E2 serum concentration in animals after treatment for 14 days with 50μg pellets. We also had autoclaved two 50μg E2 pellets as a form of sterilization prior to implanting into mice, because we believed that the heat and steam of autoclaving would sufficiently degrade the hormone and provide a negative control. The serum E2 levels from these two mice were different from controls, suggesting that the hormone was degraded. However, it was found at tissue harvest that one of the two mice had lost her pellet at some point during the treatment period.

Whole mounts were used to visually evaluate the developmental differences induced by the different pellets, and support serum results. Control pellets were filled with a cellulose matrix and these control pellets were also exposed to either EO or IR to test if
any differences that may arise between the E2 pellets was due to the degradation of E2 itself or to the degradation of the matrix (figure 2.6A). One of the 4th abdominal mammary glands was collected after 14 days of treatment for each differently sterilized pellet, fixed and stained with carmine alum. There were minimal morphological differences in the mammary glands between nulliparous control animals and those treated with an IR- or EO-sterilized control pellet. Mammary glands from the animals treated with sterilized E2 pellets had morphology comparable to pregnant day 14 mice (figure 2.6B) and were not different from the unsterilized E2 pellets. The results from the E2 serum ELISA and the morphology of the whole mounts indicates that the low percent TUNEL positive observed in the E2-treated group, and the abnormally high TUNEL values for control animals, is not due to the IR sterilization of pellets prior to implantation. Of the two mice implanted with the autoclaved E2 pellets, the animal who lost its pellet had minimal mammary gland development; the animal who retained its pellet had gland morphology similar to the E2 pellets of other sterilization techniques, supporting the suggestion that E2 is not easily degraded.

The expression of progesterone receptor (Pgr) was also quantified in the mammary gland, and the E2-pellets increased Pgr gene expression (~2.3 fold), regardless of sterilization technique, relative to the non-sterilized samples (figure 2.7, p<0.05). For the animals implanted with the autoclaved pellets, the animal who lost her pellet had low expression of Pgr, whereas the animal that retained the autoclaved E2 pellet had Pgr expression that was similar to the other E2-treated mammary glands (1.7-fold greater than control).
After the 14-day treatment period with 50μg E2 pellets, the serum E2 was equally elevated and mammary glands exhibited typical estrogen-induced branching morphology by pellets with no sterilization, IR, or EO. This suggests that the sterilization methods were not destroying the hormone. However, the qPCR data at day 14 was not robust. Also, despite a general increase in mammary gland development with hormone treatment, there were still some samples in which 50μg was not sufficient to induce extensive ductal branching and alveolar development. The variation in the extent of ductal branching corresponds to the varied E2 serum concentrations (which range between 15.14 pg/mL and 125.89 pg/mL). Our objective was to maintain sufficient pregnancy ranges of physiological E2 serum levels (60-110 pg/mL) for all animals with the E2-treatment for the entire duration of the 14-day period. We considered that low E2 serum levels may have been the result of 50μg of E2 being insufficient. To assess this, 50μg E2 pellets were compared to 100μg pellets. Animals were treated with 50μg or 100μg of E2 that had been sterilized with IR, for up to 14 days and we compared serum E2 via ELISA at day 3, 6, 9 and 12-14 (figure 2.8). Day 12-14 was the lowest for 50μg pellets, at 30.67+/−23.03 pg/mL, which is well below what is considered pregnancy levels. The 100μg E2 pellets had serum concentration levels of 303.18+/−44.93 pg/mL at day 12-14, far surpassing that of pregnancy levels.

Pgr gene expression is historically induced by estrogen, which can be seen by the immediate and significant increase at day 3 of treatment with 50μg E2 (figure 2.9A). All time points had a significant increase in Pgr gene expression relative to the control (p<0.05). This increase in gene expression peaks at day 3 (7.5-fold) followed by a downward trend to 2.6-fold at day 14, however the decreases are not significantly lower.
than day 3. When mice are implanted with the 100\(\mu g\) silastic hormone pellet, Pgr gene expression is significantly greater relative to control at all time points except for day 3, which can be attributed to an outlier at 1.78 relative Pgr gene expression \((p<0.05)\). The expression of Areg was increased at day 3 and day 9 for both the 50\(\mu g\) and 100\(\mu g\) pellets relative to control (figure 2.9B). When treated with 100\(\mu g\) E2 pellets, Areg expression was also significant on day 6 \((p=0.03)\).

Pregnancy, and the consequent elevation in the serum hormone concentration levels, induce proliferation of the mammary gland. In this respect, the 50\(\mu g\) pellets do mimic parity, as well as increase the level of proliferation relative to control animals as calculated by percent BrdU positive (figure 2.10). BrdU positive cells in the control treated animals were less than 1\% \((0.57\%)\). E2 stimulation increased proliferation as determine by BrdU incorporation such that 12.86+/−4.2\% of cells were BrdU positive by day 3 and remained elevated during the treatment period. BrdU positive levels were also counted in the mammary glands of animals treated with 100\(\mu g\). Again, proliferation levels jumped at day 3 in the 100\(\mu g\) pellets reaching 8.29+/−3.39\% and remained high for the remainder of the treatment period.

While we were testing the effectiveness of the E2 pellets and the effects of sterilization methods, we also think that we have discovered a problem with the cellulose matrix used. Previously, when we had used silastic hormone pellets in our animal models (Roman-Perez unpublished), we had included P4. The lyophilized P4 \((20mg)\) was also the packing matrix for the estrogen agonists in the silastic pellets, because a large bolus of P4 was being used. Therefore, control pellets used were empty silastic tubing. Since we wanted to compare the effects of the estrogen receptor agonists and we wanted to eliminate
the effects of progesterone in the mammary gland for these experiments, the P4 was omitted and cellulose was substituted for the matrix. Our pellets were primed in DMEM:F12 media overnight before implanting into mice. Therefore, we compared concentrations of E2 in media, priming media, and serum with an E2 specific ELISA. Surprisingly, our ELISA experiments suggested that the cellulose had background estrogenic activity (figure 2.11A). For controls we compared various media samples: media no serum (negative control), media + 300 pg/mL E2 (positive control), media + 10% CSS-FBS, and media + 10% FBS. The ELISA indicated that estrogen in the media has low E2 levels (13.5 pg/mL +/- 4.76) and adding 300 pg/mL E2 to the media is accurately detected indicating utility of the assay. There was also negligible E2 with the addition of CSS FBS (10.57 pg/mL) or standard FBS (37.83 pg/mL).

In the priming media for 50μg E2 pellets the ELISA consistently detects E2 (~240 pg/mL). But the ELISA also detects E2 in the priming media from the control pellets (~180 pg/mL). Checking serum levels from mice 3 days after implanting pellets demonstrates that E2 from the 50μg E2 pellet is present in the serum (~320 pg/mL). Two of three mice with control pellets had normal nulliparous E2 levels on day 3, but the ELISA detected high E2 levels in the third mouse (~140 pg/mL). Therefore, we are concerned that the silastic pellets or the cellulose matrix may have an estrogen-like compound that is detected by the ELISA. This compound may be metabolized much faster than E2 in most mice, but may be present at high levels in others (the third mouse). This could account for the morphology and radiation-induced apoptotic response detected in the mammary glands from the mice treated with control pellets (figures 2.3 & 2.4 respectively).
Detection of an estrogenic compound in priming media and sera does not confirm that the compound has biological activity. An ERE-luciferase assay was conducted to test the various estrogen-free control silastic capsules (figure 2.1B) including: empty silastic pellets, empty silastic pellets sealed with silicone, and silastic pellets filled with cellulose and sealed with silicone. Media with and without E2 indicates positive and negative responses, and control pellets should mimic that of media, not that of E2. In order to be successful controls, the silastic pellets should have a low estrogenic response. However, relative to media alone, both the sealed capsules and the cellulose filled capsules have estrogenic activity; ~2.3 and 10.4-fold greater activity respectively. In addition, the pellet containing cellulose has activity similar to the media with added estrogen (1nM and 10nM). Therefore, although we are not positive that the cellulose filled silastic capsules have enough sustained estrogenic activity to impact the control mice in vivo, we are also not confident that it does not have an impact.

**Discussion**

Elevated levels of E2 induce both acute proliferation and genomic surveillance pathways in the mammary gland (Becker et al. 2005). During the mouse estrous cycle endogenous estrogens peak during proestrus, decrease slightly in estrus, peak again in metestrus, and then wane during diestrus (Walmer et al. 1992). Although the acute response to estrogens require levels to remain elevated for a minimum of 72-96 hours, and is not likely to be induced during a short period of natural estrus, we did not want the potential acute response to obscure the sustained genomic responses achieved by parity. Therefore, at 15 weeks of age, mice were monitored for stage of estrous in an effort to harvest tissues during diestrus when endogenous estrogen levels are the lowest (Walmer et al. 1992).
Diestrus is naturally the longest period during the estrous cycle, so the assumption was that the majority of mice would fall during this phase of estrous (Byers et al. 2012). Upon tracking estrous throughout the course of a week, it became obvious that the mice were remaining in estrus much longer than natural. At harvest, 4 of 6 control mice in group 1 were in estrus at time of tissue harvest. Due to the fact that prolonged estrus can be caused by stimulation from attempting to stage the mice (Sinha et al. 1978), in an effort to avoid this in the second group of mice, estrous was not staged. However, 3 of the 6 of the mice in the second control group were still harvested during estrus and only one during diestrus.

Nulliparous mice that are not exposed to an estrogen receptor agonist treatment and that have never experienced pregnancy experience minimal development within the mammary gland architecture, limited to bifurcation and minimal tertiary branching (Hughes & Watson 2012). Mice that undergo pregnancy and lactation develop tertiary branching and alveolar buds that involute after weaning. It was expected that treatment with different estrogen receptor agonists would mimic pregnancy, including development of tertiary branching in the mammary gland (Dunphy et al. 2008). Mice given agonist-treatments depict morphology of the development of some tertiary branching with no alveolar development. The majority of the agonist-treated mouse glands had more extensive development than that of nulliparous control mice, but, again, did not compare to the development of pregnancy. Observing the mammary gland development and morphology after regression and quiescence, it is clear that none of the hormone treatments, E2, PPT, or ERB041, produced the same level of development as mice that underwent pregnancy. The results of this research suggest that the levels of hormones supplied by the silastic implants were not equivalent to the sustained levels of hormones during pregnancy.
Within each of the treatment groups there was a large variation in the amount of tertiary branching and mammary gland development, although no correlation can be found between the degree of tertiary branching and either the stage of estrous or the serum E2 concentration at harvest.

In parous mice, an average of 8-10% of mammary epithelial cells undergoes apoptosis in response to irradiation (Dunphy et al. 2008). This apoptotic effect is also achieved by E2 plus P4 treatment to mimic pregnancy and previous results using acute treatment with an ERβ agonist, diarylpropionitrile (DPN) + P4 shows that apoptotic responses are mediated by ERβ (Roman-Perez, unpublished). ERβ activity can also be studied using ERB041, which is an ERβ agonist that has a higher affinity for ERβ than DPN (Leitman et al. 2010). ERβ has also been seen to be anti-proliferative within cancer cells, opposing the effects and activity of ERα (Williams et al. 2008). Therefore, we hypothesized that stimulating ERβ with the agonist ERB041 for 14 days to mimic pregnancy will, like E2, enhance sustained apoptotic responses. Treatment with ERB041 resulted in an average rate of 6.96% apoptosis. While this was the highest percent TUNEL positive seen within the hormone treatments, it did not reach the levels seen in the parous mice (9.02% apoptosis). Even that of E2, the predominant endogenous hormone of pregnancy, did not reach apoptotic levels of the pregnant controls and was only 4.79%. The amount of agonist contained within each pellet was intended to exceed the level of hormones achieved by pregnancy. Again, the results suggest that not enough of each hormone was delivered systemically to the mice.

The Office of Laboratory Animal Welfare (OLAW) requires that non-pharmaceutical grade drugs are sterilized before use in living animals. Lab-made hormone
filled silastic pellets allowed easy administration of estrogen, but required sterilization to satisfy OLAW. The objective when sterilizing the pellet is to not degrade the hormone, which if that occurred, could have impacted the E2 levels. There are multiple sterilization techniques available: gamma irradiation, and sterilization with ethylene oxide (EO) or 70% ethanol (Lambert et al. 2011). Ethylene oxide (EO) can be used for sterilization but can pose problems because it can be absorbed by the sterilized material (Kowalski & Morrissey 1996). This poses a danger to the animal after implantation if the EO is then released. To avoid this problem the pellets are often given time after sterilization prior to implant for aeration. Additionally, EO is a suspected carcinogen and that can make working with it dangerous. While a viable disinfecting method, 70% ethanol is not capable of sterilization and is a known solvent for E2 that runs a high risk of leaching out the hormone. We chose to use gamma irradiation because we have access to cesium137 and because it has been shown as effective at eliminating microbial contamination without degrading estrogen at 5kGy (Mohr et al. 1999). To test the sterilization methods both control and E2 pellets sterilized with EO and IR were compared to non-sterilized pellets (figure 2.5-2.7). Results demonstrated that serum estradiol, mammary gland morphology, and gene expression was similar to that of non-sterilized pellets after 14-day E2 exposure.

Since the sterilization technique did not appear to impact results, mice were treated with either 50μg or 100μg pellets in a time course experiment, collecting samples every 3 days for 12 to 14 days. In mice, during pregnancy serum E2 concentrations rise to peak at about 60-110 pg/mL, depending on the mouse strain (McCormack & Greenwald 1974). For this reason, levels of serum E2 needed to be sustained above 100 pg/mL for at least 14 days. Animals treated with 50μg dropped below this threshold by day 9. Historically, these
type of hormone pellets usually release a spike of hormone (<1000 pg/mL) initially, but then serum estrogens rapidly decline over the course of the ensuing 14 days (Ström et al. 2012). In this test, our 100μg pellets were representative of this hormone activity, with serum E2 surpassing 1000 pg/mL initially and, although declining over the following days, remaining above the 100 pg/mL at day 14. In fact, serum E2 levels are so high with 100μg pellets, that in future experiments 75μg pellets may be preferable.

Tertiary branching and abnormally high levels of spontaneous and radiation-induced apoptosis in control treated mice indicated exposure to a potential estrogenic compound. Our results suggest that the cellulose pellets contain estrogen, resulting in estrogen-induced radiation sensitivity and mammary gland development in the controls. Silastic hormone pellets are primed for 24 hours in DMEM media at 37°C for 24 hours prior to implantation to prime estrogen release prior to implantation into the animal. This was found to be key when studying LH surges induced from implantation of E2 pellets (Johnston & Davidson 1979). Testing the media from pre-priming the pellets demonstrated biologically active estrogen was found to be released from both the E2 and control pellets. The luciferase assays reveal that the estrogen in the control pellets has transcriptional activity and may account for the demonstration of estrogenic activity in the control treated animals. Additionally, because responses in the mammary gland were lower than expected for E2-, PPT-, and possibly ERB041-treated glands in this experiment, we propose that progesterone may be necessary to elicit maximal responses. However, prior to optimizing the estrogenic responses for E2, PPT, and ERB041, we need to identify an appropriate vehicle for controls.
Treatment with our E2 pellets did not mimic parity apoptotic responses. Additionally, treatment with estrogen receptor agonists, PPT and ERB041, did not mimic parity within the mammary gland. Serum, mammary gland morphology, and gene expression indicate that the method of sterilization does not impact the 50μg E2 pellet. However, comparing serum levels of 50μg and 100μg E2 pellets demonstrates that 50μg E2 may not be sufficient to achieve the full desired parity-like effect. While there were incomplete responses seen with E2 pellets, some of the experimental controls exhibited estrogenic responses, including increases in apoptosis and tertiary branching. Further analysis of these pellets demonstrated that the cellulose matrix used was providing unexpected estrogenic activity. To further test the hypothesis, a different E2 delivery method to avoid parity-level responses in controls and an increased dosage of E2 needs to be implemented.
Table 2.1: Staging estrous to determine tissue harvest timing

Group 1 mice were staged daily for one week prior to tissue harvest in an effort to predict the day diestrus would occur.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proestrus</th>
<th>Estrus</th>
<th>Metestrus</th>
<th>Diestrus</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>E₂</td>
<td>0</td>
<td>8</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>PPT</td>
<td>0</td>
<td>7</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>ERB041</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>PPT+ERB041</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Pregnant</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2.2: Estrous cycle stage based on 14-day hormone treatment
Animals were staged via Hematoxylin and Eosin stained vaginal smears collected at time of tissue harvest.

**Endogenous Estrogens**

- **17β-estradiol**
  - ERα = ERβ

- **Estrone**
  - ERα > ERβ

- **Estriol**
  - ERα < ERβ

**Estrogen Receptor Specific Ligands**

- **PPT**
  - ERα > ERβ
  - 400-fold selectivity

- **ERB041**
  - ERα < ERβ
  - 200-fold selectivity

- **DPN**
  - ERα < ERβ
  - 70-fold selectivity

**Figure 2.1: Endogenous estrogens and estrogen specific agonists**
The body naturally produces different estrogens: 17β-estradiol (E2), estrone, and estriol. E2 is the most commonly found estrogen during reproductive years and has an equal affinity for ERα and ERβ. Estrogen receptor specific ligands can be used to study either ERα or ERβ. Propyl pyrazole triol (PPT) has 400-fold selectivity for ERα over ERβ (Frasor et al. 2003). 7-Ethenyl-2-(3-fluoro-4-hydroxyphenyl)-benzoxazolol (ERB041) and Diarylpropionitrile (DPN) have higher selectivity for ERβ over ERα of 200-fold and 70-fold selectivity, respectively.
Figure 2.2: Endogenous E2 serum levels 6 weeks after hormone removal
Serum collected at tissue harvest, 6 weeks after pellet removal, was tested on a 17-β-estradiol specific ELISA to determine serum E2 levels in mice.
Figure 2.3: Mammary gland whole mounts influenced by treatment type
Fourth abdominal mammary glands collected at tissue harvest were stained with carmine alum to visualize ductal outgrowth and branching. A. Least developed mammary gland whole mounts. B. Most developed mammary gland whole mounts.
Figure 2.4: Parous mice see significant increase in radiation-induced apoptosis

Control animals, and each treatment group, were treated with subcutaneous hormone pellets for 2 weeks and allowed 6 weeks to regress. Fourth abdominal mammary glands were formalin fixed and paraffin embedded and underwent TUNEL staining for apoptosis. – indicated no IR, + indicated IR. ***P < 0.001
Figure 2.5: Quantitative analysis of serum levels influenced by pellet sterilization technique

Groups of mice were implanted subcutaneously behind the neck with silastic pellets containing cellulose, 50µg E2, or pellet implantation was mimicked (empty). Number of animals per group indicated below graph. Pellets were sterilized by 5kGy Irradiation (IR), ethylene oxide (EO), autoclaved, or left unsterilized. Serum E2 concentrations depicted after 14-day treatment. Significant to combined controls: ***P<0.001
Figure 2.6: Mammary gland ductal and alveolar development
One fourth abdominal mammary gland was collected on day 14 of hormone treatment, fixed in 10% Neutral Buffer Formalin and stained with carmine alum. A. Hormone-free control groups B. Parous control and estradiol treatment
Figure 2.7: E2-mediated gene expression is not impacted by sterilization of the 50µg E2 pellet

Pellets were sterilized by 5kGy Irradiation (IR), ethylene oxide (EO), autoclaved, or left unsterilized, and implanted subcutaneously in mice for 14 days. Number of animals per group indicated below graph. Fold change in Progesterone receptor (Pgr) gene expression relative to control. Red point indicates outlier, 19.04, removed in analysis. Significant to combined controls: *P<0.05
Figure 2.8: Serum E2 levels of mice treated with 50µg or 100µg E2 pellets
Primed silastic pellets with 50µg or 100µg of E2 were sterilized with 5kGy irradiation and implanted subcutaneously behind the neck. Time points of serum collection and concentration indicate days since pellet implant. For each time point n=3.
Figure 2.9: Relative gene expression of 50µg and 100µg over 14-day time course
A. Progesterone receptor (Pgr) relative gene expression, B. Amphiregulin (Areg) relative gene expression. For each time point n=3. Significant to combined controls: *P < 0.05, **P<0.01, ***P<0.001
Figure 2.10: Percent BrdU positive cells in mice treated with E2 pellets
Mice treated with 50µg or 100µg E2 pellets for 14 days, fourth abdominal mammary glands were formalin fixed and paraffin embedded and underwent BrdU staining for proliferation in 3 day intervals. For each time point n=3. Significant to control: *P<0.05, **P<0.01, ***P<0.001
A. Comparison of priming media from silastic pellets and mouse sera. B. In vivo estrogenic activity of silastic capsules. Significant relative to media no serum: *P < 0.05, **P < 0.01, ***P < 0.001

Figure 2.11: Estrogen and biological activity found in control pellets
Silastic pellets are primed in media for 24 hours prior to implantation into animals, media is saved and tested on E2-specific ELISA and ERE-luciferase assay. A. Comparison of priming media from silastic pellets and mouse sera. B. In vivo estrogenic activity of silastic capsules. Significant relative to media no serum: *P < 0.05, **P < 0.01, ***P < 0.001
CHAPTER 3

ACUTE ORAL TREATMENT WITH DIFFERENT AGONISTS TO INDUCE OPTIMAL RADIATION RESPONSE

Introduction/Rationale

After the completion of aim 1 it was apparent that some part of the hormone delivery system in our animal model was not successful. The control treated animals appeared to have similar responses to the parous animals, implying that these animals had been exposed to hormone. Further testing suggested that the cellulose base may provide estrogenic activity, as a result we decided to modify our animal model. Therefore, we changed the hormone delivery method from subcutaneous silastic implants to oral administration of the desired hormones and agonists. Additionally, a collaborative study with our lab administers xenoestrogens via oral dosing with oil. Switching the hormone delivery method would allow us to compare the agonists to work done with xenoestrogens. Oral hormone delivery can also be tested and compared to the acute response in ovariectomized mice treated with daily injections (Becker et al. 2005) and silastic pellets (Roman-Perez, unpublished), figure 3.1. Orally dosing estrogen using oil as a vehicle has not been published, but other oral vehicles have been used (Table 3.1). The goal of hormone treatment is to elevate the animal’s serum estradiol levels above physiologic levels of a normally cycling mouse (5-70 pg/mL; Walmer et al. 1992). Optimally, we intend to mimic the effects in parous animals, where E2 serum levels should surpass that of natural pregnancy which reaches a maximum level of 110 pg/mL (McCormack & Greenwald 1974).
**Material and Methods**

**Animals and animal experiments:** BALB/c mice were housed in a temperature-controlled rodent vivarium with 12hr alternating day/night light cycle. All procedures were in accordance with protocols approved by the Institutional Animal Care and Use Committee. All mice were maintained on LabDiet 5058 (PicoLab St. Louis, MO, USA) 20% protein diet and water ad libitum. Animals were ovariectomized at 8 weeks of age and allotted 2 weeks to clear endogenous hormones. Mice were treated orally for four days with E2 (0.25µg/µL/g bodyweight), PPT (250µg/µL/g bodyweight), E2+P4 (25µg/µL+25µg/µL/g bodyweight), DPN(250µg/µL/g bodyweight), or vehicle (1% DMSO) control. On the fourth day of treatment mice were irradiated (5 Gy) 6 hours prior to euthanasia, and injected intraperitoneally with 70µµg/g body weight of BrdU (Sigma Aldrich) that was prepared at 10mg/ml in PBS and filter sterilized.

**Hematoxylin and Eosin Stain:** Vaginal smears were taken and spread on slides immediately at time of tissue harvest. The smears were allowed to air dry for multiple days, fixed in 10% NBF, stained in hematoxylin for 4 minutes and eosin for 12 seconds.

**Whole Mount Preparation:** Whole 3rd thoracic mammary glands were fixed in Carnoy’s Fixative (6:3:1 ethanol:chloroform:acetic acid) and then stained in carmine alum solution (2g/L; 10mM aluminum potassium sulfate). Glands were dehydrated in graded ethanol, cleared in xylenes and mounted on slides with permount. Branching was quantified using ImageJ with an overlaid grid of 30,000 pixels^2. Every duct, branching point, alveoli, and terminal end bud that was at a grid intersection was counted.

**Enzyme-Linked Immunosorbent Assay (ELISA):** Coagulated whole blood was centrifuged at 7500rpm for 5 minutes to separate serum. Serum estrogen will be
quantified sing 17β-estradiol specific ELISA (Calbiotech) as per manufacturer instruction.

**RT-qPCR:** RNA was isolated from frozen tissues using 1mL TRIZol reagent (Life Technologies) according to the manufacturers instruction. cDNA was then made using 1μg RNA and amplified using ProtoScript II first strand cDNA synthesis kit (New England Biolabs). Gene expression was quantified using SYBR (BioRad) as per manufacturer instruction. Pgr forward primer: 5’-GGTGGGCTCTCAACCATG-3’, Pgr reverse primer: 5’-GACCACATCAGGCTCAATGCT-3’, Areg forward primer: 5’-GGCTTGCAATGATTCAACT-3’, Areg reverse primer: 5’-AAGAAAAACGGACTGTGCA-3’, Esr1 forward primer: 5’-CAGCAACATGTC AAAGATCTCC-3’, Esr1 reverse primer: 5’-GCTCCTAACTTGCTCTGGAC-3’, Wnt5a forward primer: 5’-GAATCCCATTGCAACCCCTCACC-3’, Wnt5a reverse primer: 5’-GCTCCTCGGTACATTTTCTGCCC-3’, Tgfβ2 forward primer: 5’-GCAGCAATTATCTGCACATT-3’, Tgfβ2 reverse primer: 5’-GCTAATGTTGTTGCCCTCCT-3’, Cebpδ forward primer: 5’-ATGTTGGGTGTGGAAGAGAGC-3’, Cebpδ reverse primer: 5’-TCTTTGCCCCCTGCAAGTTTCT-3’. Esr2 Prime PCr Primer (BioRad)12:76145488-76158646 (chrom. Location)

**Terminal Deoxynucleotide Transferase dUTP Nick-End Labeling (TUNEL):** Four-micron paraffin embedded sections were de-paraffinized and subjected to TUNEL assay (Millipore; cat# S7101) to observe apoptosis within the mammary. Protocol was altered slightly from manufacturers suggestion to best suit mouse mammary glands, see appendix B.
Quantification and analysis: Quantification of TUNEL: A total of 1200 mammary epithelial cells were counted for each slide to determine the percent of cells that were undergoing apoptosis. Cells were counted magnified on ImageJ (http://rsb.info.nih.gov/ij/download.html). Percentages calculated as total positive out of 1200. Error bars indicate SEM.

Results

Determine optimal E2 dosage

Treating animals orally with tocopherol stripped corn oil has not been studied in our lab. In order to determine dosage and tissue harvest parameters a preliminary test was performed. Based on data collected in table 3.1 mice were treated daily with 0.25μg/uL/g bodyweight daily for 4 days. In order to determine the ideal time to harvest tissues, half the mice were harvested 6 hours after final treatment to allow for irradiation and the other half were irradiated the following day, 24 hours post final treatment. At either time of tissue harvest (6hrs or 24 hrs) serum E2 levels of the animals were above 100 pg/mL (figure 3.2). In the initial ELISA, serum levels 6 hours post final treatment were 591.66+/− 43.3pg/mL and serum levels from those harvest 24 hours post treatment dropped significantly to 323.15+/−23.05pg/mL, p-value=0.032. Decisions for the project were made based on this test. Thus, tissues were harvested 6 hours post final treatment to be certain E2 levels remain above the critical test level. However, when a second ELISA was run 5 months later, using serum from the same set of animals harvested 6 and 24 hours post treatment, levels were significantly lower than the original ELISA (90.6 pg/mL, p= 0.02 and 4.2 pg/mL, p= 0.005, respectively).

Physical Changes of Ovariectomized Mice Caused by Hormone Treatment
Ovariectomized (ovx) mice should have low serum E2 levels because removal of the ovaries removes the production of endogenous hormones. 17-β specific ELISA was used to determine serum levels after 4 days of oral hormone treatment in ovariectomized mice. E2 serum levels of control mice averaged 5.91 +/- 1.64 pg/mL (figure 3.3). In figure 3.3 statistical outliers are included and indicated in red, but the values have been removed for determining the mean, standard error, and p-values (figure 3.3 middle) or not (figure 3.3 bottom). Animals treated with PPT and DPN were also anticipated to have low serum E2 concentrations because of the specificity of the kit. The two different doses of PPT varied slightly, with 7.15 +/- 1.74 pg/mL for PPT and 1.95 +/- 0.36 pg/mL for 2xPPT. In the PPT group there was one animal whose serum E2 reading was 109.9, which was removed as a statistical outlier. Serum E2 in the DPN-treated group was low as expected (mean 3.7 +/- 1.36 pg/mL). This statistical analysis removes one outlier with 63.87 pg/mL. E2 and E+P both are anticipated to increase serum E2 levels after 4 days of hormone treatment and resulted in 46.12 +/- 15.2 pg/mL and 79.5 +/- 10.3 pg/mL respectively. Both sets of animals (E2 and E+P) were given equal doses of E2 in oil, with the only difference being the addition of progesterone. The removal of outliers confirms that treatment with both E2 and E+P cause a significant increase in serum E2 level over control, although not the desired effect of 110pg/mL serum E2.

Removal of the ovaries impacts the uterine horns and tubes of the mice. Lacking the production of endogenous hormones, the uteri regress and appear hypertrophic without the introduction of an exogenous hormone. E2, E+P, and PPT all act via ERα and would be expected to maintain proliferation in the uteri and result in larger uteri than control-treated ovx animals. Uteri from mice treated with E2 were significantly larger than control
treated animals (0.328 g, p=0.0099; (figure 3.4 A and B). E+P did not cause a significant change from the control-treated animals and uteri were about 0.20 g. Interestingly, the mice treated with DPN had uteri that were significantly smaller than the control mice (0.123 g, p=0.04). DPN acts via ERβ (Leitman et al. 2010), which could account for this further restriction on proliferation. PPT initially did not cause a difference in uterine wet weight, therefore a second test experiment was completed with 2 times the initial dosage of PPT. Oddly, these 2xPPT-treated mice exhibited a significant decrease in uterine wet weight compared to the controls (0.11 g, p=0.019).

Without exogenous hormone treatment ovx mice should have no signs of estrous when assessing vaginal smears. However, treating with E2, E+P, and PPT should all act as replacement agonists and cause vaginal smear cytology that resembles proestrus and estrus similar to a naturally cycling mouse. The mice in this experiment are mostly consistent across treatment groups (table 3.3). Most ovx control mice, lacking exogenous estrogen, had vaginal cytology representative of diestrus. The 2 of 8 control treated mice that had estrus-like cytology also had uteri weighing 0.258 g and 0.2274 g, both above average of the control 0.1811 g, and may indicate some residual presence of endogenous estrogen. One of these animals, #5359, was the removed outlier due to a serum E2 concentration of 67.79 pg/mL (figure 3.3). Aligning with the results from uterine wet weights, mice treated with PPT did not appear to have any estrogenic response as all but the serum E2 outlier (5350) had diestrus-like vaginal cytology. Animals treated with DPN also have cytology representative of diestrus. Animals treated with E2 and E+P exhibited vaginal cytology representative of the two higher stages of the estrous cycle: estrus and proestrus.
At the completion of puberty mouse mammary glands have primary and secondary ducts that spread to the edges of the fat pad (Hennighausen & Robinson 2005). Ducts begin this process of expanding at the onset of pubertal hormone release and continue until 10-12 weeks of age (Richert et al. 2000). At time of ovx, animals were 7 weeks of age, and although they were young reproductive adults, they may not have fully filled fat pads with ductal outgrowth. As the treatment period is only 4 days, any major morphological changes were not anticipated. At the end of the treatment period the majority of ducts were thin and minimally developed (figure 3.5A, Supplemental figure A.1). The control treated group should be the baseline for this minimal development, however one animal, #5342, had more advanced ductal growth. Observing hematoxylin and eosin staining of the uteri gave an explanation for the advanced mammary gland morphology found in 5342: an ovary missed during ovariectomy (figure 3.5B). This animal also was observed with estrus-like cytology but had low serum E2 concentrations, 6.46 pg/mL. Visually, PPT- and DPN-treated animals had no noticeable differences in branching from the control treated animals, which corresponds to a lack of physical impact from these agonists. However, E2- and E+P-treated animals appeared to have varied development after 4-day hormone treatment and were compared to controls by quantifying at ducts, branching points, alveoli, and terminal end buds (TEB) (figure 3.5C). Treatment with E2 significantly increased the number of branching points and alveoli compared to control treated (1.6- and 3.6-fold, respectively). E+P treatment also increased the number of alveoli compared to control (~1.6 fold), with no statistical change in the number of branching points. However, there was also a decrease in the number of TEB (1.9-fold) caused by E+P treatment that was not
seen with E2 treatment alone. There was no statistical difference between treatment with E2 and treatment with E+P.

**Gene Expression from Hormone Treatment**

Differences in the ligand binding domain of the two estrogen receptors alters how they each interact with the different agonists. One of the fourth abdominal mammary glands from each of the animals was used to make RNA and cDNA and seven genes were tested via RT-qPCR. Two of these genes are considered ERα-regulated: progesterone receptor (Pgr) and amphereregulin (Areg); three are potentially ERβ-mediated: Wnt5A, Tgfβ2, and CCAAT/enhancer-binding protein delta (Cebpδ). We compared expression for ERα (Esr1) and ERβ (Esr2) to see if there was a specific negative feedback regulation of either receptor. Pgr was significantly increased in E2-treated mice (4.1-fold) relative to the control mice (figure 3.6). E+P-treated, PPT, and DPN did not transactivate expression of Pgr. Areg relative gene expression is significantly elevated relative to the control for both E2 (4.5-fold) and E+P (2.7-fold) treated animals (p=0.0016, 0.03 respectively; figure 3.7), but PPT and DPN failed to transactivate expression of Areg. Wnt5a (figure 3.8) and Tgfβ2 (figure 3.9) are both significantly increased with E2 treatment, but E+P and the other agonists had no effect. Removal of outliers did not alter the significance with Wnt5a gene expression but it did enhance the significance of Tgfβ2 with E2 animals from p=0.043 to p=0.014. Relative gene expression of Cebpδ did not change for any treatment (figure 3.10).

For many hormone receptors, ligand-dependent receptor activation causes negative-feedback expression of the receptor expression. However, there were no significant differences in relative gene expression for Esr1, the gene encoding for ERα, (figure 3.11). Esr2 encodes for ERβ, and we observed a significant increase in relative
gene expression for 2xPPT (1.8-fold), and DPN (1.7-fold). Prior to the removal of outliers PPT also appeared to be significantly increased (figure 3.12). While E2-treatment had no effect on the expression of Esr2, E+P significantly repressed expression (p=0.04).

**Apoptosis as a Marker for Genomic Surveillance**

Parous animals have increased apoptotic levels within the mammary gland. Treatment with E2 was anticipated to mimic this increase in apoptosis as determined with the TUNEL assay. Animals treated with E2 experienced an average of 9.23% TUNEL positive cells, which was a significant increase over control (3.78-fold, figure 3.13 and supplemental figure A.2). There was some variation among E2 treated animals, ranging from 0.43% to 16.5 % TUNEL positive cells. Comparison to other tests does not provide an explanation for this large apoptotic range. The E2 treated animal with the lowest (0.43%) TUNEL positive cells, were not abnormal in comparison to the rest of the treatment group in any other test category. It was also in an estrus-like stage, with similar serum and gene expression levels. The other of the two mice with the low TUNEL positive cells (1.83%) had the lowest E2 serum level as well (7.5 pg/mL). However, this same animal had higher levels of Pgr gene expression (4.7 relative expression, figure 3.6) and high levels of mammary gland branch points and alveoli (45 and 31 respectively, figure 3.5). Interestingly, treatment with E+P resulted in 3.38% TUNEL positive cells, which is not a significant change compared to control treated animals.

Analysis of percent TUNEL positive aims at counting 1200 total cells, however, in instances where the mammary gland did not contain 1200 cells, as many cells as present were counted. Only in one case did this result in the removal of an animal, 5370, an E+P treated animal which only had 81 total cells in the stained slide. This lack of ducts likely is
related to the section used for IHC, as it was not far below average ducts within in its whole mount (62 ducts).

**Discussion**

Ovariectomizing mice comes with the expectation that all endogenous estrogens are removed and therefore anything naturally influenced by E2 should be eliminated. Therefore, seeing elevated serum E2 levels in oil-treated control ovx mice is disconcerting especially after detecting estrogenic activity in the cellulose used as the previous vehicle. We use Calbiotech’s ELISA to assess serum E2 levels, and a study done comparing E2 serum detection kits deemed this kit as one of the most successful (Haisenleder et al. 2011). Even so, these investigators observed serum levels in ovx mice that were unexpectedly elevated and claimed an inability to distinguish serum E2 between ovx and intact animals when intact animals were at lower E2 stages (ie not in proestrus). Because of these results, the average serum levels of ~6 pg/mL in control animals, is reasonable because it would be unreasonable to expect an average of 0 pg/mL.

Treatment with E2 consistently changed responses compared to control treated animals. Acute, 4-day treatment, was able to significantly increase uterine wet weights (1.8-fold) and serum concentrations (7.8-fold) compared to control-treated animals. This short-term treatment of E2 was also able to induce increased mammary gland development, significantly increasing the branching points and alveoli over the control-treated animals. Additionally, it was able to significantly increase relative gene expression in the mammary glands for 4 of the 7 genes tested: Pgr, Areg, Wnt5a, and Tgfβ2.

E2 treatment also increased radiation-induced apoptosis compared to control (p=0.025). With an average of 9.23%, E2-treatment induced levels of apoptosis similar to
historical values of parity (8-12%). Previous work done within our lab showed that acute
treatment with PPT, an ERα agonist, was able to increase apoptosis levels and proliferation
levels. Alternatively, treatment with ERβ agonist DPN only induced increased apoptosis
(Roman-Perez unpublished). Both of the ER agonists in this experiment did not appear to
cause an induction of apoptosis, averaging around 2% TUNEL positive. In fact, increasing
the PPT dosage decreased the percent positive cells from 2.75% to 1.89% (1.3-fold). DPN
was almost identical to control percent positive, 2.25%. There was one outlier in this set,
indicated in red, with 8.5% TUNEL positive cells.

Intact control treated animals historically have 2-3% radiation-induced apoptotic
cells. The animals in this experiment fell approximately within this range, with an average
of 2.46% except for one statistical outlier which had 22.92% TUNEL positive. While this
is the animal that retained an ovary, there is no clear explanation for this exceptionally high
level of apoptosis. Additionally, this animal was removed from analysis of mammary gland
morphology because of the influence of the ovary, and had 52 alveolar points. Despite the
retention of one ovary, the serum E2 level of this animals was within the accepted range,
2.24 pg/mL.

Addition of progesterone to E2 treatment resulted in a lack of morphological
changes. The uteri of the E+P-treated animals had similar wet weights to that of control-
treated ovariectomized uteri. Additionally, treatment with E+P does not result in a
significant transcriptional change with Pgr gene expression. However, in both cases
treatment with E2 alone resulted in significant change over the control animals. These
findings suggest that progesterone modulated the function of estrogen in vivo. Progesterone
has been shown to oppose estrogen-mediated proliferation within the uterus (Kim &
Chapman-Davis 2010 & Clark & Sutherland 1990). Furthermore, both progesterone receptors (PR-A and PR-B) are able to act as ligand dependent repressors of transcriptional activity mediated by estrogen receptors (Kraus et al. 1995). The ability of E2 treatment to increase uterine weight and gene expression while E+P treatment fails to do so supports the opposing functionality of progesterone. E+P treatment, however, was able to increase the number of alveoli in the mammary gland indicating an increase in proliferation. Additionally, E+P treatment did not alter radiation-induced apoptosis.

Treating animals orally for 4 days suggests that PPT might not be absorbed through oral treatments. The first indicator was a lack of observable difference in uterine wet weights, which led to the addition of a second treatment set given at double the dosage. Additionally, PPT is an ERα agonist (Leitman et al. 2010) and should result in an increase in relative gene expression for ERα regulated genes. However, there was no response from the ERα regulated genes tested: Pgr or Areg. Interestingly, when the dose of PPT was increased, Esr2 gene expression was increased relative to the control (p=0.004).

Treating with DPN in the past has been done either with daily injections (Frasor et al. 2003) or silastic implants (Roman-Perez unpublished), and orally dosing was previously untested. While the effectiveness of PPT came into question immediately upon measuring uterine wet weights, we were intrigued by the significant decrease in uterine size of DPN-treated animals compared to control treated animals (1.5-fold, figure 3.4). A decrease in uterine wet weights not only implied that DPN was successfully absorbed via oral treatment, it also implied an impact on uterine growth. This is contradictory to Frasor et al. (2003) who found that DPN does not impact uterine weight at any of their tested dosages. However, they did find that DPN was able to antagonize the increased uterine weight
induced with PPT treatment by 30%. Further analysis of DPN treated animals via oral
dosing resulted in no significant changes with any of the 7 genes tested. This, as well as
the TUNEL results, indicates that DPN was not sufficient to induce a response when treated
orally at 250µg/µL/g bodyweight.

Orally dosing animals eliminated the cellulose matrix, and provided to be a
successful vehicle for the control animals. Additionally, it was a successful vehicle for E2-
treatment and was able to highlight differences seen with the addition of progesterone to
E2 treatment. Using a new method of treatment brings into question whether the agonists
were unable to be absorbed via oral dosing or if oral agonist delivery is not the right choice
to test my hypothesis.
### Table 3.1: Estradiol delivery in various mouse experiments

A compilation of information from other mouse experiments were used to design a new E2 delivery method, daily oral dosing in oil.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum Levels</th>
<th>What we should do</th>
<th>Source</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.12ug/30g mouse/day</td>
<td>20-120pg/mL fluctuation through day</td>
<td>3-5x this dosage to reach pregnancy levels</td>
<td>Ström et al., 2012</td>
<td>This is about 0.037ug/gBW Nutella administration</td>
</tr>
<tr>
<td>56ug/kg BW/day</td>
<td>physiological range, averages below 50pg/mL closer to pregnancy levels in short term, reading right after administration</td>
<td>Increase – we want above pregnancy level throughout entire treatment period, not just immediately post treatment</td>
<td>Ingberg et al., 2012</td>
<td>This is equivalent to 0.056ug/gBW, or 1.68ug/30g/day for comparison to Ström paper Nutella administration</td>
</tr>
<tr>
<td>0.054ug/g BW/day</td>
<td>Physiological changes in mammary glands (serum not assessed)</td>
<td></td>
<td>Berryhill et al., 2016</td>
<td>E2 in water ad lib</td>
</tr>
</tbody>
</table>

### Table 3.3: Estrous cycle stage in ovariectomized mice treated with agonists for 4 days

Vaginal smears were collected at time of harvest after 4-day treatment and stained with hematoxylin and eosin to evaluate estrous cycle-like cytology.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proestrus</th>
<th>Estrus</th>
<th>Metestrus</th>
<th>Diestrus</th>
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<tr>
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<td>2</td>
<td>0</td>
<td>6</td>
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<tr>
<td>E2</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>E+P</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PPT</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>2xPPT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>DPN</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure 3.1: Previous work with acute hormone delivery in ovariectomized mice

Work done previously by A. Becker et al. (2005) and B. Roman-Perez (unpublished) can be used to make comparisons about the success with the new oral hormone delivery method.
A small sub-set of animals were treated with E2 orally for 4 days and then harvested either 6 hours after final treatment (n=2) or 24 hours later (n=2) in order to determine final harvest time. Serum E2 levels of the treatment oils were also calculated. A. Pre-test 1, B. Pre-test 2; tests were conducted 5 months apart.
Figure 3.3: 17-β estradiol serum concentrations
Serum was collected after 4 days of hormone treatment and was tested for E2 concentration on a 17-β estradiol specific ELISA (Calbiotech). Each point represents one animal (ctrl n=8; E2, E+P, PPT, 2xPPT, DPN n=6), ***P < 0.001. Outliers removed for comparison are shown in red. Table below graph shows mean and p-value with the outlier (red) removed (middle) or included (bottom).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>E2</th>
<th>E+P</th>
<th>PPT</th>
<th>2xPPT</th>
<th>DPN</th>
</tr>
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<tbody>
<tr>
<td>Without Outliers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>5.91</td>
<td>46.12</td>
<td>79.51</td>
<td>7.15</td>
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<td>3.70</td>
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<td>0.376</td>
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<tr>
<td>Outliers Included</td>
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<tr>
<td>mean</td>
<td>13.65</td>
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<td>79.51</td>
<td>24.28</td>
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<td>P-value</td>
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<td>0.5508</td>
<td>0.2279</td>
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Figure 3.4: Uterotrophic response to orally dosed animals
A. Uterine wet weights taken at time of tissue harvest. Number of animals per treatment notes on graph. B. Uteri prior to fixation. Significant to control, *P<0.05, **P<0.01
Third thoracic mammary glands were fixed in Carnoy’s fixative at time of harvest, and then stained with Carmine Alum. A. Representative image from each treatment group. Magnification 40X. B. 5342 Control mammary gland and H&E of missed ovary, magnification 40X and 100X respectively. C. Quantification of mammary structures, significant to control *P<0.05, **P<0.01
Figure 3.6: Progesterone receptor relative gene expression
Control n=8; E2, E+P, PPT, 2xPPT, DPN n=6. TTest to control, **P<0.01
Outliers removed for comparison are shown in red, calculated by 1.6 x StDev. Tables below graph shows mean and p-value with the outlier (red) removed (middle) or included (bottom).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>E2</th>
<th>E+P</th>
<th>PPT</th>
<th>2xPPT</th>
<th>DPN</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>mean</td>
<td>0.69</td>
<td>2.828</td>
<td>0.7115</td>
<td>0.525</td>
<td>0.393</td>
<td>0.2922</td>
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<tr>
<td>P-value</td>
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<tr>
<td>mean</td>
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<td>0.7115</td>
<td>0.6197</td>
<td>0.393</td>
<td>0.2922</td>
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<td>0.4436</td>
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Figure 3.7: Ampheregulin relative gene expression
Control n=8; E2, E+P, PPT, 2xPPT, DPN n=6. TTest to control, *P<0.05, **P<0.01

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>E2</th>
<th>E+P</th>
<th>PPT</th>
<th>2xPPT</th>
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<tr>
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Figure 3.8: Wnt5a relative gene expression
Control n=8; E2, E+P, PPT, 2xPPT, DPN n=6. TTest to control, **P<0.01
Outliers removed for comparison are shown in red, calculated by 1.6 x StDev. Tables below graph shows mean and p=value with the outlier (red) removed (middle) or included (bottom).
Figure 3.9: Tgfβ2 relative gene expression
Control n=8; E2, E+P, PPT, 2xPPT, DPN n=6. TTest to control, *P<0.05
Outliers removed for comparison are shown in red, calculated by 1.6 x StDev. Tables below
graph shows mean and p=value with the outlier (red) removed (middle) or included (bottom).

<table>
<thead>
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</thead>
<tbody>
<tr>
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<td>2xPPT</td>
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<tr>
<td>mean</td>
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<td>1.623</td>
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<tr>
<td>P-value</td>
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<td>E+P</td>
<td>PPT</td>
<td>2xPPT</td>
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<tr>
<td>mean</td>
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<td>1.623</td>
<td>1.1696</td>
<td>1.027</td>
<td>0.6605</td>
</tr>
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<td>P-value</td>
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<td>0.422</td>
<td>0.6494</td>
<td>0.2423</td>
<td>0.6926</td>
</tr>
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</table>

Figure 3.10: Cebpδ relative gene expression
Control n=8; E2, E+P, PPT, 2xPPT, DPN n=6. TTest to control, no significance
Figure 3.11: Esr1 relative gene expression
Control n=8; E2, E+P, PPT, 2xPPT, DPN n=6. TTest to control, no significance
Figure 3.12: Esr2 relative gene expression
Control n=8; E2, E+P, PPT, 2xPPT, DPN n=6. TTest to control, *P<0.05, **P<0.01.
Outliers removed for comparison are shown in red, calculated by 1.6 x StDev. Tables below
graph shows mean and p-value with the outlier (red) removed (middle) or included (bottom).
Figure 3.13: TUNEL staining for apoptotic cells
TUNEL IHC conducted on 4th abdominal mammary glands, brown cells indicate apoptotic cells. A. Percentage of TUNEL positive cells, outliers removed for comparison are shown in red, calculated by 1.6 x StDev. Tables below graph shows mean and p-value with the outlier (red) removed (middle) or included (bottom). B. representative images from each treatment type. *P<0.05
CHAPTER 4

COMPARE RADIATION SENSITIVITY IN MAMMOSPHERES FROM PAROUS AND NULLIPAROUS MAMMARY GLANDS

Introduction/Rationale

Determining the mechanism for the protective effect of early parity would allow for the development of preventative methods to decrease the risk of breast cancer development. The protective effect of pregnancy has also been observed in mice and rats resulting in a 75% decrease in carcinoma development as compared to controls (Medina 2005). Morphological changes that occur in the mammary gland during pregnancy may alter the behavior of mammary stem cells (MaSCs) (Meier-Abt et al. 2013, Wagner et al. 2002, Boulanger et al. 2005). In the mouse, parity results in drastic changes in subpopulations of mammary epithelial cells suggesting that luminal progenitors (Luminal Sca1+) are reduced, but stem cells are not (figure 4.1). Opposing this hypothesis, other work suggests that parity causes persistent changes in the mammary gland and results in a new subpopulation of luminal progenitor epithelial cells referred to as PI-MECs (parity-induced mammary epithelial cells, Wagner et al. 2002, Boulanger et al. 2005). PI-MECs develop during the first pregnancy and have self-renewal capacities to assist in alveologenesis and ductal formation during subsequent pregnancies. Changes in mammary epithelial progenitor populations could account for the protective effect of pregnancy. We hypothesize that parity will alter the radiation sensitivity of MaSCs. Considering that the age at first pregnancy is key to this protection in women, it is practical to believe that temporal exposure to pregnancy hormones will also impact protection in rodents. Varying the age of parity in the mice tested allows me to test whether the age at pregnancy is
impacting mammary stem cells and radiation sensitivity. To be certain that involution had occurred completely we waited 5-6 weeks post involution, based on Meir-Abt et al. (2013) who confirmed that involution is completed between 28 and 40 days post-weaning.

The dissertation of Luwei Tao demonstrated that one mammosphere was equivalent to one MaSC by performing an *in vitro* differentiation assay and transplantation assay (Unpublished). Using this information, I can count mammospheres and assume an approximate 1:1 ratio of mammospheres to MaSCs and study the differences in radiation sensitivity in nulliparous and parous animals. Dr. Tao’s preliminary work also demonstrated that parity does not affect the ability of mammosphere-initiating cells to self-renew. However, when exposed to irradiation, mammospheres from parous mice experience a decrease in population as compared to age-matched nulliparous mice (figure 4.2).

Wnt signaling is a key pathway in the regulation of MaSCs (Roarty & Rosen 2010). Wnt4 is localized the luminal compartment of the mammary gland and is required to mediated side branching of mammary ducts that occurs during pregnancy. Parous cells have been shown to have a 3.4-fold downregulation of Wnt4 (Meier-Abt et al. 2013). Secreted frizzled-related protein 1 (Sfrp1) acts as a Wnt inhibitor and belongs to a family of cysteine rich endogenous regulators of Wnt (Finch et al. 1997).

The TM40A cell line is a mammary epithelial cell line derived from BALB/c mice and retains wild-type p53 (D.J.J., unpublished data). This cell line can be used to grow mammospheres *in vitro* allowing us to study progenitor cell behavior with treatments in media preliminarily to animal studies. Because Wnt4 and Sfrp1 are both implicated in regulating mammary progenitor cells, our objective is to compare their effect on
mammosphere forming capability with and without irradiation in mammary cells. By doing this in both TM40A cells and cells dissociated from mammary glands of nulliparous BALB/c mice we hope to have a more complete understanding of their impact on mammosphere initiating cells.

**Materials and Methods**

**Animals:** BALB/c mice were housed in a temperature-controlled rodent vivarium with 12hr alternating day/night light cycle. All procedures were in accordance with protocols approved by the Institutional Animal Care and Use Committee. All mice were maintained on LabDiet 5058 (PicoLab St. Louis, MO, USA) 20% protein diet and water ad libitum. Female mice were put with males at 5, 9, and 24 weeks of age. Pups were removed at day 4 of lactation and dams were given 5 to 6 weeks for complete involution.

**Mammary gland harvest and digestion:** Both fourth abdominal mammary glands were minced and digested in MEM (Gibco) supplemented with 5% fetal bovine serum (Gibco, Paisley, UK), 2mg/mL collagenase (Worthington Biochemicals, Freehold, NJ), 100u/ml hyaluronidase (Sigma), 100u/ml antibiotic/antimicotic (Gibco) and 100μg/ml gentamicin (Gibco) for 6 hours at 37℃. Cells were further dissociated with 1mL pre-warmed 0.25% trypsin-EDTA (Gibco) and 200μL 1mg/mL DNase1, Grade II (Roche). Cell suspensions were sieved through a 40μm cell strainer to achieve a single cell suspension.

**Mammosphere Culture:** Primary single cells were seeded on ultra-low attachment dishes or plated at a density of 20,000 viable cells/mL in serum-free mammary epithelial growth medium (HuMEC, Gibco) supplemented with B27 (Gibco), 20ng/ml EGF (Sigma), 20ng/ml bFGF (Sigma), 4μg/mL heparin (Sigma), 100u/mL antibiotic/antimitotic, 5μg/mL gentamicin. To passage mammary stem cells, mammospheres were collected after 7 days
and centrifuged at 800rpm and dissociated with 0.25% trypsin-EDTA and 60μL DNase1 and seeded at 1000 cells/well in a 96-well ultra-low attachment plate. To test IR-responses of mammosphere-initiating cells, single cell suspensions received 0-Gy (control) or 5-Gy dose (radiation response) of γ-irradiation from a cesium-137 irradiation source before being plated. After 7 days mammospheres were counted at 100x magnification. When treated, mammospheres were treated with 100ng/mL rWnt4 (R&D Systems), 1μg/mL rSFRP-1 (R&D Systems), or media alone as primary mammospheres for 7 days. Half were re-treated as secondary mammospheres for an additional 7 days.

**TM40A cell culture:** TM40A cells were maintained in Mammary Epithelial Cell Line (MECL) complete media: DMEM:F12 supplemented with 2% adult bovine serum (Gibco), 20ng/mL mEGF, 100u/mL antibiotic/antimicotic, 5μg/mL gentamicin, 10μg/mL insulin (Gibco). Cells were expanded as a 2D culture and treated with 100ng/mL rWnt4, 1μg/mL rSFRP-1, or media alone for 3 days. Cells were then dissociated into a single cell suspension with 0.05% Trypsin-EDTA. Cells were switched to HuMEC media supplemented with B27 (Gibco), 20ng/ml EGF (Sigma), 20ng/ml bFGF (Sigma), 4μg/mL heparin (Sigma), 100u/mL antibiotic/antimitotic, 5μg/mL gentamicin and treated again with 100ng/mL rWnt4 (R&D Systems), 1μg/mL rSFRP-1 (R&D Systems), or media alone and half were exposed to 5-Gy of γ-irradiation from a cesium-137 irradiation source prior to plating at 1000 cells/well in a 96-well ultra-low attachment plate. After 7 days mammospheres were counted at 100x magnification.
**Results**

**Parous and Nulliparous Mammosphere Radiation Response**

Female mice were put with males at 5, 9, or at 24 weeks and after involution, tissues were harvested at 13, 18, and 32 weeks, (figure 4.3 & 4.4, table 4.1). AMV mice for both 5 week and 9 week groups resulted in similar levels of secondary mammospheres, 67 and 65.8 per 1000 cells respectively, in the non-irradiated group. However, the non-irradiated mammospheres from the 24 week AMV group were significantly fewer than 5 week and 9 week AMV (47.5, p=0.0006 and p=0.0002 respectively). The non-irradiated mammospheres from the parous animals bred at 9 weeks had a significantly increased number (89.08), compared to those bred at 5 weeks (56.29, p=8.5E-11) and 24 weeks (36.53, p=1.4E-20).

While age at harvest may impact some differences in mammospheres quantities, there are still notable differences between parous and AMV non-irradiated mammospheres of the same age. Mammospheres from mice bred at 5 weeks and at 24 weeks produced significantly fewer than their AMV (p=0.04 and p=0.003 respectively). Interestingly, in the 9 week breeding group there were significantly more non-irradiated parous mammospheres than their age-matched virgin counterpart (p=1.4E-05).

Irradiation of secondary mammospheres from females bred at 5 and 9 weeks induced a significant decrease in mammospheres in the parous mammary glands (p=0.0017 and 0.0011, respectively), but not in the age-matched virgins (figure 4.3). This supports the hypothesis that parity causes a change in radiation sensitivity of mammospheres resulting in a decrease in stem cell self-renewal. However, although the mice bred at 24 weeks had the fewest mammospheres, and fewer mammospheres than their AMV counterpart, they
were not sensitive to irradiation, suggesting that radiation-sensitivity is only implemented by early parity.

The first set of mice completed were those bred at 9 weeks and this experiment was extended to include tertiary mammospheres (figure 4.4). Upon irradiation, tertiary parous mammospheres had a decrease in quantity, 41.06 compared to 53 non-irradiated mammospheres per 1000 cells. However, this difference is not statistically significant, p=0.06. Interestingly, there is a statistical decrease between AMV irradiated mammospheres and irradiated parous mammospheres (p=0.02). Tertiary mammospheres produce lower overall numbers than secondary mammospheres. Although the trends persist, the significance does not, therefore, only secondary mammospheres were counted after the completion of mice bred at 9 weeks.

**TM40A Mammosphere Response**

To investigate the effects of Wnt on mouse MaSCs, TM40A mouse cells were treated with 100 ng/mL rWnt4 or 1 μg/mL rSfrp1, a Wnt agonist and antagonist respectively. Cells were treated as a 2D culture before being plated as mammospheres. Early parity is known to decrease Wnt4 which causes a restriction to the proliferative abilities of MaSCs (Meier-Abt et al. 2013). This lends to the possibility that the addition of Wnt4 would increase proliferation. Surprisingly, in this 2D culture, both treatment with Wnt4 and Sfrp1 significantly decreased the amount of cells/mL compared to the control cell population (figure 4.5, p=0.001 and 3.1E-5 respectively). Sfrp1 is an agonist to Wnt4, therefore adding it was expected to inhibit natural Wnt and result in the decrease in cells.

After counting the 2D culture, cells were transferred to low-attachment plates to grow as mammospheres. The objective was to reveal the role Wnt4 plays in the radiation
sensitivity of parous animals. Half of the cells were re-treated with fresh treatment, and half were removed from the treatment media and plated in media alone. To try and mimic the radiation sensitivity seen in parous animal each set of cells was broken into irradiated and non-irradiated. However, mammosphere forming assays were not successful. When plated on low-attachment plates cells clumped together in a compilation of single cells rather than coherent mammospheres (figure 4.6).

**Treatment of Nulliparous Mammospheres**

Due to the inability to form mammospheres with TM40A cells, the experiment was repeated with mMECs. For this experiment, both 4th abdominal mammary glands were collected from 3 nulliparous animals and pooled to create a larger population of mMECs. When plating for primary mammospheres, cells were either treated with plain media, 100ng/mL Wnt4 media, or 1μg/mL Sfrp1 media. Upon passaging for secondary mammospheres, half of the pre-treated cells were re-treated with the same dosage. At this point, half of each treatment group was exposed to 5Gy irradiation, and plated for 7 days. Pre-treating the cells with either Wnt4 or Sfrp1 resulted in a significant increase over the control mammospheres, whether irradiated or not (figure 4.7 & 4.9). There were also significantly greater Sfrp1 non-irradiated mammospheres than Wnt4-treated non-irradiated mammospheres (124.25 to 111.62 per 1000 cells, p=0.038). Control and Wnt4 pre-treated mammospheres were not radiation sensitive. However, treatment with Sfrp1 induced radiation sensitivity, decreasing the number of mammospheres (p=0.003).

Since we were not sure whether mammospheres would need to be continuously treated to undergo any alterations, we had a second set of mammospheres that were re-treated with the same dosages upon plating for secondary mammospheres (figure 4.8 &
As with the pre-treated mammospheres there were significantly more Wnt4-treated, non-irradiated and irradiated mammospheres than there were control mammospheres. For the Sfrp1-treated mammospheres there were significantly less non-irradiated mammospheres (p<0.001) and there was no change between irradiated and non-irradiated as seen with the pre-treatment alone. However, the re-treated cells were in falcon tubes at room temperature for an extended period of time, and required more handling. Upon plating, there were some concerns about the reliability of the cells and their survival rate. For this reason, the data collected from the re-treated mammospheres needs to be repeated before any conclusions can be made.

**Discussion**

It is accepted that mice and humans gain protection against developing mammary tumors when they have a full-term pregnancy early in reproductive life. The mechanism behind this however is elusive. During pregnancy, the mammary gland undergoes a variety of changes that could alter the function of MaSCs (Meier-Abt et al. 2013, Wagner et al. 2002, Boulanger et al. 2005). Therefore, it was hypothesized that MaSCs alter during pregnancy and retain a long-term sensitivity to DNA damage. Using a mammosphere assay, and irradiation as a conduit for cellular damage, MaSCs from parous and nulliparous animals can be compared.

Female mice who began breeding at 5 and 9 weeks of age saw a decrease in mammospheres after exposure to irradiation, indicating that parity causes an increased sensitivity to cellular damage. This decrease in mammospheres is equivalent to a decrease in MaSCs, as shown by a mammospheres ability to fully regenerate a normal mammary gland when implanted into a cleared fat pad (Liao et al. 2007). Mammary stem cells have
extensive proliferation potential and a random mutation, even in this population, could result in tumor formations. Therefore, the decrease in MaSCs identified in parous mammary glands from 5 and 9 week breeders, but not 24 weeks breeders, correlates to the belief that there is a connection to the protective effect of an early pregnancy.

Interestingly, those bred at 9 weeks were the only parous animals who had an increased number of non-irradiated parous mammospheres over their AMV. Parity-induced mammary epithelial cells (PI-MECS) appear after pregnancy and lactation and they lead the repopulation of the mammary gland in following pregnancies (Wagner et al. 2002 & Boulanger et al. 2005). In Wagner & Boulanger’s (2005) study detecting PI-MECS, mice were 7-12 weeks of age and they did not test mice that were either younger or older. For this reason, it is not clear as to whether PI-MECS only occur within this 7-12 week age range. Mice in their earliest reproductive period, 5 weeks, have had minimal mammary gland expansion prior to hormone exposure, potentially limiting the induction of PI-MECS in comparison to mice in their prime reproductive period. These mice, represented by the 9 week age group, have already achieved full mammary gland expansion and would allow for maximum PI-MEC formation. Older mice, although reproductive between 2 and 10 months, have declining hormones. Additionally, in some mouse strains there is a 50% decline in fecundity at 7 months (Shimizu & Yamada 2000). If with increasing reproductive age mammary epithelium also declines, parity may produce fewer PI-MECS. This would explain why 9 week parous animals saw a striking increase in mammospheres that 5 and 24 week animals did not.

Decreasing Wnt4 in MCF7 cells was found to decrease proliferation by 30% and hinder cell growth and expansion (Vouyvitch et al. 2016). In 2D culture of TM40A cells,
the addition of Wnt4 decreased cell growth and proliferation (figure 4.5). Unfortunately, without the ability to treat mammospheres we cannot determine if this decrease is related to the progenitor cell population of TM40A cells. The inability of the TM40A cells to form mammospheres has no clear explanation. Unlike in past experiments done in this lab by Dr. Tao (2010), cells remained separated and did not form spheroid structures. The only difference in the control treated cells between past and recent TM40A cell experiments is that Tao et al. (2010) used that the TM40A cells were infected with DsRed-let7c-sensor.

As the TM40A cells failed to form mammospheres, we decided to treat nulliparous mouse mammary epithelial cells (mMECs) with Wnt4 or Sfrp1 in a similar manner as the TM40A. Wnt4 has been shown to increase anchorage-independent growth in mammary cell lines (Vouyovitch et al. 2016). This corresponds to the significant increase observed in mammospheres treated with Wnt4 (p<0.001 for No IR and IR). Wnt4 is also seen to be increased in mammary carcinomas, which could correspond to this increase in proliferative ability of mammosphere initiating cells. Because Wnt4 expression is decreased in parous animals (Meier-Abt et al. 2013), it was hypothesized that Sfrp1 would decrease the Wnt expression in the nulliparous mammospheres and result in mimicking radiation sensitivity to that of parous mammospheres. Although the non-irradiated mammospheres increased in number due to Sfrp1-treatment, there was a significant decrease in irradiated mammospheres such that those treated with Sfrp1 averaged 124.25 per 1000 cells and irradiated mammospheres decreased to 100.63 mammospheres per 1000 cells (p=0.003). Control treated mammospheres had similar numbers of secondary mammospheres to 5 and 9 week AMV, which supports reproducibility and allows comparison across experiments. Because there is a decrease in Wnt4 following parity (Meier-Abt et al. 2013), treating
parous mMECS with Wnt4 should negate the protective effect of parity and return radiation response to that of the AMV. Future work should investigate the effects of Ent4 and Sfrp1 on parous mammospheres.
Table 4.1: Secondary mammospheres from females bred at varying ages
Tabular representation of figure 4.3: Balb/c female mice put with a male at 5, 9, or 24 weeks. Pups were removed 4 days after parturition and females were given 5-6 weeks for complete regression of the glands, at which time they were harvest and plated as mammospheres. Secondary mammospheres from parous animals were compared to age match virgin animals (AMV). Number of animals indicated on graph. *P < 0.05, **P < 0.01, ***P < 0.001

Figure 4.1: Parity-induced changes in epithelial cell populations
Taken from Meier-Abt et al. 2013
Figure 4.2: Parous mammary stem/progenitor cells are susceptible to radiation

Taken from Luwei Tao dissertation 2011
Figure 4.3: Secondary mammospheres from female bred at varying ages

Balb/c female mice put with a male at 5, 9, or 24 weeks. Pups were removed 4 days after parturition and females were given 5-6 weeks for complete regression of the glands, at which time they were harvest and plated as mammospheres. Secondary mammospheres from parous animals were compared to age match virgin animals (AMV). Number of animals indicated on graph. *P < 0.05, **P < 0.01, ***P < 0.001
Figure 4.4: Tertiary mammospheres from female mice put with a male at 9 weeks of age
Mammospheres from females bred at 9 weeks were taken out to tertiary mammospheres under the same conditions as secondary mammospheres. *P < 0.05

Figure 4.5: Cells/mL in 2D culture after 3-day treatment
Cells were plated at 50,000cells/mL and then were treated with 100 ng/mL rWnt4 or 1 μg/mL Sfrp1 in MECL media and cultured for 3 days before counted. ***P<0.001
Figure 4.6: Unsuccessful treated TM40A Mammospheres
TM40A cells plated for mammosphere formation never formed true mammospheres. Cells were treated with control media, 100 ng/mL rWnt4, or 1 μg/mL Sfrp1 in MECL prior to plating and half of the Wnt4 and Sfrp1 cells were re-treated on plates destined for mammosphere formation.
Figure 4.7: Wnt4 and Sfrp1 treatment of nulliparous mammospheres
Balb/c female nulliparous mice were harvested at 7 weeks, both 4th abdominal mammary glands were collected and pooled from 3 mice and plated as mammospheres. Cells were plated as primary mammospheres with control media, media + 100ng/mL Wnt4, or media +1µg/mL. After seven days mammospheres were passaged and plates at 1000 cells/well, controls n=16, pre-treated n=8. Significance: *P < 0.05, **P < 0.01, ***P < 0.001
Figure 4.8: Nulliparous mammospheres treated at primary and secondary levels
When passaging mammospheres from primary to secondary spheres, half of the pre-treated spheres were re-treated with the same protein to hit all stages of development when plated as secondary spheres at 1000 cells/mL under the same pre-treatment conditions: control media, media + 100ng/mL Wnt4, or media +1μg/mL. Significance: **P < 0.01, ***P < 0.001
Figure 4.9: Secondary Mammospheres
Primary mammospheres were passaged to secondary and were plated at 1000 cells/mL for 7 days. Imaged at 200X
CHAPTER 5

SUMMARY AND FUTURE

Apart from age and genetics, reproductive history has the greatest impact on the development of breast cancer. It is well accepted that an early in life pregnancy results in postmenopausal protection against the development of breast cancer (Weinberg 2004). Understanding the mechanism behind this protection would provide a means for better understanding the development of breast cancer and the prevention of breast cancer in women.

The most common estrogen during reproductive years functions via two estrogen receptors, ERα and ERβ. We hypothesized that ERβ mediates the protective effect of breast cancer by increasing genomic surveillance in the mammary gland. Initially, this was tested by treating mice for 2 weeks with E2, PPT as an ERα agonist, or ERB041, an ERβ agonist. Using physical changes in the mammary glands and TUNEL staining for apoptotic responses we were able to determine that dosing was inconsistent and that control treated animals were exposed to estrogens. Further testing indicated that the control vehicle, cellulose, was providing biologically active estrogen, nullifying the controls.

Moving forward, an acute 4-day treatment was given orally using tocopherol stripped corn oil as a vehicle. This provided a successful control to compare and E2 as well as E+P treatment were functional. However, the two agonists, PPT and DPN, were variable in their impact. While DPN decreased uterine wet weights, and both PPT and DPN altered gene expression relative to controls in a few tested genes, there was not enough evidence to determine whether these agonists were in circulation or had estrogenic effects in the mammary gland. In the future, we need to address the best agonist to use to test ERβ, and
confirm that it is deliverable orally. Once this is determined, the persistent effects of estrogen receptor agonists, as in chapter 2, can be repeated. This would provide data to compare treatment with agonists to the impacts of parity and further test the hypothesis of ERβ mediating genomic surveillance.

Parity also increases genomic surveillance in stem cells, inducing radiation-sensitivity in parous mammospheres, whereas nulliparous mammospheres remain radiation-insensitive. This is an age-related response, that occurs only when the first full-term pregnancy occurs early in life. The radiation-sensitivity of parous mammospheres indicates that after parity, mammary stems cells are less proliferative or more easily eliminated in response to genomic damage. This can help to explain the protective effect of early in life pregnancy. To further test the mechanism behind this radiation sensitivity, nulliparous mammospheres were treated with Wnt4 and Sfrp1. Sfrp1 antagonizes wnt and we chose this pathway to mimic the reduced wnt-signaling in parous mice. An increased radiation sensitivity was observed in Sfrp1 treated mammospheres, however both irradiated and non-irradiated Wnt4 and Sfrp1 treated mammospheres produced more secondary mammospheres than control mammospheres. If pregnancy provides protection against breast cancer via a decrease in wnt, it would be reasonable that there would be fewer Sfrp1-treated mammospheres than control treated after irradiation. To further understand this, parous mammospheres should be treated with Wnt4 and Sfrp1, hypothesizing that Wnt4 would eliminate the radiation sensitivity seen in parous mammospheres. This would also help to further elucidate the reason behind and overall increase in mammospheres induced by both retreatments.
It would be exceptionally informative to treat mammospheres *in vitro* with estrogen receptor specific agonists, PPT and DPN. This would allow extended studies of whether ERβ mediates the protective effect of pregnancy. Additionally, it would allow for a succinct continuation and extension of the various parts of this work.

Treatment with Wnt4 and Sfrp1 was initially done with TM40A cells, however, these cells were unable to form mammospheres. Despite this, future testing can use let-7-ds-red TM40A cells, which would allow identification of progenitor cells without the need for mammospheres. Cells treated in 2D culture can then be fluorescence-activated cell sorted to identify the DS red progenitor cells.

As studies into the changes induced by parity continue, we get closer to comprehending the underlying mechanism behind the protective effect of parity. Using radiation, we were able take a step closer to understanding the differences between nulliparous and parous animals. Highlighting the differences between E2 and E+P treatment has increased our understanding of the changes induced by mimicking pregnancy. Furthermore, studying mammospheres indicated that progenitor cells are key in understanding the protective effect of pregnancy.
APPENDIX A

SUPPLEMENTAL FIGURES

Control

Treated

E2 Treated
E+P Treated

PPT Treated
Figure A.1: Wholemount 3rd Mammary Gland from 4d oil Treatment
Glands collected at tissue harvest were fixed in Carnoy’s Fixative and stained with Carmine Alum. Images organized by treatment (control, E2, E+P, PPT, 2xPPT, DPN), and separated by mouse tissue ID number. Images taken at 40X.
Control Treated

E2 Treated
E+P Treated

5366, 2.67%
5367, 4%
5368, 2.06%
5369, 3.07%
5370, removed
5371, 5.11%

E+P Treated

5347, 1.65%
5348, 3.56%
5349, 3.67%
5350, 2.75%
5351, 3.08%
5352, 1.81%

PPT Treated
Fourth abdominal mammary gland collected at tissue harvest were fixed in 10% NBF and formalin fixed paraffin embedded, then TUNEL stained. Images organized by treatment (control, E2, E+P, PPT, 2xPPT, DPN), and separated by mouse tissue ID number. Images taken at 200X.

**Figure A.2: TUNEL 4th Mammary Gland from 4d oil Treatment**
APPENDIX B

PROTOCOLS

BrdU Injection

Prepared at 10mg/ml in PBS and filter sterilized. BrdU is a mutagen and must be marked, incinerated, and disposed of properly.

1. Mice were weighed immediately before injection to determine injection volume:

\[ 70\mu g/g \text{ body weight} \times \text{average 21.5g mouse would require} \ 1,500\mu g \text{ of BrdU} / 10,000 \mu g/mL \text{ solution} = \sim 150\mu L \text{ of [10mg/mL BrdU] injected} \]

BrdU Assay

When a cell is proliferating the DNA replicates by unwinding the double stranded DNA and using each existing strand as a template for a new strand. In vivo, BrdU incorporates into the DNA during synthesis by replacing the nucleic acid thymine and linking with adenosine. In vitro, formalin-fixed paraffin embedded mammary gland tissue sections are incubated with an antibody conjugated with horseradish peroxidase (HRP). The antibody is specific for the BrdU that has been incorporated into the DNA. When diaminobenzidine (DAB) is added to the tissue sections, the HRP oxidizes the DAB and the substrate becomes brown (figure B.1)

1. Deparaffinize tissues
   a. 3 changes of xylenes, 3 minutes each
2. Rehydrate Tissues
   a. 3 changes of 100% EtOH, 3 minutes each
   b. 2 changes 95% EtOH, 3 minutes each
   c. 70% EtOH, 3 minutes
d. dH2O, 5 minutes
3. Quenching Solution
4. 1 30% H2O2 : 9 absolute methanol
   a. Submerge slides, incubate 10 minutes
   b. Rinse in 3 changes PBS, 2 minutes each
5. Trypsin
   a. Add 1 drop trypsin concentrate (1A) : 3 drops trypsin dilutant (1B)
   b. Add 2+ drops to each, incubate in humidity chamber 3-10 minutes
   c. Rinse in 3 changes dH2O, 2 minutes each
6. Add 2 drops denaturing solution (2) to each
   a. Incubate room temperature 20-30 minutes
   b. Rinse in 3 changes PBS, 2 minutes each
7. Apply 2 drops blocking solution (3) to each,
   a. Incubate at room temperature for 10 minutes
   b. Drain/blot solution
8. apply 2 drops biotinylated mouse anti-BrdU (4) to each
   a. Incubate room temperature 30-60 minutes
   b. Rinse in 3 changes PBS, 2 minutes each
9. Ab mixture
   a. 1 drop substrate buffer concentrate (6A), DAB concentrate (6B), 0.6% H2O2 (6C):1mL dH2O
      i. Protect from light
   b. Apply 2 drops to each slide, 2-5 minutes
   c. Rinse in H2O
10. Counterstain with Hematoxylin
    a. 4 minutes
    b. Wash in tap H2O
    c. PBS, about 30 seconds until blue
    d. Rinse in dH2O
11. Dehydrate slides
    a. 2 changes 95% EtOH, 2 minutes each
    b. 2 changes 100% EtOH, 3 minutes each
    c. 3 changes of xylenes, 3 minutes each
    d. Coverslip with cytoseal

**ELISA**

1. Bring all reagents to room temperature
2. Leave desired number of anti-estrogen antibody coated wells in the holder
3. Dispense 25μL of standards, specimens and controls into appropriate wells
   a. Two wells per sample
4. Add 100μL of working reagent of E2 enzyme conjugate into each well
5. Mix well by placing on shaker for 10-20 seconds
6. Incubate room temperature 2 hours
7. Remove liquid from all wells
8. Wash 3 times with 300μL if 1x wash buffer, blot on bench diaper
9. Dispense 100μL of TMB reagent into each well. Gently mix for 10 seconds
10. Incubate at room temperature for 30 minutes
11. Add 50μL stop solution to each well
12. Gently mix 30 seconds, make sure all blue color has completely changed to yellow
13. Read absorbance at 450nM with microplate reader within 15 minutes
**Hematoxylin & Eosin Stain**

Done for vaginal smears
1. Fix air dried slides in 10% Neutral Formalin Buffer for 1 minute
2. Rinse in 70% EtOH
3. Rinse in d H2O
4. Hematoxylin, dip for 4 minutes
   a. stains negatively charged nucleic acids in the nucleus of the cell
5. Wash with cold tap water until it runs clear
6. Set Hematoxylin stain with 2% glacial acetic acid
7. Dip in H2O
8. Dip in 0.5% NH3OH
9. Dip in H2O to fix hematoxylin
10. Rinse in 95% EtOH
11. Eosin, dip for 12 seconds
   a. binds to the positively charged amino acids in the cytoplasm
12. Rinse in 95% EtOH
13. Dip in H2O
14. Dehydrate
   a. 95% EtOH, 2 minutes
   b. 100% EtOH, 3 minutes
   c. 3 changes of xylenes, 3 minutes each

**ImageJ Counting Program**

1. Download Image J
2. File ➔ Open image
3. Plugins ➔ analyze ➔ cell counter
4. Zoom in to 75%
5. Choose the paint tool
6. Select a counter (“type 1, 2, 3...”)
   a. Each counter is a different color so choose a color that works with your stain
   b. For TUNEL (Aliza) uses:
      i. Type 4 (pink) for TUNEL positive (brown)
      ii. Type 8 (yellow) for TUNEL negative (purple or green depending on stain)
7. Click on each cell, the counter will count for you
8. Only count cells that are a part the duct (they are the round ones, don’t count the slightly
   oval perimeter cells or any in the surrounding tissue or center of duct itself)
9. When you’re done zoom back out so that you can see all of the counted slides at once
   (usually 33 or 50%)
10. Click “results” and place the window over the picture but not blocking the counted cells
11. Screen shot the picture with the markers and the results
Isolating Mouse Epithelium and Mammosphere Culture

I. Reagents

A. Digestion Media

<table>
<thead>
<tr>
<th>Reagent and [stock]</th>
<th>500mL</th>
<th>100mL</th>
<th>[Final]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM:F12</td>
<td>500mL</td>
<td>94mL</td>
<td></td>
</tr>
<tr>
<td>Antibiotic/Antimitotic [100x]</td>
<td>1 mL</td>
<td>[100U/mL]</td>
<td></td>
</tr>
<tr>
<td>Collagenase type 3 (Worthington Cat# LS004180)</td>
<td>200 mg</td>
<td>[2mg/mL]</td>
<td></td>
</tr>
<tr>
<td>Hyaluronidase (Sigma H3506)</td>
<td>7.3 mg</td>
<td>[100U/mL]</td>
<td></td>
</tr>
<tr>
<td>Gentamycin [50mg/mL]</td>
<td>0.2 mL</td>
<td>[100ug/mL]</td>
<td></td>
</tr>
<tr>
<td>Charcoal Stripped FBS</td>
<td>FILTER</td>
<td>STERILIZE</td>
<td>Store at 4°C</td>
</tr>
</tbody>
</table>

B. Culture Media for Mammospheres

<table>
<thead>
<tr>
<th>Reagent and [stock]</th>
<th>1 L</th>
<th>500 ml</th>
<th>[Final]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuMEC basic serum-free media (Gibco 12753-018)</td>
<td></td>
<td>482mL</td>
<td></td>
</tr>
<tr>
<td>B27 Supplement [50x] (Gibco 17504-044)</td>
<td></td>
<td>10mL</td>
<td></td>
</tr>
<tr>
<td>mEGF [5ug/mL]</td>
<td></td>
<td>2mL</td>
<td>[20ng/mL]</td>
</tr>
<tr>
<td>bFGF [10ug/mL]</td>
<td></td>
<td>1mL</td>
<td>[20ng/mL]</td>
</tr>
<tr>
<td>heparin [4mg/mL]</td>
<td></td>
<td>0.5 mL</td>
<td>[4ug/mL]</td>
</tr>
<tr>
<td>Antibiotic/Antimitotic [100x]</td>
<td></td>
<td>5mL</td>
<td></td>
</tr>
<tr>
<td>Gentamycin</td>
<td></td>
<td></td>
<td>[15ug/mL]</td>
</tr>
</tbody>
</table>

C. Basic Fibroblast Growth Factor (bFGF) Sigma F0291 [10mg/ml]

<table>
<thead>
<tr>
<th>Reagent and [stock]</th>
<th>1 L</th>
<th>500 ml</th>
<th>25 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>5mM sterile Tris, pH7.6</td>
<td>2.5 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To prepare 5mM sterile Tris, pH7.6 from 1M Tris, pH7.6:
Add 50 mL of 1M stock Tris to 10mL of MQ H₂O
\[ V_1 C_1 = V_2 C_2 \]
\[ V_1 = ((0.005M)(10mL))/(1M) = 0.05 mL \]
Filter sterilize through 0.2 µM filter

D. Heparin Sigma H3149 [4mg/ml]

<table>
<thead>
<tr>
<th>Reagent and [stock]</th>
<th>1 L</th>
<th>500 mL</th>
<th>100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>16 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MQ H₂O</td>
<td>4 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Filter sterilize through 0.2 µM filter

<table>
<thead>
<tr>
<th>DNase (Sigma 10104159001)</th>
<th>0.001g</th>
</tr>
</thead>
<tbody>
<tr>
<td>depc H₂O</td>
<td>1mL</td>
</tr>
</tbody>
</table>

store -20 ~1 week

II. Equipment
- Bio-containment hood and standard tissue culture materials
- Sterile surgical tools (scissors, 2 curved forceps, 1 straight forceps, 2 scalpel blades)
- Autoclaved 2” cotton squares
- 60mm dishes (1 low adhesion, 1 regular)
- 40µm cell strainer
- Shaker at 37°C (or rotating hybridization oven)

III. Procedures
A. The day before
   1) Autoclave materials
   2) Prepare mammosphere culture media

B. Harvest Glands
   1) Weigh 50mL conical + 30 mL PBS (one per animal), place one ice
   2) Euthanize mice (CO₂ inhalation)
   3) Immerse entire mouse in 70% EtOH, move to BSL2 hood
   4) Cut out lymph nodes and remove 4th mammary glands
   5) Place mammary glands into a conical with sterile PBS, weigh and calculate weight of the mammary glands (both 4th glands from mouse into the same)

C. Mammary Gland Digestion
   1) Make digestion media
      ▶ 10mL/g mammary gland tissue
   2) Aspirate PBS and put tissues in the top lid of the regular 60mm dish, using sterile techniques chop mammary glands with two scalpels for 5 minutes (pieces <1mm)
      ▶ Note: Tissues can also be shopped with a razor blade, and on a sterile plastic block as long as all of the cut gland can be collected
      ▶ Be careful to transfer all of the gland, easiest to scoop with blade
   3) Transfer minced mammary fragments from both mammary glands of one animal into a 15mL conical tube with digestion media
      ▶ Amount of digestion media should be calculated based on weight of glands so that 10mL of media is added per 1g of tissue
   4) Shake vigorously
5) Place on shaker at 37°C for 6-8 hours, shaking vigorously break clumps periodically (about 1/hour)
   ▶ Note: The hybridization oven can also be used
   ▶ Set the shaker to 115-125rpm
6) Vortex and pipet 3-5 times to break apart clumps
7) Centrifuge at 1200 rpm for 5 min. Remove supernatant.
8) Wash pellet with 3mL PBS 2 times
   • Resuspend and centrifuge to repellet at 1200 rpm for 5 min
9) Add 1mL pre-warmed 0.05% Trypsin-EDTA to the cell pellet, gently pipet to break apart using P1000. Stop the reaction by adding 10mL of 2% PBS-FBS with AB/AM
   ▶ Note: if big clusters of cells can still be seen incubate at 37° for 1-5 minutes
10) Centrifuge 1200 rpm for 5 min to re-pellet cells
11) Aspirate supernatant of the pellet
   ▶ Note: At this point cells have begun to die and DNA will be released, causing a “stringy mass”, be very cautious when aspirating while still removing as much supernatant as possible
12) Add 200µL/ 1mg/mL DNase I and pipette to further dissociate cell clumps.
13) Dilute the cell suspension with 10mL PBS. Centrifuge at 1200rpm for 5 min, aspirate supernatant
   ▶ Note: Pellet will be smaller at this point because the DNase has broken up the dead cells and so the pellet will now contain less
14) Resuspend cell pellet in 5mL mammosphere culture media and sieve the suspension through 40µm cell strainer.
   ▶ Note: Can take more than one pass through the sieve to really break the cells apart
15) Count the cells using Trypan Blue, if more clusters are seen repeat mechanical dissociation and sieving (more than ~5%)?
16) Plate single cells on 60mm Corning ultra-low attachment dish at a density of 20,000 cells/mL in primary culture
   ▶ To Count: Plate 0.5mL per well on low attachment 24-well plate (equates to 10,00 viable cells or 500 mammospheres per well). Measure and count spheres after 7 days in culture.
17) Allow to grow at 37°C
D. Passage Mammospheres
1) After 7-10d collect mammospheres by gently centrifuging at 800rpm for 5 min
2) Enzymatically dissociate with 1mL 0.05% trypsin-EDTA and 60µL 1mg/mL DNase1. Incubate at 37°C for 10-15 min, pipetting every 5 min until single cell suspension is achieved
3) Inactivate trypsin with 10mL of 2% PBS-FBS with AB/AM
4) Centrifuge at 1200rpm for 5 min
5) Aspirate and resuspend in mammosphere culture media
6) Sieve cells through 40µm cell strainer
7) Plate at 1,000 cells/mL in passages


Preparation of pellets

Pellets were made using silastic tubing (Fisher, # 11-189-15H) sealed with silicone (DAP Inc, # 070798006881) approximately 1.2 cm long (figure B.2). These were packed with one of the five compounds: control (cellulose; Fischer, # AC382312500), E2 (50µg; Sigma Aldrich; Cat# E27858), PPT (400µg; R&D Systems, Cat# 1426-50), ERB041 (400µg; R&D Systems; Cat# 4276-50), and ERB041+PPT (400µg), each packed in cellulose. The proper ratio of cellulose to hormone was combined prior to packing the capsules and then measured in a pre-tared capsule to 0.015g so each would contain the appropriate concentration of the specified agonist. The pellets were sterilized with 5kGy gamma irradiation and primed in standard phenol red-free DMEM:F12 media (Sigma Aldrich; # D2906) for 24 hours prior to implantation. This priming media will also be tested to ensure the starting concentration of hormone is appropriate (higher concentration than the peak produced during pregnancy in mice, >100pg/ml E2).

Surgical Protocol—Pellet implant and removal

The mice were anesthetized using isoflurane and ear notched for identification. Fur was shaved at the base of the neck and cleaned with ethanol. A small incision was made and a pocket was formed just under the skin. The pellet was then inserted into this pocket and the incision was closed using surgical staples. Bupivicaine was used at a maximum of 1mg/kg body weight/per mouse at the site of implantation to relieve pain. The mice were then removed from isoflurane, and monitored each day for one week post-surgery.

Surgical Protocol—Ovariectomy

Anesthetize mice with isoflurane. Shave fur on back between hind legs and mid back, clean with betadine and then 70% ethanol. Make a small incision through the skin
and peritoneum. Gently pull out fat pad surrounding ovary, tie off blood flow to the ovary and remove the ovary from the body. Close wound and treat animals with buprenorphine subcutaneously to relieve pain, a second dose is given the following day. Remove mice from isoflurane and monitor each day for one week post-surgery.

**Tissue Harvest**

Six hours prior to tissue harvest mice were irradiated with 5Gy cesium IR, after completing the first group a decision was made to irradiate the entire second group. Non-irradiated mice were part of the original outline to be used as negative controls. The first set of mice provided enough for statistical analysis. The complete irradiation of group 2 allows more samples to be studied for surveillance. The mice were euthanized with carbon dioxide followed by cervical dislocation. At the time of euthanasia tissues were collected including whole blood for serum and vaginal smears. The lymph node was removed from the 4th mammary gland prior to being flash frozen on dry ice to use for RNA. The other 4th mammary gland, uterus, colon, and skin were also collected. These were all fixed in 10% neutral buffer formalin overnight and then transferred to 70% ethanol until paraffin embedded for immunohistochemistry. The paraffin embedded 4th mammary gland will be used for immunohistochemical procedures including a BrdU proliferation assay, an apoptosis assay, and H&E stain for morphology. The 3rd mammary gland was harvested for whole mount and fixed in Carnoy’s fixative, stained in Carmine Alum, dehydrated with Xylenes and mounted with permount (Fisher; cat# SP15-100). The whole mount will be assessed with a dissecting microscope to be sure that the mice all went through regression and that the mammary glands are completely involuted and quiescent. The coagulated whole blood will be centrifuged at low speed to separate the serum. Serum estrogen will
be quantified using a 17β-estradiol ELISA (Calbiotech; Cat# Es1805-100). This is used to confirm stage of estrus. Additionally, it will be used to quantify how much estrogen was present at tissue harvest and whether this had an effect on responses in the mammary gland.

Follow ELISA protocol that comes in kit from Calbiotech.

**TUNEL Assay**

The fourth mammary gland was fixed in 10% NBF, paraffin embedded, and then sectioned for use. 5Gy-gamma-irradiation causes damage to the cells and any cells that cannot efficiently repair the damage should enter apoptosis. During apoptosis endonucleases fragment the DNA into nucleosomes. In vitro, the mammary gland tissue sections on slides are treated with a terminal transferase enzyme (TDT-enzyme), which incorporates biotinylated nucleotides (biotin-dUTP) to link onto the fragmented DNA. Avidin-HRP links onto the biotin-dUTP and the HRP causes the oxidation of diaminobenzidine. As with the BrdU assay, a brown color appears in the nuclei of any cell that was going through apoptosis at time of harvest. Initially the TUNEL assay was counterstained using hematoxylin, staining the cytoplasm a dark purple. This color was very similar to the brown nuclei indicating apoptosis, and therefore the counter stain was switched to methyl green, which has a contrasting light green/blue color (figure B.3).

1. Deparaffinize tissues
   a. 3 changes of xylenes, 5 minutes each
   b. 2 changes of 100% EtOH, 5 minutes each
   c. 95% EtOH, 3 minutes
   d. 70% EtOH, 3 minutes
   e. Wash in PBS, 5 minutes

2. Prepare dilution of 10mg/mL Proteinase K into 2ng/mL

3. Permeabilization of tissues with proteinase K
   a. Prepare fresh 20μg/mL dilution of proteinase K
      i. 1μL 2ng/mL stock + 99μL of 10mm Tris for each slide
   b. Arrange slides in humidity chamber without sponges
   c. Add 100μL of proteinase K directly to the tissue
d. Incubate 12 minutes, don’t allow to dry out

e. Wash slides in 2 changes of dH2O, 2 minutes each

4. Quench Exogenous Peroxidase
   a. In 100mL graduated cylinder prepare 3% hydrogen peroxide in PBS
      i. 10mL 30% hydrogen peroxide
      ii. 90mL PBS
   b. Pour into histology bucket and add slides
   c. Incubate 5 minutes, do not over incubate
   d. Rinse in 2 changes of PBS, 5 minutes each

5. Apply Equilibrium Buffer
   a. Tap of excess liquid and dry around edges of tissue
   b. Apply 75μL equilibrium buffer to tissues
   c. Incubate at least 10 seconds, up to 1 minute

6. Apply working strength TdT enzyme
   a. Prepare microfuge tube with 77μL reaction buffer (2 slides/tube)
   b. 2 slides at a time
      i. Tap off excess liquid and wipe around tissue
      ii. Add 33μL TdT enzyme to microfuge tube
      iii. Add 55μL diluted TdT enzyme to each slide
      iv. Incubate in humidity chamber with sponges at 37°C, use a piece of parafilm over tissue to keep moisture in, 1 hour (up to 1.5 hours)
      v. Thaw stop/wash buffer on ice

7. Apply Stop/wash buffer
   a. Prepare 100mL of working strength stop/wash buffer in coplin jar
      i. 3mL stop/wash and 102mL dH2O
   b. Incubate slides in coplin jar agitating, 15 seconds
   c. Incubate at room temperature, 10 minutes

8. Apply Anti-digoxigenin Conjugate
   a. Wash in 3 changes of PBS, 1 minute each
   b. Tap off excess and wipe around tissue
   c. Apply 65μL room temperature anti-digoxigenin conjugate
   d. Cover with parafilm and incubate in humidity chamber with sponges at room temperature, 30 minutes

9. Wash with PBS
   a. 4 changes, 2 minutes each

10. Prepare working strength peroxidase substrate
    a. 147μL DAB dilution buffer and 3μL DAB substrate, per 2 slides

11. Develop color in peroxidase substrate
    a. Tap off excess liquid and wipe around tissues
    b. Cover tissue with 75μL peroxidase substrate
    c. Stain 3-6 minutes at room temperature
    d. HAZARDOUS WASTE DISPOSAL

12. Counter Stain with Hematoxylin
    a. Cover tissues in 75μL methyl green
    b. Incubate on slide warmer, 5 minutes
    c. Rinse in dH2O
d. rinse in EtOH
   i. dip 10 times in 95%
   ii. dip 10 times in 100%, 2 changes
13. Dehydrate slides
   a. 3 changes of xylenes, 3 minutes each
   b. coverslip with cytoseal

Whole Mount

1. Fix and Stain tissues
   a. Press tissues between slides and fix in Carnoy’s fixative, at least an hour
   b. Transfer tissues to cassettes and fix in Carnoy’s fixative, at least an hour
   c. Rinse in 70% EtOH for 15 minutes
   d. Rinse in distilled water
   e. Stain overnight in carmine alum at 4°C
2. Dehydrating and Mounting tissues
   a. Dehydrate at room temperature in 75% EtOH, 30min-1hr
   b. Dehydrate at room temperature in 95% EtOH, 30min-1hr
   c. Dehydrate at room temperature in 100% EtOH for 30min-1hr
   d. Clear in xylenes for 1hr+
   e. Clear in fresh xylenes for 30 min
   f. Mount slides and coverslip with permount
Figure B.1: BrdU incorporation
Left: *in vivo*, Right: *in vitro*

Figure B.2: Preparation of pellets
Silastic tubing is stuffed with hormone and cellulose matrix, tubes are sealed at the ends with silicone.

Figure B.3: TUNEL incorporation
Left: *in vivo*, Right: *in vitro*
BIBLIOGRAPHY


