Tumor suppressor regulation
of the cancer stem cell niche

BY

Kaitrin Kramer
B.S., University of Illinois, Urbana-Champaign 2008

THESIS

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Thesis Committee
Dr. David Crowe, Chair and Advisor
Dr. Anne George
Dr. Tom Hart
Dr. Charles Zhou
Dr. Alan Diamond, Department of Pathology
I dedicate my dissertation work to my family and friends. Words cannot express how appreciative I am for your words of encouragement and support throughout this process. Thank you for accepting all of the times I was unable to attend events, parties, football games and for still attempting to include me nonetheless. I look forward to joining in on the fun in the future.

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KK
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>INTRODUCTION</td>
</tr>
<tr>
<td>1.1 Background</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Significance</td>
<td>4</td>
</tr>
<tr>
<td>1.3 Specific Aims</td>
<td>5</td>
</tr>
<tr>
<td>1.4 Hypotheses</td>
<td>6</td>
</tr>
<tr>
<td>2.</td>
<td>REVIEW OF LITERATURE</td>
</tr>
<tr>
<td>2.1 Human Breast Cancer</td>
<td>7</td>
</tr>
<tr>
<td>2.2 Biology of the Mammary Gland</td>
<td>9</td>
</tr>
<tr>
<td>2.3 Breast Cancer Stem Cells</td>
<td>18</td>
</tr>
<tr>
<td>2.4 Tumor Angiogenesis and Breast Cancer</td>
<td>19</td>
</tr>
<tr>
<td>2.5 Molecular and Cellular Biology of microRNAs</td>
<td>22</td>
</tr>
<tr>
<td>2.6 microRNA and Breast Cancer</td>
<td>25</td>
</tr>
<tr>
<td>2.7 Molecular Biology of PPARs</td>
<td>31</td>
</tr>
<tr>
<td>2.8 PPARγ and Breast Cancer</td>
<td>38</td>
</tr>
<tr>
<td>3.</td>
<td>METHODOLOGY</td>
</tr>
<tr>
<td>3.1 Methods and Statistical Analysis</td>
<td>42</td>
</tr>
<tr>
<td>3.2 IRB/ACC Approval</td>
<td>49</td>
</tr>
<tr>
<td>4.</td>
<td>RESULTS</td>
</tr>
<tr>
<td>4.1 Results</td>
<td>50</td>
</tr>
<tr>
<td>5.</td>
<td>DISCUSSION</td>
</tr>
<tr>
<td>5.1 Discussion</td>
<td>84</td>
</tr>
<tr>
<td>5.2 Limitations of the Study</td>
<td>91</td>
</tr>
<tr>
<td>5.3 Future Directions</td>
<td>94</td>
</tr>
<tr>
<td>6.</td>
<td>CONCLUSION</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>CITED LITERATURE</td>
<td>97</td>
</tr>
<tr>
<td>VITA</td>
<td>127</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Loss of PPAR(\gamma) expression reduces tumor latency, expands the tumorigenic CD24+/CD49f(^{hi}) stem cell population, and increases proliferation in Wnt1 induced mammary cancer.</td>
<td>51</td>
</tr>
<tr>
<td>2. Loss of PPAR(\gamma) correlates with increased angiogenesis in Wnt1 mammary tumors.</td>
<td>55</td>
</tr>
<tr>
<td>3. Decreased PPAR(\gamma) expression correlates with increased angiogenesis in human breast cancer.</td>
<td>57</td>
</tr>
<tr>
<td>4. miR-15a and Angpt1 regulate angiogenesis in Wnt1 mammary tumors.</td>
<td>59</td>
</tr>
<tr>
<td>5. The 5' flanking region of miR-15a gene has promoter activity and a DR1 element that is activated by PPAR(\gamma).</td>
<td>62</td>
</tr>
<tr>
<td>6. MSC from MMTV-Cre;PPAR(\gamma)(^{ff});Wnt1 mammary tumors are sensitive to anti-angiogenic chemotherapy.</td>
<td>65</td>
</tr>
<tr>
<td>7. MMTV-Cre;PPAR(\gamma)(^{ff});Wnt1 mammary tumors are resistant to cyclophosphamide chemotherapy.</td>
<td>68</td>
</tr>
<tr>
<td>8. MSC from MMTV-Cre;PPAR(\gamma)(^{ff});Wnt1 mammary tumors are sensitive to sunitinib/cyclophosphamide chemotherapy.</td>
<td>72</td>
</tr>
<tr>
<td>9. CD61+ luminal progenitor cell expansion in chemotherapy treated MMTV-Cre;PPAR(\gamma)(^{+/+});Wnt1 and MMTV-Cre;PPAR(\gamma)(^{ff});Wnt1 mammary tumors.</td>
<td>76</td>
</tr>
<tr>
<td>10. Chemotherapy treated CD61+ luminal progenitor cells are tumorigenic but unipotent and not angiogenic</td>
<td>80</td>
</tr>
<tr>
<td>11. Validated mechanism for PPAR(\gamma) regulation of angiogenesis</td>
<td>83</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF1/2</td>
<td>Activation domain 1 or 2</td>
</tr>
<tr>
<td>Ago</td>
<td>Argonaute protein</td>
</tr>
<tr>
<td>Aldh1</td>
<td>Aldehyde dehydrogenase 1</td>
</tr>
<tr>
<td>Angpt1</td>
<td>Angiopoietin 1</td>
</tr>
<tr>
<td>Angpt2</td>
<td>Angiopoietin 2</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Bak1</td>
<td>Bcl-2 antagonist killer 1</td>
</tr>
<tr>
<td>BM11</td>
<td>Polycomb group RING finger protein 4</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>Breast Cancer 1 early onset</td>
</tr>
<tr>
<td>BRDU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>CD24</td>
<td>Heat stable antigen</td>
</tr>
<tr>
<td>CD49f</td>
<td>Integrin alpha 6</td>
</tr>
<tr>
<td>CD61</td>
<td>Integrin beta 3</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy DNA</td>
</tr>
<tr>
<td>ChiP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic leukocytic leukemia</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
</tr>
<tr>
<td>Csn2</td>
<td>Casein Beta 2</td>
</tr>
<tr>
<td>CTX</td>
<td>Cylophosphamide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DGCR8</td>
<td>DiGeorge Syndrome Critical Region Gene 8</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-Dimethylbenz(a)anthracene</td>
</tr>
<tr>
<td>Dsc1</td>
<td>Desmocollin 1</td>
</tr>
<tr>
<td>Dsc3</td>
<td>Desmocollin 3</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Eukaryotic Initiation factor 4E</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ERBB2/HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>ERBB3</td>
<td>Human epidermal growth factor 3</td>
</tr>
<tr>
<td>ESA</td>
<td>Epithelial cell adhesion molecule</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>GATA3</td>
<td>Trans-acting T-cell-specific transcription factor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hemotoxylin and Eosin</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<td>HMGA</td>
<td>High mobility group protein</td>
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<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
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<tr>
<td>LP</td>
<td>Luminal progenitor</td>
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<tr>
<td>miR/miRNA</td>
<td>Micro Ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonating Imaging</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MSC</td>
<td>Mammary Stem Cell</td>
</tr>
<tr>
<td>MUC</td>
<td>Mucin 1</td>
</tr>
<tr>
<td>NRAS</td>
<td>Neuroblastoma- ras</td>
</tr>
<tr>
<td>PAX8</td>
<td>Paired box 8 gene</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Pdcd4</td>
<td>Programmed cell death protein 4</td>
</tr>
<tr>
<td>PGC1α</td>
<td>Peroxisome Proliferator Activated receptor γ coactivator 1 alpha</td>
</tr>
<tr>
<td>PPARd</td>
<td>Peroxisome Proliferator Activated Receptor Delta</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome Proliferator Activated Receptor Gamma</td>
</tr>
<tr>
<td>PPRE</td>
<td>Peroxisome Proliferator response element</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RXRα</td>
<td>Retinoid X Receptor alpha</td>
</tr>
<tr>
<td>Sca1</td>
<td>Stem cell antigen 1</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>Sprr4</td>
<td>Small proline rich protein 4</td>
</tr>
<tr>
<td>Stat5</td>
<td>Signal transducer and activator of transcription 5</td>
</tr>
<tr>
<td>Sunit</td>
<td>Sunitinib</td>
</tr>
<tr>
<td>TDLU</td>
<td>Terminal Ductal Lobular Unit</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal End Bud</td>
</tr>
<tr>
<td>TGFb</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIE2</td>
<td>Angiopoietin receptor</td>
</tr>
<tr>
<td>TPM1</td>
<td>Tropomyosin 1</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal Deoxynucleotidyl Drasferase dUTP Nick End Labeling</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Factor</td>
</tr>
<tr>
<td>ZEB1/ZEB2</td>
<td>Zinc finger E box binding homeobox</td>
</tr>
</tbody>
</table>
SUMMARY

Mammary stem cells (MSC) expansion is associated with aggressive human breast cancer. Tumorigenic MSC expansion is correlated with increased angiogenesis and poor clinical prognosis in human breast cancer. The mammary tumor suppressor peroxisome proliferator activated receptor γ (PPARγ) was previously shown to have anti-angiogenic effects, but the mechanisms for these observations were not completely characterized. Recently, anti-angiogenic therapy failed to extend patient survival in cancer clinical trials. To better understand these results, we genetically deleted expression of PPARγ in the mammary epithelium of a tumor prone in vivo model. Loss of PPARγ expression reduced tumor latency, expanded the CD24+/CD49f hi MSC population, and increased tumor cell proliferation. PPARγ null mammary tumors exhibited a dramatic angiogenic phenotype which also was detected in human breast cancer. In vivo genetic inhibition of a miR-15a/angiopoietin-1 pathway blocked not only increased angiogenesis but also MSC expansion and cell proliferation. PPARγ activated a response element in the 5’ flanking region of the miR-15a gene. PPARγ null mammary tumors were resistant to targeted anti-angiogenic or cytotoxic chemotherapy. However, normalization of tumor vasculature resulted in an objective response to cytotoxic chemotherapy. Chemotherapy treated PPARγ null mammary tumors exhibited dramatic expansion of unipotent CD61+ luminal progenitor cells which gave rise to luminal
SUMMARY (continued)

adenocarcinomas. These results have important implications for case selection and clinical response to anti-angiogenic therapy in breast cancer patients.
1. INTRODUCTION

1.1 Background

Mammary stem cells (MSC) are the progenitor population for all breast epithelia (for review see Crowe et al., 2004; Sleeman et al., 2007; Visvader, 2009; van Keymeulen et al., 2011). MSC have been isolated from both humans and mice using cell surface markers (Gudjonsson et al., 2002; Dontu et al., 2003; Stingl et al., 2006; Shackleton et al., 2006). The MSC population is expanded in some mouse mammary cancer models (Li et al., 2003; Liu et al., 2004), and tumorigenic progenitor populations have been isolated from human breast cancers (Al-Hajj et al., 2003; Ponti et al., 2005). A tumorigenic luminal progenitor population capable of regenerating all tumor cells was discovered in the MMTV-Wnt1 model (Vaillant et al., 2008). MSC expansion is associated with aggressive human breast cancer (Pece et al., 2010).

Tumorigenic MSC expansion has been associated with increased angiogenesis and poor clinical prognosis in human breast cancer (Nieto et al., 2007; Fantozzi et al., 2014). Tumorigenic MSCs expressed increased levels of vascular endothelial growth factor (VEGF; Sun et al., 2013), and circulating endothelial cells have been associated with poor prognosis in human breast cancer (Naik et al., 2008; Goon et al., 2009). However, anti-angiogenic therapy has not increased survival in breast cancer patients (Chekhonin et al., 2013). Anti-angiogenic agents may increase the breast cancer stem cell fraction by
creating hypoxic conditions in tumors (Conley et al., 2012), resulting in clinical relapse.

Ligands for the group of nuclear hormone receptors known as peroxisome proliferator activated receptors (PPAR; for review see Lehrke and Lazar, 2005; Bensinger and Tontonoz, 2008) have anti-angiogenic effects via reduction of VEGF expression (Asano et al., 2001; Fauconnet et al., 2002; Panigraphy et al., 2002), inhibition of endothelial cell proliferation (Xin et al., 1999), and induction of endothelial cell apoptosis (Bishop-Bailey and Hla, 1999; Bishop-Bailey and Swales, 2008). PPARs have functional domains for ligand binding and interact with recognition sequences that are found in the promoter regions of their target genes in order to regulate transcription. Natural ligands such as long chain saturated and unsaturated fatty acids, leukotrienes, and prostaglandins selectively activate PPARs. Synthetic ligands include the thiazolidinedione class which have been shown to inhibit endothelial cell proliferation (Dong et al., 2009; Park et al., 2009). Breast cancers express reduced levels of PPARγ compared to normal mammary tissue consistent with its tumor suppressor function (Jiang et al., 2003; Watkins et al., 2004). PPARγ ligands inhibit human breast cancer cell proliferation (Mueller et al., 1998; Thoennes et al., 2000; Lapillonne et al., 2003; Qin et al., 2003; Veliceasa et al., 2008; Catalano et al., 2011) and delay mammary tumor formation in animal models (Suh et al., 1999; Pighetti et al., 2001; Crowe and Chandraratna, 2004; Han and Crowe, 2010). PPARγ ligands enhanced chemotherapy mediated growth inhibition in human breast cancer cells and animal models (Yu et al., 2008; Tikoo et al., 2009). However, mutant forms
of PPARγ promoted tumor growth in animal models (Saez et al., 2004; Tian et al., 2009), and polymorphisms in PPAR genes were associated with increased breast cancer risk in humans (Golembesky et al., 2008).

The mutant PPAR/PAX8 fusion protein was shown to regulate thyroid cancer angiogenesis via microRNA-122 (Lacroix et al., 2005; Reddi et al., 2013). MicroRNAs (miRNA) are single-stranded small non-coding RNA molecules (for review see O’Day and Lal, 2010). miRNAs regulate the stability of their target mRNAs by binding to their 3’ untranslated regions and inducing degradation. miRNAs can inhibit multiple mRNA targets in human breast cancer which regulate stem cell expansion, epithelial-mesenchymal transition, angiogenesis, differentiation, proliferation, metastasis, drug resistance, and apoptosis (Majumder and Jacob, 2011; Howe et al., 2012; Mattiske et al., 2012; Wang and Wang, 2012; Chen et al., 2013; Endo et al., 2013; Tekiner and Basaga, 2013). Circulating tumor associated miRNAs are elevated in breast cancer patients and are associated with poor prognosis (Roth et al., 2010).

Previous in vitro studies used human cell lines to investigate PPARγ mediated tumor suppression in breast cancer. Despite potent anti-tumor effects in these studies, synthetic PPARγ ligands and other anti-angiogenic therapies failed to extend patient survival in cancer clinical trials. To better understand these results, we genetically deleted PPARγ expression in the mammary epithelium of a tumor prone in vivo model. We discovered a novel mechanism by which tumorigenic MSCs regulate the angiogenic niche in mammary cancer that is relevant to human breast cancer. Molecular inhibition of this pathway disrupts
the angiogenic niche and suppresses the MSC expansion and reduced tumor latency associated with increased angiogenesis in this model. Targeted preclinical chemotherapy in this model may inform the use of anti-angiogenic agents in human breast cancer, and how chemotherapy mediated expansion of other tumorigenic cell fractions may result in poor clinical outcomes.

1.2 **Significance**

Breast cancer is a disease that affects more than 2.9 million women. Understanding biologic factors that influence response to chemotherapy has significant implications on disease outcomes. Previous studies characterized PPARγ function using human breast cancer cell lines and by expressing mutant PPARγ proteins in cells and animal models, however results from these studies were often conflicting. Studies have shown that PPARγ has tumor suppressor function in mammary tumorigenesis, but the mechanisms by which PPARγ exerts this function are not completely characterized. We hypothesized that PPARγ functions as a tumor suppressor in breast cancer by inhibiting expansion of the mammary stem cell population. We created a novel preclinical model by selectively deleting PPARγ from the mammary epithelium in a Wnt1 oncogene driven mouse tumor model. We demonstrated that loss of PPARγ influences the tumor microenvironment creating an angiogenic niche that responds differently to targeted anti-angiogenic and conventional chemotherapy. These results have important implications for case selection and clinical response to anti-angiogenic chemotherapy in breast cancer patients.
1.3 **Specific Aims**

**Specific Aim 1**
To characterize mammary cancer phenotype in Wnt1;MMTV-Cre;PPARγ+/+ and conditional Wnt1;MMTV-Cre;PPARγf/f mice.

Subaim 1A
To identify CD49f<sup>hi</sup>/CD24<sup>+</sup> stem cells in PPARγ+/+ and PPARγ f/f mammary tumors.

Subaim 1B
To characterize changes in PPARγ target genes in mammary tumors from Wnt1;MMTV-Cre;PPARγ+/+ and conditional Wnt1;MMTV-Cre; PPARγf/f mice.

**Specific Aim 2**
To characterize tumorigenicity and the chemotherapy response of CD49f<sup>hi</sup>/CD24<sup>+</sup> stem cells from Wnt1;MMTV-Cre; PPARγ+/+ and conditional Wnt1;MMTV-Cre; PPARγ f/f mammary tumors.
1.4 Hypothesis

PPARγ functions as a tumor suppressor in mammary epithelium by inhibiting expansion of mammary cancer stem cells.
2. REVIEW OF LITERATURE

2.1 Human Breast Cancer

In the United States, cancer is the second most common cause of death exceeded only by heart disease. Breast cancer is the most commonly diagnosed cancer in women. The American Cancer society estimated 232,340 new cases of breast cancer were diagnosed in 2013. One in 8 women will develop invasive breast cancer during their lifetime. In 2013 there were more than 2.9 million women living with a history of breast cancer. For women, breast cancer is the second most common cause of cancer death after lung cancer. An estimated 39,620 women were expected to die in 2013 from breast cancer (ACS Cancer Facts 2013).

Previous studies demonstrate that genetic and environmental factors affect the risk of developing breast cancer. The highest relative risks are associated with age (greater than 65), inherited genetic mutations (BRCA1/BRCA2, PTEN), and a history of breast cancer. There is an elevated relative risk associated with alcohol consumption, early menarche, late age at first term pregnancy, late menopause, having a first degree relative with breast cancer and obesity (ACS Breast Cancer Facts 2013; Kauff et al., 2007; Bougnouz et al., 2006).

Breast cancer typically presents without any symptoms when tumors are smallest and easiest to treat, stressing the importance of early breast cancer screening, including imaging such as mammography or MRI, and clinical exams. Currently, there are three main ways to treat breast cancer. The main treatment
for breast cancer is breast conserving surgery or mastectomy. The goal of surgery is to remove the tumor from the breast and clinically stage the disease to determine if additional therapy is needed. Radiation therapy is another form of treatment and is often used as an adjunct to surgical treatment. The third type of treatment is systemic therapy, which includes hormone therapy, targeted therapy, and chemotherapy. Systemic therapy can be administered before or after surgical treatment and is the main form of treatment for metastatic disease (ACS Breast Cancer Facts 2013).

There has been a decline in breast cancer mortality in women since the late 1980s that have been attributed to early detection and improvements in targeted therapies. Despite improvements in diagnosis and treatment, patient response to therapy remains variable. This is partly due to the biological heterogeneity of breast cancer (Perou et al., 2000).

Human breast tumors are diverse in their presentation, etiology, and response to treatment. The Carolina Breast Cancer Study identified 6 major subtypes of breast cancer using gene expression analysis; luminal A, luminal B (estrogen receptor positive/progesterone receptor negative), human epidermal growth factor receptor 2 (HER2) positive, and basal like breast cancers (Carey et al., 2006; Perou et al., 2000). Another subtype was identified, the claudin-low subtype, which is characterized by low expression of epithelial markers such as claudin and E-cadherin and high expression of mesenchymal markers such as vimentin (Hershkowitz et al., 2007). By classifying breast cancer based on
subtype, therapy can be personalized based on the predicted response to different chemotherapy regimens.

2.2 **Biology of the Mammary Gland**

In women, the mammary gland is an organ that provides nutrition to offspring through production of milk. The mammary gland is composed of epithelial cells, fibroblasts, adipocytes, lymph nodes, immune cells, and blood vessels (Watson and Khaled, 2008). Mammary gland development occurs mainly after birth and is under the control of steroid and peptide hormones. Mammary gland development includes embryonic, pre-puberty, pubertal, pregnancy, lactation, and involution stages. Reciprocal signaling between the epithelium and the mesenchyme govern initial stages of mammary development and are independent of systemic cues (Hennighausen and Robinson, 2001).

The mammary gland is composed of a branching network of ducts and lobulo-alveolar structures consisting of three epithelial cell lineages: luminal, alveolar, and myoepithelial (Stingl et al., 2001). Luminal cells line the ducts within the mammary gland. Ducts branch into smaller ducts and terminate in lobules composed of alveolar cells that are responsible for milk production during lactation. Myoepithelial cells or basal cells surround the luminal cells and contract in response to hormone signaling aiding in milk release during lactation.

The human breast is characterized by a mammary tree of a branching network of ducts that end in small clusters of ducts termed terminal ductal lobular units (TDLUs). The majority of human breast cancers arise within the TDLUs.
The stromal component of the mammary gland, referred to as the mammary fat pad, is composed of endothelial cells, fibroblasts, macrophages, and adipocytes (Neville et al., 1998). In contrast, mouse mammary glands do not have TDLUs, but instead have terminal end buds (TEB) that are formed during each estrous cycle while ductal branching and elongation occurs from TEBs during puberty. Mice have less fibrous connective tissue and more adipocytes within their mammary glands. However, there are important similarities to human breast tissue that make the mouse mammary gland an ideal model to study mammary gland development and cancer. For example global gene expression analysis of mouse mammary stem cells revealed similarities to human breast cancer gene expression (Williams et al., 2009). Studies have also shown conservation of gene expression pathways between human and mouse. For example, the mouse mammary tumor model MMTV-Neu and MMTV-PyMT tumors correlated with a luminal signature in human breast cancer while MMTV-Wnt1 mouse tumors correlated with the basal subtype of human breast cancer (Lim et al., 2010).

Mammary epithelial cells undergo extensive proliferation and differentiation during puberty, pregnancy, and lactation. These cellular processes are regulated by mammary stem cells (MSC). MSCs are self-renewing, multi-potent mammary epithelial cells. Early studies demonstrated that clearing the mammary fat pad allowed for transplantation and growth of normal mammary cells under the influence of a normal physiological environment (DeOme et al., 1959). Mammary stem cells have been identified along the
entire mammary tree and are represented in different developmental stages of the mammary gland (Smith and Medina, 1988). An important study demonstrated that fragments of mammary epithelium tagged with mouse mammary tumor virus (MMTV) were able to regenerate the mammary gland upon transplantation (Kordon and Smith, 1988). The mammary gland also contains long-lived unipotent stem cells that have been identified within the luminal and basal epithelial compartments (Van Keymeulen et al., 2011).

Mammary gland stem cells were classically identified by labeling with tritiated thymidine or BrdU, stem cell antigen 1 (Sca1) expression, or exclusion of Hoechst dye (for review see Wang, 2006). Due to variability in results from these techniques, newer techniques that involve dissociating tissue into single cell suspensions and using cell surface markers to study and identify mammary stem cells have been used (Eirew et al., 2012; Visvader et al., 2011). Combining micro-dissection with functional assays has been key to identifying stem cells within the human breast and mouse mammary gland. A seminal paper published in 2006 demonstrated the ability to isolate mouse mammary stem cells based on cell surface markers and showed that a single MSC can reconstitute a complete functional mouse mammary gland in vivo (Shackleton et al., 2006). Additional studies have identified subsets of mouse mammary cells that regenerated the entire gland including luminal and myoepithelial cells within 6 weeks. This self-renewing cell population was found in the basal position of the mammary epithelium (Stingl et al., 2006; Sleeman et al., 2006).

A number of studies have demonstrated evidence of stem cells in human
breast tissue. Ducts were enriched for a subpopulation of cells with the capacity for self-renewal, clonal growth, and bipotency (Villadsen et al., 2007). Epithelial cells expressing a mucin negative/epithelial specific antigen positive profile within the luminal lineage were shown to function as precursor cells of the terminal duct lobular unit, the resident site of MSCs in humans (Gudjonsson et al., 2002). In humans, studies using Hoechst dye efflux revealed a flow cytometric side population isolated from human mammary gland (Clarke et al., 2005; Smalley et al., 2005). An in vitro system for propagating self-renewing cells based on the ability to proliferate in non-adherent culture conditions has been developed. These mammosphere cultures grown in suspension were enriched for MSCs and were able to generate multi-lineage colonies when grown in adherent culture conditions (Dontu et al., 2003). Mouse mammary stem cells have also been grown as mammospheres (Liao et al., 2007).

The current gold standard for identifying stem cells is the ability to recapitulate the mammary gland upon transplantation into an immunodeficient mouse. However, there are limitations to studying human mammary stem cells in vivo using this method. In a seminal paper, Kuperwasser and colleagues were able to overcome these issues by humanizing the mouse fat pad by transplanting the stromal and epithelial components from human mammary glands into the cleared fat pads of immunodeficient mice. Using this model they were able to demonstrate the outgrowth of benign and malignant tumors in a heterogeneous environment similar to that of the human breast (Kuperwasser et al., 2004). Other groups also have transplanted stem cells with stromal components under
the capsule of the kidney in immunodeficient mice, a highly vascularized recipient site (Eirew et al., 2008).

Early studies demonstrated that the mammary gland is responsive to signaling from the stromal parenchyma, which influences ductal morphology (for review see Sakakura, 1991; Silberstein, 2001). Stromal mammary gland macrophages have been shown to support stem and progenitor cell differentiation, as well as proliferation, indicating the important role of this niche in MSC function (Gyorki et al., 2009). The importance of the mammary stroma was demonstrated when non-mammary stem cells transplanted into the MSC niche synthesized milk proteins and expressed estrogen and progesterone receptors (Booth et al., 2008; Boulanger et al., 2009). Additionally, pregnancy has been shown to increase the MSC population (Asselin-Labat et al., 2010).

The stroma of the mammary gland regulates a number of transcription factors and molecular pathways that play a role in the development of the mammary gland and regulation of the MSC population (Hennighausen and Robinson, 2005). Transcription factor Gata3 regulates differentiation along ductal and alveolar lineages. Gata3 deficiency leads to the accumulation of luminal progenitor cells that are unable to form ductal structures (Asselin-Labat et al., 2007). Transcription factor Elf5 is crucial for commitment from a luminal progenitor to the alveolar lineage (Oakes et al., 2008). Signal transducer and activation of transcription 5 (Stat5) is important for establishment and maintenance of luminal progenitor cells and alveolar cells during pregnancy (Yamaji et al., 2009). The transmembrane signaling protein Notch restricts
expansion of MSCs (Bouras et al., 2008). Constitutive activation of the Hedgehog growth factor signaling pathway, led to decreased MSC activity (Moraes et al., 2007). BMI1 oncogene and Hedgehog signaling have been shown to be important in the self-renewal of mammary stem cells as well as breast cancer stem cells (Liu et al., 2006; Tanaka et al., 2006). The mammary stem cell population does not express progesterone receptor (PR), estrogen receptor (ER), or Her2 (Asselin-Labat et al., 2006). However MSCs respond to both estrogen, which regulates ductal growth and proliferation, and to progesterone, which regulates the secretory alveolar development. Oophorectomy diminished the mammary stem cell population while MSC activity was increased in response to treatment with estrogen and progesterone (Asselin-Labat et al., 2006). Estrogen has been shown to induce expression of amphiregulin, which plays an important role in epithelial cell proliferation and ductal elongation (Ciarloni et al., 2007). Studies in different cancer types have demonstrated that a hypoxic microenvironment enriches for tumorigenic stem cells (Das et al., 2007). Together these studies indicate the important role that the stem cell niche plays in positively and negatively regulating the MSC population.

Newer techniques in studying the mammary stem cell population involve using lineage tracing. Transplantation studies have been shown to have limitations. A study showed that hair follicle stem cells differentiate into epidermal lineages following transplantation, but under physiological conditions are only able to regenerate the hair follicle (Blanpain et al., 2009). This reveals
an important distinction between normal stem cell behavior under physiological conditions and stem cell capacity in a non-physiological environment.

Lineage tracing studies identify different cell types by allowing in vivo dynamic analysis of labeled cells. A particular cell can be labeled with fluorescent protein or β-galactosidase and when this cell divides all of its progeny maintain the label, which allows them to be analyzed over time. Lineage tracing aids in determining if a cluster of labeled cells originates from a single cell. This is typically done using transgenic mice that express the Cre recombinase enzyme under the control of a cell-specific promoter. Mice are crossed to a second mouse expressing a reporter gene that will only be expressed in cells where the LoxP site (stop codon) is excised by Cre recombinase allowing for expression of the reporter. There are various ways to control the expression of Cre. Cre can be constitutively expressed in cells of interest under the control of a cell specific promoter. Cre can also be induced by tetracycline or under the control of a mutated hormone receptor such as the progesterone or estrogen receptor. Administration of a ligand for either receptor will activate Cre expression, excise the LoxP sites and lead to the expression of the reporter gene in the cell of interest (for review see Van keymeulen and Blanpain, 2012; Alcolea and Jones, 2013). An important limitation to this approach is the need to have a specific promoter that targets the cell population of interest in order to properly analyze subsequent labeled cells. Additionally, because there is heterogeneity within the epithelium, it is important to also consider the existence of additional stem cell populations within the epithelium that are not labeled by one promoter.
Lineage tracing has been used to identify stem cells in the prostate, upper airway epithelium, intestine (Blackwood et al., 2011; Rock et al., 2009; Sato et al., 2009). Lineage tracing studies in the ovary revealed Lgr5 positive cells as a stem and progenitor cell marker that contributes to the formation of ovary surface epithelium. G-protein coupled receptor Lgr5 positive cells function in repair and maintain homeostasis of the ovary surface epithelium (Ng et al., 2014). Lineage tracing revealed Lgr5 positive cells actively cycle and exhibit long-term self-renewal potential in the hair follicle, small intestine and the colon (Barker et al., 2009). In the mammary gland, lineage-tracing studies demonstrated Lgr5 positive progeny undergo a switch from the luminal to myoepithelial compartment early in development (de Visser et al., 2012). Multicolor lineage tracing identifies multiple stem cells clones and labels all of the progeny derived from these individual clones so that they can be distinguished easily by microscopy. This technique was utilized to identify stem cells in the lingual epithelium (Tanka et al., 2013).

In the mammary gland, inducible Cre expressed in luminal cells (keratin 8/18) or myoepithelial cells (keratin 5/14) is typically used to study mature epithelial cells. Studies have demonstrated that the mature mammary gland contains long-lived unipotent stem cells from both luminal and myoepithelial lineages. These cells are shown to undergo expansion following pregnancy and have the ability to self renew (Van keymeulen et al., 2011). A novel 3-dimensional confocal imaging technique combined with lineage specific markers has also been used to identify bipotent mammary stem cells that provide
additional evidence of the hierarchalical organization of the mammary gland (Rios et al., 2014).

Lineage tracing has important implications for identifying cancer stem cell populations. By using an inducible Cre model targeted to Lgr5 positive cells with a multicolor reporter to excise the APC gene involved in intestinal adenomas, the differentiation of clonal and polyclonal intestinal tumors was demonstrated (Schepers et al., 2012). Additionally, some tumors were unlabeled, indicating the presence of other tumor initiating cells. Lineage tracing has also been employed to demonstrate that basal cell plasticity is regulated by intrinsic and extrinsic factors when the microenvironment changes. Using an inducible model of a cytokeratin 5 promoter regulating expression of a cre-estrogen receptor fusion protein (CK5-CreER) transgenic mouse with an rosa26- yellow fluorescent protein reporter (R26R-YFP) a group was able to identify a small population of bipotent prostate basal cells that function during tissue homeostasis and regeneration (Wang et al., 2013).

Future lineage tracing studies will need to combine knowledge of normal development with understanding mutations leading to a tumorigenic environment. A combination of the use of transplantation into immunodeficient mice and lineage tracing will help to identify the differentiation patterns of mammary stem cells.
2.3 **Breast Cancer Stem Cells**

Cancer stem cells (CSCs) were first identified in hematopoietic cancers (Bonnet and Dick, 1997). The cancer stem cell hypothesis proposes that a small subset of cells within the tumor has the ability to self-renew and to recapitulate all cells that comprise the tumor (Clarke et al., 2006). Early studies in hematopoietic cancers have indicated that the tumor-initiating cell is most commonly the stem or progenitor cell. These cells may be a target for transformation due to their longevity allowing for accumulation of mutations (Bonnett and Dick, 1997). The cell of origin for most types of breast cancer is largely unknown, however, tumor-initiating cells for some breast cancer subtypes have been explored. The cell of origin for the claudin-low subtype resembles the mammary stem cell (Prat et al., 2010). Breast cancers with BRCA1 mutations were initially proposed to be of a basal subtype due to absence of hormone receptors and Her2 expression, however, studies have shown that the luminal progenitor population was transformed and expanded in these tumors (Molyneux et al., 2010; Liu et al., 2008). In Wnt1 oncogene driven mammary tumors, both MSC and luminal progenitor cells are tumorigenic with the MSC population exhibiting greater expansion (Vaillant et al., 2008).

Cancer stem cells in breast cancer have been classified by several phenotypes. First, cancer stem cells can recapitulate all cells within the tumor following transplantation into immunodeficient mice. As few as 100 breast cancer cells, isolated by flow cytometry using cell surface markers CD44+CD24- can form tumors upon transplantation into immunodeficient mice (Al-Hajj et al.,
Cancer stem cells have also been identified in transgenic mouse breast cancer models, such as the MMTV-Wnt1 mouse (Cho et al., 2008). p53 null mice develop mammary tumors that were enriched for a small subset of cells that formed tumors on transplantation and demonstrated tumorsphere forming ability in vitro (Zhang et al., 2008).

Cancer stem cells have been shown to metastasize and are resistant to anti-proliferative cancer therapies. Residual breast cancer cells following chemotherapy treatment were enriched for tumorigenic subpopulations and increased mesenchymal gene expression indicative of cells with potential to undergo epithelial to mesenchymal transformation (EMT) and metastasis (Creighton et al., 2009; Li et al, 2008). Cancer stem cell expansion is correlated with poorly differentiated and clinically aggressive tumors (Pece et al., 2010). CSC expansion has been shown to correlate with loss of tumor suppressor function. Loss of p53 expression resulted in expansion of human breast cancer cells as demonstrated by CD44+ expression (Godar et al., 2008; Mizuno et al., 2010). BRCA1 plays an important role in breast epithelial differentiation. Therefore, loss of this tumor suppressor results in accumulation of unstable stem cells which are potential targets for oncogenic events (Liu et al., 2008).

2.4 Tumor Angiogenesis and Cancer

The development of new blood vessels from differentiating endothelial cells is termed vasculogenesis and is important during embryogenesis. Angiogenesis is the formation of new blood vessels from existing ones.
Physiological angiogenesis plays an important role in female menstruation and wound healing. Uncontrolled angiogenesis promotes tumor formation and helps to sustain tumor growth. Tumors acquire nutrients and oxygen via the vasculature. Classical studies demonstrated that angiogenesis was required for tumor growth when cancer cells grafted to rabbit cornea recruited new blood vessels (Gimbrone et al., 1972). Angiogenesis is important for tumor progression with endothelial cells and pericytes playing an important role in creation of the tumor microenvironment (for review see Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). During tumor development, cancers undergo an angiogenic switch (for review see Hanahan and Folkman, 1996). This switch is defined by a progressive increase in vascularization in order to sustain tumor growth. The angiogenic switch is regulated by a balance of angiogenic and anti-angiogenic factors within the tumor microenvironment (Baeriswyl and Christofori, 2009).

In response to increased production of angiogenic cytokines and the hypoxic environment of the tumor, blood vessel growth becomes abnormal. This abnormal architecture leads to increased hypoxia (for review see Jain, 2005). This situation poses a significant therapeutic issue related to drug delivery. Targeted angiogenic therapy has been proposed to normalize and improve the structure of abnormal tumor vessels thereby making the tumor more susceptible to chemotherapy (Tong et al., 2004; Winkler et al., 2004).

Angiopoietin 1 (Angpt1) is a vascular stabilizing factor first shown to be expressed by vascular smooth muscle cells and pericytes. Angpt1 regulates
angiogenesis through interaction with its receptor Tie2 (tyrosine kinase with immunoglobulin and EGF-like domains) on endothelial cells. Upon binding of Angpt1 to Tie2 the receptor dimerizes or oligomerizes and is auto-phosphorylated at its tyrosine residues (Kim et al., 2005; Yu et al., 2013). Angpt1 binding to Tie2 activates a number of downstream targets including PI3 kinase/AKT which promotes endothelial cell survival (Papapetropoulos et al., 2000). Angpt1 mediated Tie2 activation activates various effector proteins including Dok-R activation, GRB2, and SHP2 (Jones et al., 1998; Huang et al., 1995). The Angpt1 null mutation is embryonic lethal due to blood vessel dysfunction and myocardial defects (Suri et al., 1996). Angpt1 functions in blood vessel stabilization and induces final blood vessel maturation through pericyte recruitment and prevention of plasma leakage (Holash et al., 1999; Thurston et al., 2000; Stoeltzing et al., 2003; Augustin et al., 2009). Studies have shown that Angpt1 mediates endothelial cell adhesion independent of Tie2 activation (Carlson et al., 2001). Angpt1 has been shown to inhibit apoptosis of endothelial cells by inhibiting caspase function (Harfouche et al., 2002). Angpt2 is an antagonist of Angpt1, which binds Tie2 but does not activate the receptor (Maisonpierre et al., 1997). In studies of rabbit ischemic hind limbs, Angpt1 was able to rescue angiogenesis (Shyu et al., 1998).

Vessel density is an important prognostic factor for breast cancers. High microvessel density correlated with metastasis and poor prognosis (Weidner et al., 1991). Later studies showed that Angpt1 regulates proliferation and growth of ER negative breast cancers (Harfouche et al., 2011). Angpt1 expression
correlates with BRCA1 expression and triple negative breast cancer in human patients (Danza et al., 2013). Overexpression of Angpt1 expression in colorectal carcinoma correlated with increased tumor microvessel density, however Angpt1 did not have prognostic value (Chung et al., 2006). Some studies have shown that ectopically expressing Angpt1 inhibits xenograft tumor growth due to increased pericyte coverage (Hawighorst et al., 2002). Other studies show that a systemic injection of Angpt1 promoted lung metastasis in transplanted lung cells, however this may have been due to increased blood vessel diameter (Holopainen et al., 2009).

Studies have revealed that downstream effects of Angpt1/Tie2 signaling are context dependent. Matrix bound Angpt1 induced endothelial cell adhesion and cell-matrix contacts, while activated ERK signaling led to proliferation and migration (Fukuhara et al., 2008). Cell in contact with other cells displayed Angpt1 induced Tie2 translocation and formation of complexes that activated PIP kinase signaling reducing capillary permeability. Additionally, cells in contact activated AKT signaling leading to endothelial cell survival (Saharinen et al., 2008). Therefore the stabilization and maturation effects of Angpt1 have different effects on the vasculature depending on whether the vessels are new or existing.

2.5 **Molecular and Cellular Biology of microRNA**

microRNAs (miRs or miRNA) are small 20-25 nucleotide endogenous non-coding RNAs that regulate gene expression. miRs were first identified as small temporal RNAs that regulate development in *C. elegans* (Pasquinelli et al.,
The mature miRNA sequence exists in a double stranded hairpin structure termed primary miRNA (pri-miRNA) created by RNA polymerase II or RNA polymerase III. This sequence is excised by Drosha, a nuclear endoribonuclease that with dsRNA binding protein DGCR8 are responsible for the first cleavage of the pri-miRNA releasing precursor or pre-miRNA that has a 3’ overhang. The pre-miRNA is then exported to the cytoplasm via Exportin-5 that works in complex with Ran-GTP. In the cytoplasm pre-miRNA undergoes a second endoribonuclease cleavage by Dicer which creates a ~22 nucleotide double stranded RNA with a 3’ overhang at both ends. The miRNA is then loaded onto an Argonaute (Ago) protein. One strand is complementary to the target mRNA (guide strand) is selected to form the miRNA effector as part of the miRISC complex (miRNA induced silencing complex). The second strand or the passenger strand is released and degraded (for review see Yates et al., 2013).

miRNA binds target 3’ UTR with imperfect complimentary binding and function mainly as translational repressors. miRNA target sites are found in the 3’ UTR of mRNAs where recognition occurs via base pairing in nucleotide positions 2-8 of the miRNA sequence. The overall degree of the miRNA:mRNA complementary binding is the key determinant of mRNA regulation (for review see Bartel, 2009).

miRNA regulation of gene expression is mediated by translational inhibition as demonstrated by early studies that showed effects on protein expression, but no changes to mRNA levels (Wightman et al., 1993, Olsen et al., 1999). Models for miRNA mediated gene repression involve mRNA degradation
and translational repression (Coller et al., 2005; Djuranovic et al., 2011). However these processes are very closely linked as early studies showed translational repression leads to mRNA degradation (Schwartz et al., 1999). Inhibition of translation initiation is the first and primary target of miRNA. miRNA assembled with Argonaute 2 (Ago2) binds to the 7-methyl guanosine cap found on eukaryotic mRNA, preventing interaction with eukaryotic initiation factor 4E (eIF4E; Kiriakidou et al., 2007; Mathonnet et al., 2007) leading to inhibition of translation. For example, miRNA Lin-4 base pairs with the 3’ UTR of Lin-14 mRNA in C. elegans that results in post-transcriptional repression (Nottrott et al., 2006). A similar mechanism has been shown with miR2 mediated repression in Drosophila (Thermann et al., 2007; Zdanoqicz et al., 2009). De-adenylation of the 3’ poly-adenylated tail of mRNA further contributes to miRNA mediated translational repression and follows Ago2 mediated 7-methyl guanosine cap binding (Fabian et al., 2009). miRNA also play a role in translational repression and cleavage of mRNA transcript in plants (Hake et al., 2006).

miRNA differ from other small RNAs in their processing and regulation. The primary function of miRNA is to regulate gene expression. They are processed from stem loop precursors and have imperfect base pairing. Most animal miRNA bind with mismatches and bulges, but a key 2-8 nucleotide span of Watson-Crick base paring is required for function (for review see Meister and Tuschi., 2004; Tomari and Zamore, 2005). miRNA regulate about 5,300 genes, which translates to relatively 30% of the genome (Lewis et al., 2005). Using the mirBase database, roughly 700 human miRNAs have been predicted or
confirmed (Griffiths-Jones, 2008). Each miRNA can regulate hundreds of genes associated with cellular function, proliferation, and differentiation.

2.6 **microRNA and Breast Cancer**

Given the role miRNA play in regulating physiologic cell processes, aberrant expression of miRNA has been shown to play a critical role in tumorigenesis. Altered expression of miRNA has been observed in several human malignancies including chronic lymphocytic leukemia, gastric cancer, lung cancer, lymphomas, and breast cancers (for review see Croce, 2009). Many miRNA loci localize to genomic regions associated with human cancers (for review see Croce, 2009). Studies that demonstrate mapping of miRNA genes to specific chromosomal regions associated with frequent deletions and amplification provide important clues for the roles of miRNA in cancers (Calin et al., 2004; Iorio, 2005). In cancer, copy number alterations of miRNAs and their target genes are highly prevalent (Zhang et al., 2006). These studies have demonstrated that some miRNA are upregulated or downregulated in different tumors, correlating with their role as tumor suppressors and oncogenes (Gaur et al., 2007).

miRNA play important roles in regulating stem cell fate, including self-renewal, differentiation, and programming (for review see Choi et al., 2013). Deletion of miRNA processing proteins has been shown to alter transition from G1-S phase and proliferation of embryonic stem cells. When cells lacking Dicer and Drosha were transplanted into immunodeficient mice, they formed more
aggressive tumors (Murchison et al., 2005; Wang et al., 2008). Inhibition of miRNA processing proteins resulted in an alteration of miRNA expression, creating cells that formed more aggressive tumors with decreased latency (Kumar et al., 2007). miRNAs are down-regulated in human and mouse mammary stem cells and breast cancer stem cells (Shimoto et al., 2009).

One proposed method for miRNA regulation of breast cancer cells is through histone deacetylases (HDACs). Treatment of breast cancer cell lines with HDAC inhibitors rapidly alters expression of miRNAs (Scott et al., 2006). miR-335 is downregulated in metastatic breast cancer tissue. miR-355 was shown to be regulated via promoter DNA hypermethylation and genomic loss of copy number in metastatic breast cancer cells (Png et al., 2011).

miRNAs show differential expression during pregnancy and lactation when compared to virgin and involuted glands (Avril-Sassen et al., 2009). miRNA expression can also be used to differentiate cancer tissue from normal tissue. miRNA expression has been correlated with hormone receptor and tumor suppressor expression, lymph node metastasis, and proliferation index (Iorio et al., 2005). Aberrant miRNA expression occurs in tumor cell lines and tumors isolated from human cancers (Calin et al., 2006). Hierarchical clustering revealed grouping of different molecular subtypes of breast cancer based on individual miRNA expression (Mattie et al., 2006). For example, miRNAs can identify basal vs. luminal subtypes (Blenkiron et al., 2007).

Generalized decreases in miRNA expression are seen in tumors compared to normal tissue, suggesting a possible role as tumor suppressors (Lu
et al., 2005). The miR-200 family has been shown to be downregulated in cells that have undergone the epithelial to mesenchymal transition through targeting of the E-cadherin repressors ZEB1 and ZEB2 (Park et al., 2009; Korpal et al., 2008). These studies correlated with invasive breast cancer cell lines demonstrating a decrease in miR-200 expression (Gregory et al., 2008). Triple negative breast cancer cell lines expressed lower levels of miR-34 compared to normal cell lines. Studies found miR-34 was required for a normal cellular apoptosis in response to DNA damage with loss of function leading to an increase in radiation sensitivity (Kato et al., 2009). miRNA let-7 has been shown to function as a tumor suppressor by regulating oncogenes Ras and high mobility group A (HMGA) expression in lung cancer where low levels of let-7 expression correlates with high oncogene expression (Johnson et al., 2005; Lee et al., 2007). Lentiviral transduction of breast cancer stem cells with let-7 lead to decreased mammosphere formation, decreased proliferation and decreased tumor formation upon xenotransplantation (Yu et al., 2007). In addition to let-7, expression of miR-30 is reduced in breast cancer stem cells. Ectopic expression of miR-30 leads to blockage of self-renewal capacity and reduces tumorigenicity upon transplant into immunodeficient mice. Inhibiting miR-30 expression leads to enhanced tumor formation and metastasis (Yu et al., 2010). miRNA let-4 expression is reduced in breast cancer stem cells, but increased in differentiated cells. Exogenously expressed miR-125a and miR-125b suppressed ERBB2 and ERBB3 expression leading to impaired anchorage, decreased cell invasion and migration (Scott et al., 2007). Ectopic expression of miR-31 reduced invasion,
motility, and diminished resistance to anoikis in metastatic breast cancer cell lines. When these cells were xenografted into immunodeficient mice, the miR-31 expressing cell lines demonstrated decreased metastasis compared to controls (Valastyan et al., 2009). miR-15a and miR-16-1 were discovered as tumor suppressors in CLL with loss of these miRs associated with roughly 70% of CLL cases (Calin et al., 2002). miR-15a was shown to be decreased in breast cancer samples as determined by PCR compared to normal adjacent tissues (Luo et al., 2013).

A number of miRNAs have been demonstrated to function as oncogenes. miRNA-155 is overexpressed in breast cancer and functions as an oncogene, inhibiting expression of tumor suppressors in breast tissue and breast cancer cell lines. Overexpression of miR-155 in breast cancer cells activates JAK/STAT signaling leading to an increase in expression of genes associated with inflammation (Jiang et al., 2010). miR-21 has been shown to downregulate Pdcd4, a tumor suppressor in colorectal cancer. This leads to an increase in tumor invasion and metastasis (Asagnani et al., 2008). miR-373 is upregulated in metastatic human breast cancer tissue. miR-373 expression and miR-520 expression correlated with cell migration and invasion (Huang et al., 2008). Suppression of miR-21 inhibits tumor cell growth in vitro and in vivo mediated by an increase in tumor suppressors TPM1 and cell death protein PDCD4. Compared to normal tissue miR-21 is overexpressed in tumor tissues (Zhu et al., 2007; Frankel et al., 2008). miR-10b is overexpressed in metastatic breast cancer cells and human tumors. miR-10b initiates tumor invasion and distant
metastasis in vivo (Li et al., 2007). An increase in TGFβ expression leading to enhanced tumorsphere forming cells is mediated by the miRNA-181 downregulation of the tumor suppressor ataxia telangiectasia mutated (ATM) (Wang et al., 2011). miR-17-5p also inhibited anchorage independent growth of cells mediated by IGF (Hossain et al., 2006). miR-125a has been shown to function as a tumor suppressor in breast cancer by inhibiting cell proliferation and migration mediated by RNA-binding protein HuR expression (Guo et al., 2009). miR-17-5p has been shown to downregulate AIB (amplified in breast cancer protein) leading to decreased ER positive cells and decreased proliferation of breast cancer cells. miR-17-5p also inhibited anchorage independent growth of cells mediated by IGF (Hossain et al., 2006).

High throughput miRNA expression profiling identified several miRNAs as potential biomarkers for breast cancers. miRNAs have been shown to correlate with prognosis (Calin et al., 2005; Schetter et al., 2008). miR-30c is a prognostic marker for breast cancer that correlates inversely with IL-11 expression. Low level of IL-11 expression correlates with relapse-free survival in breast cancer patients (Bockhorn et al., 2013). miR-335 and miR-126 are decreased in primary breast tumors and loss of expression is associated with metastatic breast cancer (Tavazole et al., 2008). miR-206 represses ERα mRNA and protein expression and was upregulated in ER negative tumor lines (Adams et al., 2007). miR221/222 negatively regulate ERα expression with inhibition of their expression restoring ERα expression (Zhao et al., 2008).
miRNAs have been associated with relapse-free survival and prognosis of different types of breast cancer (Buffa et al., 2011). miR-126 and miR-335 are downregulated in primary breast tumors in patients who experienced relapse. Loss of expression of miR-126 and miR-335 is correlated with increased metastasis supporting their role as tumor suppressors in miRNAs (Tavazole et al., 2008). miR-141 and miR-200c strongly regulate epithelial differentiation in breast cancer cells (Burk et al., 2008). miR-10b expression is correlated with breast cancer progression (Ma et al., 2007).

Studies have shown that a number of miRNAs influence cellular response to chemotherapy. miR-221/222 expression is upregulated in Her2/Neu positive human breast cancer tissue. Breast cancer cell lines that ectopically express miR-221/222 become resistant to tamoxifen through inhibition of cell cycle protein p27 (Miller et al., 2008). miR-125b was upregulated in chemotherapy resistant breast cancer cells. miR-125b targets pro-apoptotic Bcl-2 antagonist killer (Bak1), and inhibiting miR-125b restored Bak1 expression leading to increased taxane sensitivity (Zhou et al., 2010). miR-451 regulates expression of the multidrug resistance gene, increasing sensitivity of breast cancer cells to chemotherapy treatment (Kovalchuk et al., 2008). miR-214 downregulates tumor suppressor PTEN and induces cell survival and chemotherapy resistance in ovarian cancer cells (Yang et al., 2008).

There have been a limited number of studies using miRNA replacement therapy as a possible therapeutic intervention. Efficacy of therapy is mainly dependent on the delivery method. The main method is adenoviral delivery, but
liposome based delivery as well as polymer carriers have also been used (Esquela-Kerscher et al., 2008; Wiggins et al., 2010; Ibrahim et al., 2011). One study used vector-based plasmids to deliver miR-15a/miR-16-1 to colon cancer cells and found a significant inhibition of tumor growth and angiogenesis when cells were transplanted into immunodeficient mice (Dai et al., 2012).

miRNAs play an important role in regulating vasculogenesis in normal physiology as well as angiogenesis of tumors. Deletion of Dicer in endothelial cells effects miRNAs leading to diminished angiogenic activity in response to VEGF, wounding and ischemia (Suarez et al., 2008). miR-9 upregulation in breast cancer cells downregulates E-cadherin leading to increased β-catenin signaling which leads to increased tumor angiogenesis mediated by an increase in VEGF (Ma et al., 2010). miR-145 targets VEGF-A and N-RAS and acts as tumor suppressor in breast cancer by inhibiting angiogenesis (Zou et al., 2012). miR-155 regulates angiogenesis in breast cancer as an oncogene by down-regulation of the von Hippel-Lindau tumor suppressor. Additionally, miR-155 expression correlates with poor prognosis and lymph node metastasis (Kong et al., 2014). Ectopic expression of miR-148 and miR-98 in vitro and in vivo models inhibited cell proliferation, survival, and angiogenesis in breast tumors (Yu et al., 2011; Siragam et al., 2012).

2.7 Molecular Biology of PPARs

Peroxisome proliferator activated receptor gamma (PPARγ) is a member of the class 1 nuclear receptor subfamily. Other members of this family include
thyroid hormone receptors, vitamin D receptors, and retinoic acid receptors (for review see Aranda and Pascual, 2001). PPARγ is a 57.6 kD protein made up of 9 exons and 505 amino acids (Astarci et al., 2009). PPARγ was first identified in *Xenopus* using homology cloning (Dreyer et al., 1992) and later in mice (Zhu et al., 1993). Early studies identified a factor termed ARF6 that regulated the adipocyte P2 (aP2) gene via binding to response elements adipocyte response element 6 and 7 (ARE6 and ARE7). ARE6 was detected in nuclear extracts derived from adipocytes. Introducing mutations in the ARE6 sequence led to a decrease in aP2 enhancer activity (Graves et al., 1992). PPARγ was shown to demonstrate preferential binding to ARE6 and ARE7 (Brun et al., 1996). Another group attempted to identify targets for thiazolidinedione (TZDs), a group of drugs used to regulate insulin sensitivity and treat diabetes. These studies identified TZDs as potent ligands for PPARγ (Lehmann et al., 1995). Early studies also demonstrated that PPARγ binds to its obligate heterodimer retinoid X receptor α (RXRα) and together they form a transcription complex that regulates gene expression in response to agonist binding (Kliewer et al., 1992).

PPARγ consists of a NH2 terminal activation domain (AF1), a conserved DNA binding domain (DBD), a linker region, a ligand-binding domain (LBD) within the carboxy-terminal region that contains an activation domain AF2 (for review see Tontonoz and Speigelman, 2008). The N-terminal domain has transcriptional regulatory function and the carboxyl terminal domain is responsible for ligand binding, heterodimerization with RXRα, and transactivation.
The PPARγ protein has two N terminal variants that are formed by alternate promoter usage: PPARγ1 and PPARγ2. PPARγ2 has 30 additional amino acids in its N terminus and is expressed in a more adipocyte specific manner (Zhu et al., 1995; Vidal-Puig et al., 1996). The N terminal activation domain AF1 domain has transcriptional activity (Castillo et al., 1999). Modification of this domain by MAP kinase phosphorylation or mutation of Ser112 to Asp reduces ligand binding activity, indicating the important role of the N terminal domain in regulating transcriptional activity (Shao et al., 1998). Fusion of the N terminus of PPARγ to the PPARδ DNA binding domain promotes adipocyte differentiation via PPARδ (Hummasti et al., 2006). Loss of the N-terminus leads to nonspecific inactivation of target genes indicating the important role it pays in receptor activity and selectivity (Hummasti et al., 2006).

PPARγ binds to its obligate heterodimer RXRα in response to ligand activation. PPARγ/RXRα display half site reverse polarity on DNA where PPARγ binds the 5’ extended half site of the response element and RXR binds the 3’ half site (IJpenberg et al., 1997). Reverse polarity binding differentiates PPARγ/RXRα from RXR and other nuclear hormone receptor heterodimerization. Together, PPARγ/RXRα form permissive heterodimers whereas ligands for either receptor have a synergistic effect on the transcription of reporter genes. However, mutation of the PPARγ AF2 domain inhibits the ability of PPARγ/RXRα hetereodimers to respond to ligands from either receptor (Schulman et al., 1998). The ligand binding domain of PPARγ interacts with its DNA binding domain and RXRα to enhance response element binding (Chandra et al., 2008). RXR
homodimers have been shown to selectively bind PPREs (peroxisome proliferator response element) and induce transactivation, but only when stabilized through ligand dependent interaction with co-activators (Ijpenberg et al., 2004).

PPARγ has been shown to bind a response element on DNA termed a PPRE. Analysis of previously identified PPARγ response elements led to identification of the consensus sequence AGGTCANAGGTCA as the canonical PPRE direct repeat 1 sequence (DR1; Palmer et al., 1995). The PPARγ DNA binding domain consists of α helices that interact directly with the AGGTCA sequence. Hydrogen bonding stabilizes the interaction between the amino acids of the DBD and the major groove in the DNA of the half sites for each receptor. PPARγ and RXRα DNA binding domains interact within the minor groove of the spacer in the DR1 sequence (Chandra et al., 2008). Two sets of zinc fingers contribute to the specificity and polarity of the interaction between the DBD and DNA (Hihi et al., 2002). Zinc deficiency has been shown to negatively affect PPARγ signaling, indicating the important role of the conserved zinc fingers in DNA binding (Meerarani et al., 2003). Downregulation of PPARγ led to the discovery of a number of target genes involved in adipogenesis that were shown to interact with PPARγ via the PPRE (Perera et al., 2006).

Recent studies have identified a number of PPARγ epigenetic regulators, including histone acetylases, deacetylases, and DNA methyltransferases (for review see Sugii and Evans, 2011). Using ChIP on chip analysis, a transcriptionally inactive form of PPARγ residing on the promoter region of genes
associated with adipogenesis was identified. Several of these genes contained novel, imperfect PPREs, suggesting the possibility of additional PPARγ targets outside of the canonical DR1 sequence (Nakachi et al., 2008). The estrogen receptor has been shown to bind its half site suggesting that PPARγ may in fact tether to atypical PPREs and recruit other regulatory factors using a different mechanism (Carroll et al., 2005). Post-translational regulation of PPARγ plays an important role in regulation of normal cellular processes as well as in cancer.

The crystal structure of PPARγ revealed the ligand-binding domain contains a large T-shaped cavity composed of 13 alpha helices and four beta pleated sheets. PPARγ contains an additional H2' helix which, in contrast to other nuclear receptor family members, allows it to interact with a wider range of ligands. Highly conserved glutamate and lysine resides form a charge clamp that interacts with a conserved LXXLL motif found on co-activators such as PGC1α and SRC1 (Nolte et al., 1998; Li et al., 2008). This “mouse trap” model describes a mechanism where the C terminal AF2 domain closes on the ligand-biding site in response to binding of a ligand and leads to the transcriptionally active form of PPARγ. More recent crystallography studies indicate that additional residues within the hydrophobic pockets of the LBD are potential targets for PPARγ mediated drug therapy (Montanari et al., 2008). Four different amino acids groups contribute to the ligand independent stabilization of helix 12 of the PPARγ LBD and these are highly conserved and stabilize the interaction with co-activator proteins (Molnar et al., 2005).
In addition to the well-established class of TZD synthetic ligands, PPARγ has a number of endogenous ligands including eicosanoids, prostaglandin derivatives, and poly-unsaturated fatty acids (Kliewer et al., 1994; Kliewer et al., 1995; Kliewer et al., 1997; Tontonoz et al., 1994b). Endogenous ligands differentially regulate transcriptional activity of PPARγ through recruitment of co-activators and repressors (Thoennes et al., 2000). Synthetic ligand GW0072 acts as a potent antagonist of PPARγ activity, but did not interact with the AF2 domain of PPARγ and instead occupies a different binding site within the LBD (Oberfield et al., 1999). Serotonin metabolites act as agonists for PPARγ, and regulates adipogenesis and macrophage function (Waku et al., 2010). Retroviral expression of PPARγ stimulates adipocyte differentiation, which demonstrates the critical role PPARγ plays in lipid metabolism (Tontonoz et al., 1994a). Responses to PPARγ ligands are dependent on the abundance of PPARγ isotypes, sequence of the PPRE, and heterodimerization of RXR and co-activators/repressors (for review see Qi et al., 2000).

Nuclear receptors regulate target genes by undergoing conformational changes in response to ligand binding that allows dissociation from co-repressors and recruitment of co-activators. PPARγ interacts with a number of transcriptional co-activators and co-repressors. One of the most well characterized co-activator is PPARγ coactivator-1α (PGC1α). PGC1α is a well-established co-activator of PPARγ that lacks intrinsic enzymatic activity. Early studies demonstrated that upon interaction with PGC1α, PPARγ undergoes a conformational change that leads to the recruitment of histone acetyltransferases
that alter chromatin structure and increase transcriptional activity (Puigserver et al., 1999). Specificity of co-activator binding in response to ligand is significantly influenced by allosteric effects of heterodimerization. PPARγ also has been shown to interact with co-activators p160/SRC1 family and TRAP220 (DRIP205) in a ligand specific manner (Yang et al., 2000). Classical PPARγ target genes like aP2 are constitutively associated with co-activators in contrast to functional target genes like glycerol kinase (Gyk) which are occupied by nuclear receptor corepressors. Ligand binding has been shown to dismiss histone deacetylase complexes and recruit co-activators such as PGC1-α to the GyK gene (Guan et al., 2005). Down-regulation of co-repressors NCoR and SMRT in vitro increased PPARγ expression leading to increased adipocyte differentiation (Yu et al., 2005). NCoR was shown to repress PPARγ leading to a decrease in transcriptional activity (Li et al., 2011). UHRF1 cofactor negatively regulates PPARγ through recruitment of histone deacetylases and increasing DNA methylation at the PPARγ promoter (Sabatino et al., 2012). Inhibition of MEK signaling decreases PPARγ activity in response to insulin and TZDs (Zhang et al., 1996). Sumoylation of PPARγ recruits NCoR HDAC3 complexes and prevents removal of co-repressor complexes that are required for gene activation (Pascual et al., 2005).

Several mutations and polymorphisms have been identified in PPARγ. Dominant negative mutations in human PPARγ within the ligand-binding domain lead to the early onset of diabetes mellitus, hypertension and severe insulin resistance (Barroso et al., 1999). Somatic mutations leading to loss of function
have been shown to influence sporadic colon cancer formation (Sarraf et al., 1999). A Pro12Ala variant of the PPARγ2 gene promotes sensitivity to TZDs and minimizes insulin resistance (Saraf et al., 2011). In a study of 121 obese patients, a Pro115Gln mutation resulted in phosphorylation of Ser114 in PPARγ2, which accelerated the differentiation of adipocytes and accumulation of triglyceride (Ristow et al., 1998). However, mutations in the PPARγ gene in human cancers are rare (Ikezoe et al., 2001).

2.8 **PPARγ and Breast Cancer**

The PPARγ/RXRα heterodimer was reported to be biologically active in human breast cancer cells (Crowe and Chandraratna, 2004). PPARγ is expressed in human breast cancer cell lines and tissues from human breast cancer (Kilgore et al., 1997; Mueller et al., 1998). PPARγ is also expressed in normal and tumorigenic tissue in murine mammary glands, with decreased expression observed during lactation and pregnancy (Gimble et al., 1998). Early studies investigated the role of TZDs and endogenous ligands on breast cancer cell lines and breast cancer tissue from patients. These studies indicated that treatment with PPARγ ligands lead to growth arrest and apoptosis in vitro (Elsner et al., 1998; Yin et al., 2001; Clay et al., 1999). Ligand activation of PPARγ led to terminal differentiation of breast cancer cells (Mueller et al., 1998). Studies have shown that combining conventional chemotherapy with PPARγ ligands lead to improved outcomes, including increased apoptosis and increased
chemotherapy/radiation sensitivity in ovarian cancer, lung cancer, and melanoma (Brautigam et al., 2011; Freudlsperger et al., 2007; Girnun et al., 2008).

PPARγ activation in vitro leads to cell cycle arrest in G1 phase through proteasome dependent degradation of cyclin D1 (Qin et al., 2003). Cyclin D1 normally inhibits PPARγ through HDAC recruitment (Fu et al., 2005). PPARγ also inhibits proliferation through the reduction of the binding of cell cycle transcription factor E2F/DP (Altiok et al., 1999). PPARγ suppresses β-catenin signaling and carcinogenesis in colon and prostate cancer (Girnun et al., 2002; Wei et al., 2007). PPARγ activation also induces apoptosis in liver cancer cells via caspase 3 activation (Toyoda et al., 2002). PPARγ has chemopreventative activity through signaling that leads to an increase in transcription of tumor suppressor genes such as PTEN (Patel et al., 2001) and BRCA1 (Pignatelli et al., 2003; Margalit et al., 2012).

A phase 2 clinical trial with PPARγ ligands for refractory breast cancer treatment found no significant effect, but this was mainly due to the adverse side effects of TZDs (Burnstein et al., 2003). However, in clinical trials for other malignancies, TZD treatment lead to histological and biochemical differentiation of tumors and enhanced efficacy of drug delivery (Demetri et al., 1999; Hisatake et al., 2000; Kebebew et al., 2006).

Studies using transgenic models of PPARγ overexpression have lead to conflicting conclusions regarding the role of PPARγ in tumorigenesis. The PPARγ null mutant mouse is embryonic lethal and embryos die at E9.5-10 due to disruption of placental formation (Barak et al., 1999; Kubota et al., 1999).
Therefore, most animal models used to study PPARγ activity use transgenic mice. In carcinogen driven mouse models, PPARγ ligands were shown to reduce tumor number, weight, and incidence (Suh et al., 1999). PPARγ ligands have a chemopreventative effect on carcinogen driven mouse mammary tumors (Mehta et al., 2000; for review see Kopelovich et al., 2002). TZDs in the diet stopped tumor growth or lead to regression of mammary tumors in DMBA mediated carcinogenesis and were as effective as tamoxifen in preventing additional tumor development (Pighetti et al., 2001). The synthetic PPARγ ligand GW7845 delayed tumor formation while PPARδ ligands accelerated tumor in DMBA mediated carcinogenesis. Additionally, tumors treated with PPARγ ligands appeared histologically different than PPARδ tumors indicating the PPARγ specific effect of ligands on carcinogenesis (Yin et al., 2005). Mice with constitutively active PPARγ do not exhibit a tumorigenic phenotype. However, when crossed to a tumor prone MMTV-PyMT, mouse exhibit markedly accelerated mammary tumorigenesis, possibly mediated by Wnt1 signaling (Saez et al., 2004). However, genes are induced in this mouse model that are not related to PPARγ activation (Li et al., 2002). A dominant negative PPARγ receptor was created by a fusion with Paired box 8 transcription factor. Dominant negative MMTV-Pax8/PPARγ mice were more susceptible to tumor formation and insensitive to the chemopreventive effects of PPARγ ligands in DMBA mediated carcinogenesis (Yin et al., 2009). A heterozygous PPARγ model was generated by breeding PPARγ floxed mice with the EIIA–Cre transgenic mouse in a DMBA mediated carcinogenesis model. The PPARγ
heterozygous mice exhibited a 3-fold increase in primary mammary adenocarcinoma (Nicol et al., 2004).
METHODOLOGY

3.1 Methods and Statistics

Mouse Breeding and Procedures. To inhibit PPARγ expression in mammary epithelium, we bred MMTV-Cre;PPARγf/f;Wnt1 and MMTV-Cre;PPARγ+/+;Wnt1 mice. All mouse strains were obtained from The Jackson Laboratories (Bar Harbor, ME). PPARγ expression is deleted in mammary epithelium of these mice by Cre mediated recombination of exons 1 and 2. Mammary tumorigenesis is driven by Wnt1 oncogene expression, which is a model of basal subtype breast cancer, exhibits MSC expansion and contains tumorigenic MSC and luminal progenitor cells (Valliant et al., 2008). All mice were genotyped using PCR amplification of extracted tail DNA according to Jackson Laboratories protocols. Twenty tumors were obtained from each group for analysis. The latency, number, and volume of tumors were recorded for each mouse. Complete necropsy was performed on each mouse. Portions of each tumor were fixed in 10% formalin, flash frozen for storage at -80°C, and trypsin dissociated for cryopreservation in liquid nitrogen. For chemotherapy experiments, tumor bearing mice were treated with five daily doses of the angiogenesis inhibitor sunitinib (60 mg/kg; Sigma, St. Louis, MO), three doses of the cytotoxic chemotherapeutic drug cyclophosphamide (200 mg/kg on alternating days; Sigma, St. Louis, MO), sunitinib followed by cyclophosphamide, or vehicle. A minimum of five tumors per group were harvested as described above for analysis. Data were analyzed by t test.
**RT-PCR.** RNA was extracted from sorted MSC from MMTV-Cre;PPARγf/f;Wnt1 and MMTV-Cre;PPARγ+/+;Wnt1 tumors using a commercially available kit (RNeasy, Qiagen, Germantown, MD) and reverse transcribed according to manufacturer’s instructions (Invitrogen, Carlsbad, CA). Adipose tissue from wild type mice was used as the positive control for PPARγ expression. PCR reactions without cDNA template were used as the negative control. cDNA was amplified using mPPARγ primers 5’-AGCTGAATCACCCAGAGTCC-3’ and 5’-TGCAATCAATAGAAGGAACG-3’. β-actin was amplified using primers 5’-AAAAGCCACCCCACTCCTAAG-3’ and 5’-TCAAGTCAGTGTACAGGCCAGC-3’. PCR was performed using thermal cycling parameters of 94°C for 25 seconds, 55°C for 1 minute, and 72°C for 1 minute (Stratagene, La Jolla, CA). Products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

**Histopathology, Immunohistochemistry, and Immunofluorescence Microscopy.** Fixed tumor tissue was dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Sections were deparaffinized and stained with hematoxylin and eosin. For immunohistochemistry and immunofluorescence studies, sections were rehydrated in phosphate buffered saline (PBS, pH 7.4) and blocked with 10% serum. For immunohistochemistry studies, sections were incubated with anti-proliferating cell nuclear antigen (PCNA), keratin 5 (K5), or keratin 18 (K18) primary antibodies overnight at room temperature. Following washing in PBS, sections were incubated with biotinylated secondary antibody and streptavidin conjugated horseradish peroxidase. Antigen-antibody
complexes were detected by incubation with peroxide substrate solution containing aminoethylcarbazole chromogen followed by hematoxylin counterstaining. The percentage of PCNA+ cells in 10 random high power fields was determined by counting. Data were analyzed by t test. For immunofluorescence studies, sections were incubated with anti-CD24, -CD49f, -CD31, -PPARγ, or -Angpt1 (Abcam, Cambridge, MA) primary antibodies overnight at room temperature. Sections from 70 human breast cancer cases and matching normal breast tissue (Novus Biologicals Littleton, CO) were incubated with anti-PPARγ and –CD31 antibodies overnight at room temperature. After washing in PBS, sections were incubated with secondary antibodies conjugated to AlexaFluor 488 or AlexaFluor 555 and visualized by fluorescence microscopy following coverslipping with anti-fade mounting medium containing DAPI (Vector, Burlingame, CA). Data were analyzed by Fischer exact test or t test.

**Fluorescence Activated Cell Sorting.** Enzymatically dissociated tumor cells were incubated with phycoerythrin conjugated anti-CD24 and AlexaFluor 488 conjugated anti-CD49f antibodies (Stem Cell Technologies, Vancouver, BC, Canada), washed in PBS, and the CD24+/CD49f hi MSC fraction sorted by flow cytometry (MoFlo Astrios, Becton Dickinson, Franklin Lakes, NJ). The CD24+/CD49f lo/CD61+ luminal progenitor fractions were sorted in separate experiments. Data were analyzed by t test.

**Cell Death Analysis.** Tumor tissue sections were incubated with terminal deoxynucleotidyl transferase and dUTP-fluorescein for 1 hour at 37°C according
to manufacturer’s recommendations (Roche Applied Sciences, Indianapolis, IN). After washing, apoptotic cells were visualized by fluorescence microscopy following coverslipping with anti-fade mounting medium containing DAPI. The percentage of fluorescent cells in 10 random high power fields was determined by counting. Data were analyzed by t test.

**Cell Culture, Lentiviral Transduction, and Transplantation.** $10^4$ sorted MSCs from MMTV-Cre;PPARγf/f;Wnt1 and MMTV-Cre;PPARγ+/+;Wnt1 tumors were cultured in 3:1 Dulbecco’s modified Eagle medium:F12 medium containing 1X B27 supplement, 10 ng/ml epidermal growth factor, 25 ng/ml basic fibroblast growth factor, 0.2% heparin, 40 µg/ml gentamicin, 2.5 µg/ml amphotericin B (MSC medium) at 37°C in a humidified atmosphere of 5% CO₂. For tumorsphere analysis, clonogenicity and proliferation was determined by counting and tumorsphere diameter measurements were made every second day for 2 weeks. Tumorspheres were photographed using phase contrast microscopy.

miR-15a interaction with Angpt1 mRNA (Chi et al., 2009) was identified using TarBase and DIANA software. $10^4$ sorted MSCs from MMTV-Cre;PPARγf/f;Wnt1 tumors were cultured in MSC medium and separately transduced with Angpt1 shRNA, miR-15a, or control lentiviruses with 5 µg/ml polybrene overnight according to manufacturer’s protocol (ThermoFisher Scientific, Waltham, MA) at 37°C in a humidified atmosphere of 5% CO₂. MSC medium was replaced and the cells were cultured for 24 hours. Two µg/ml puromycin was added to the media and cells were incubated for 48 hours. MSC
medium was replaced and cells were injected into the fat pads of 2 month old immunocompromised NU/J mice. Mice were examined weekly for tumor formation for up to 6 months. The latency and volume of tumors were recorded for each mouse. Complete necropsy was performed on each mouse. Portions of each tumor were fixed in 10% formalin, flash frozen for storage at -80°C, and trypsin dissociated for cryopreservation in liquid nitrogen. Tumors were processed for histopathology, immunohistochemistry, immunofluorescence, FACS, and cell death analysis. Data were analyzed by t test.

**Gene Expression Analysis.** Total RNA was extracted from sorted MSC from MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 tumors. The integrity of the ribosomal RNA bands was confirmed by Northern gel electrophoresis. Total RNA (1 µg) was converted to labeled cRNA targets. The biotinylated cRNA targets were then purified, fragmented, and hybridized to mouse genome 2.0 ST expression arrays (Affymetrix, Santa Clara, CA) to interrogate transcript abundance in each sample. Affymetrix GCOS software was used to generate raw gene expression scores and normalized to the relative hybridization signal from each experiment. All gene expression scores were set to a minimum value of 2 times the background determined by GCOS software in order to minimize noise associated with less robust measurements of rare transcripts. Data were analyzed by t-test with a value of p<0.005 followed by ratio analysis (minimum 2-fold change).

**Western Blot.** Sorted MSC from MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 tumors were lysed in 1X Laemml buffer. Sorted MSC from
lentiviral transduced MMTV-Cre;PPARγf/f;Wnt1 mammary tumors were analyzed in separate experiments. Fifty µg total cellular proteins were separated by SDS-PAGE. Proteins were electroblotted to PVDF membranes (Roche Applied Sciences, Indianapolis, IN). Blots were incubated with blocking solution followed by anti-Angpt1 and anti-β-actin antibodies for 16 hours at 4°C. After washing in Tris buffered saline containing 0.1% Tween 20, blots were incubated for 30 minutes at room temperature with anti-IgG secondary antibody conjugated to horseradish peroxidase. Bands were visualized by the enhanced chemiluminescence method and quantitated by densitometry. Data were analyzed by t test.

**Chromatin Immunoprecipitation.** The putative PPAR response element in the miR-15a promoter was identified using the UCSC Genome Browser. MSC from MMTV-Cre;PPARγ+/-;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 mammary tumors were fixed in 1% formaldehyde for 10 minutes followed by native lysis and DNA fragmentation. Purified chromatin was immunoprecipitated using anti-PPARγ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A/G agarose beads. Eluted DNA fragments were purified for PCR templates. The input fraction prior to immunoprecipitation was amplified as the positive control. Control IgG immunoprecipitates were used as the negative control. DNA templates were amplified with primers 5’-TTCAAAAAAGTATCCCATTTCTG-3’ and 5’-TGTCCTATTTCCTTCCTCAA-3’ flanking the putative PPAR response element in the miR-15a gene. The 197 bp fragment was separated by agarose gel electrophoresis and visualized by ethidium bromide staining.
**Promoter Cloning and Reporter Gene Analysis.** A 1.8 kb fragment of the promoter region of the mouse miR-15a gene was amplified by PCR from mouse genomic DNA using primers 5’-

\[\text{CGGGGTACC} \text{ACTAGATAACAGCCATGGGAGACAC} \text{-3’ and 5’-}
\]

\[\text{CCGCTCGAGAGTATGGCCTGCACCTTTTCAACA-3’}.\] This fragment was cloned into the KpnI and XhoI sites of the pGL3 luciferase vector. We generated a mutant miR-15a promoter construct with a 3 bp substitution in the putative PPARγ response element using the QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA). Wild type and mutant constructs were confirmed by sequencing. Promoter constructs were transfected with mouse PPARγ expression vector or control plasmid and Renilla luciferase reporter vector pRL-TK into the mouse mammary tumor cell line NF639 (American Type Culture Collection, Manassas, VA) using Lipofectamine (Life Technologies, Grand Island, NY). Promoter activity was determined using the Dual Luciferase Assay kit (Promega, Madison, WI) and luminometry (Berthold, Bad Wildbad, Germany). miR-15a promoter activity was normalized to TK promoter activity. Data were analyzed by ANOVA.
3.2 IRB Approval

March 7, 2014

David Crowe
Center for Molecular Biology & Oral Diseases
M/C 860

Dear Dr. Crowe:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on 2/18/2014. The protocol was not initiated until final clarifications were reviewed and approved on 2/25/2014. The protocol is approved for a period of 3 years with annual continuation.

Title of Application: Mammary Stem Cell Fate in Obesity Related Breast Cancer

ACC Number: 13-233

Initial Approval Period: 2/25/2014 to 2/18/2015

Current Funding: Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol. UIC is the only performance site currently approved for this protocol.

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW’s “What Investigators Need to Know about the Use of Animals” (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/6R
cc: BRL, ACC File

Phone (312) 996-1972 • Fax (312) 996-9088 • www.research.uic.edu
4. RESULTS

To determine the effects of loss of PPARγ expression on mammary tumorigenesis, we created the MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 mouse in which exons 1 and 2 of the PPARγ gene is selectively deleted in mammary epithelium via Cre mediated recombination. Wnt1 mammary tumors model human basal subtype breast cancer (Li et al., 2003). PPARγ expression in MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 and MMTV-Cre;PPARγ<sup>+/+</sup>;Wnt1 control MSC is shown in Fig. 1A. MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 mammary tumors developed with shorter mean latency of 140 days compared with 182 days for MMTV-Cre;PPARγ<sup>+/+</sup>;Wnt1 cancers (P<0.04; Fig. 1B). Both genotypes developed poorly differentiated adenocarcinoma as determined by histopathologic analysis (Fig. 1C,D). Mammary adenocarcinomas of both genotypes were composed both of basal and luminal epithelial cells. MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 mammary tumors showed 36% relative expansion of the MSC population (P<0.02, Fig. 1E) as determined by FACS (Fig. 1F,G). MSC were localized in MMTV-Cre;PPARγ<sup>+/+</sup>;Wnt1 and MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 tumors by CD24/CD49f immunofluorescence (Fig. 1H,I). MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 mammary tumors exhibited increased cell proliferation (36% vs. 24%; P<4x10<sup>-6</sup>; Fig. 1J) as shown by PCNA immunohistochemistry (Fig. 1K,L). No significant differences in apoptotic cells were observed in mammary tumors of either genotype (Fig. 1M) as shown by TUNEL analysis (Fig. 1N,O). These results indicate that loss of PPARγ expression decreases tumor latency, expands the MSC population, and increases cell proliferation in Wnt1 mammary tumors.
Fig. 1. Loss of PPARγ expression reduces tumor latency, expands the tumorigenic CD24+CD49f^hi stem cell population, and increases proliferation in Wnt1 induced mammary cancer. (A) PPARγ expression in adipocytes (+ control), MMTV-Cre;PPARγ/f/f;Wnt1 and MMTV-Cre;PPARγ/+;Wnt1 mammary
tumors is shown by RT-PCR. β-actin amplification was used as the loading control. DNA size marker is shown at left. (B) Decreased tumor latency in MMTV-Cre;PPARγf/f;Wnt1 mammary cancer. Kaplan-Meier analysis of MMTV-Cre;PPARγf/f;Wnt1 and MMTV-Cre;PPARγ+/+;Wnt1 mammary tumor latency shows percent tumor free mice over time (days). (C,D) Histopathologic analysis of MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 mammary tumors indicates poorly differentiated adenocarcinomas as shown by H&E staining. Scale bar = 10 µm. (E) The tumorigenic CD24/CD49f stem cell population is expanded in MMTV-Cre;PPARγf/f;Wnt1 compared to MMTVCre;PPARγ+/+;Wnt1 mammary tumors. Quantitation of tumor stem cell fraction is shown for both genotypes. Representative flow cytometric analysis of tumor stem cell fraction in MMTV-Cre;PPARγ+/+;Wnt1 (F) and MMTV-Cre;PPARγf/f;Wnt1 (G) is shown. Immunofluorescence microscopy shows CD24/CD49f stem cells in MMTVCre;PPARγ+/+;Wnt1 (H) and MMTVCre;PPARγf/f;Wnt1 (I) mammary tumors. CD24 and CD49f expression is shown by phycoerythrin and fluorescein conjugated antibodies (red and green respectively). DAPI nuclear counterstaining is shown (blue). Merged images are shown at lower right. Scale bar = 5 µm. (J) Loss of PPARγ expression increases cell proliferation compared to MMTV-Cre;PPARγ+/+;Wnt1 tumors as demonstrated by PCNA immunohistochemistry. Representative images of PCNA expression in MMTV-Cre;PPARγ+/+;Wnt1 (K) and MMTV-Cre;PPARγf/f;Wnt1 (L) mammary tumors. Sections were counterstained with hematoxylin. Scale bar = 10 µm. (M) Quantitation of apoptotic cells using TUNEL analysis in MMTV-
Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 tumors. Representative TUNEL images of apoptotic cells in tumors from MMTV-Cre;PPARγ+/+;Wnt1 (N) and MMTV-Cre;PPARγf/f;Wnt1 (O) are shown. TUNEL positive cells are shown by dUTP-fluorescein labeling (green) and DAPI nuclear counterstain (blue). Merged images are shown at lower left. Scale bar = 5 µm.

To determine the effects of loss of PPARγ expression on cancer stem cell clonogenicity and proliferation in vitro, CD24+/CD49fhi MSC from MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 mammary tumors were grown as tumorspheres in mammary stem cell medium on low attachment plates. We observed no significant differences in MSC clonogenicity (Fig. 2A) or tumorsphere proliferation (Fig. 2B) between cells from MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 mammary tumors. Tumorspheres appeared morphologically similar by phase contrast microscopy (Fig. 2C,D). These results indicate that loss of PPARγ expression does not affect in vitro clonogenicity and proliferation of tumor derived MSC.

The absence of differences in clonogenicity and proliferation between MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 MSC in vitro suggests that microenvironmental effects in PPARγ null tumors may account for MSC expansion observed in vivo. To understand these effects, we performed gene expression analysis on MSC from both genotypes. Interestingly, the expression of genes associated with angiogenesis was upregulated in MMTV-Cre;PPARγf/f;Wnt1 MSC (Angpt1, 2 fold; miR-15a, -2 fold). There were no
differences in VEGF, VEGFR, or Tie2 expression between genotypes (data not shown). We searched public databases for miR-15a target mRNAs and identified an Angpt1 interaction in a previous genome wide screen (Chi et al., 2009; Fig. 2F). MMTV-Cre;PPARγf/f;Wnt1 mammary tumors expressed increased Angpt1 protein as shown by western blot (2-4 fold; Fig. 2G). To determine if elevated Angpt1 expression correlated with increased angiogenesis in MMTV-Cre;PPARγf/f;Wnt1 mammary tumors, we localized capillary endothelium by CD31 expression. We observed a 4 fold increase in capillary density in MMTV-Cre;PPARγf/f;Wnt1 mammary tumors (P<0.007; Fig. 2H-J). These results indicate that loss of PPARγ expression correlates with increased angiogenesis in MMTV-Cre;PPARγf/f;Wnt1 mammary tumors.
**Fig. 2.** Loss of PPARγ correlates with increased angiogenesis in Wnt1 mammary tumors. (A) In vitro clonogenicity of MSC from MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγff;Wnt1 mammary tumors. (B) In vitro proliferation of MSC from MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγff;Wnt1 mammary tumors. Percent small (20-49 µm) and large (50-100 µm) tumorspheres from each genotype is shown. Representative phase contrast photomicrographs of tumorspheres from MMTV-Cre;PPARγ+/+;Wnt1 (C) and MMTV-Cre;PPARγff;Wnt1 (D) MSC. Scale bar = 10 µm. (E) Gene expression changes in MMTV-Cre;PPARγff;Wnt1 compared to MMTV-Cre;PPARγ+/+;Wnt1 mammary tumors. (F) Complementary sequences of miR-15a and Angpt1 3′-
UTR. (G) Angpt1 protein expression is increased in MMTV-Cre;PPARγf/f;Wnt1 tumors. Angpt1 expression in representative MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 mammary tumors is shown by western blot. β-actin expression is used as the loading control. (H) Increased angiogenesis in MMTV-Cre;PPARγf/f;Wnt1 mammary tumors. Representative CD31 immunofluorescence images of capillary endothelium in MMTV-Cre;PPARγ+/+;Wnt1 (I) and MMTV-Cre;PPARγf/f;Wnt1 (J) mammary tumors. Nuclei were counterstained with DAPI (blue). Scale bar = 20 μm.

To determine if loss of PPARγ expression correlated with angiogenic phenotype in human breast cancer, we examined PPARγ and CD31 expression by immunofluorescence microscopy in 70 human breast cancers and normal breast tissue (Fig. 3A). All normal human breast epithelium demonstrated diffuse PPARγ nuclear staining with minimal stromal capillaries (Fig. 3B). In contrast, human breast cancer specimens were predominantly negative for PPARγ expression (68/70; 97%). These PPARγ negative breast cancer specimens were highly angiogenic (54/70; 77%; P<0.05; Fig. 3D). The two breast cancer samples that expressed PPARγ did not exhibit the angiogenic phenotype (Fig. 3C). These PPARγ expressing human breast cancer specimens showed a different pattern of nuclear localization compared to normal breast epithelium. PPARγ expression in these cancers was confined to the nuclear periphery, a region associated with transcriptional repression (Heesen and Fornerod, 2007; Steglich et al., 2013).
These results indicate that loss of PPARγ expression correlates with increased angiogenesis in human breast cancer.

Fig. 3. (A) Decreased PPARγ expression correlates with increased angiogenesis in human breast cancer. PPARγ and CD31 expression in control human mammary gland (B) and human breast cancers (C,D). Representative immunofluorescence images of CD31 (red), PPARγ (green), and DAPI nuclear counterstain (blue). Merged images are shown at lower right. Scale bar = 20 µm.
To determine if altered Angpt1 and miR-15a expression was responsible for the angiogenic phenotype of MMTV-Cre;PPARγf/f;Wnt1 mammary tumors, we stably transduced MMTV-Cre;PPARγf/f;Wnt1 MSC with Angpt1 shRNAs, miR-15a, or control lentiviruses followed by transplantation to mammary fat pads of immunodeficient mice. Angpt1 shRNAs reduced Angpt1 protein expression in MMTV-Cre;PPARγf/f;Wnt1 tumors by 90% as determined by western blot (Fig. 4A). miR-15a lentivirus reduced Angpt1 protein expression in MMTV-Cre;PPARγf/f;Wnt1 tumors by 80%. Mammary tumors derived from Angpt1 shRNAs, miR-15a, and control transduced MSC were classified as poorly differentiated adenocarcinomas by histopathologic analysis (Fig. 4B-D). The angiogenic phenotype was dramatically suppressed in tumors derived from transplanted MSC transduced with Angpt1 shRNAs or miR-15a compared to control lentivirus as determined by CD31 immunofluorescence microscopy (75-80% reduction; P<0.002; Fig. 4E-H). Reduced angiogenesis correlated with 3-fold decreases in the CD24+/CD49fhi MSC fractions of tumors derived from Angpt1 shRNA and miR-15a transduced stem cells as determined by FACS (P<0.02; Fig 4I-L). No significant differences were observed in cell proliferation (Fig. 4M-P) or apoptosis (Fig. 4Q-T) in tumors derived from Angpt1 shRNA or miR-15a transduced MSC compared to control mammary cancers. These results indicate that Angpt1 and miR-15 regulate the angiogenic phenotype of MMTV-Cre;PPARγf/f;Wnt1 mammary tumors. Inhibition of the angiogenic phenotype dramatically suppressed MSC expansion in these cancers.
**Fig. 4.** miR-15a and Angpt1 regulate angiogenesis in Wnt1 mammary tumors. 

(A) Angpt1 protein expression in MMTV-Cre;PPARγf/f;Wnt1 MSC transduced with control, Angpt1 shRNAs, and miR-15a lentivirus is shown by western blot. 

β-actin is expression used as the loading control. 

Histopathologic analysis of mammary tumors from control (B), Angpt1 shRNAs (C), or miR-15a lentivirus (D).
transfected MMTV-Cre;PPARγf/f;Wnt1 MSC indicates poorly differentiated adenocarcinomas as shown by H&E staining. Scale bar = 10 µm. (E) Decreased angiogenesis in mammary tumors from Angpt1 shRNAs and miR-15a lentivirus transduced MMTV-Cre;PPARγf/f;Wnt1 MSC. Representative CD31 immunofluorescence images of capillary endothelium are shown in tumors from control (F), Angpt1 shRNAs (G), and miR-15a (H) lentiviral transduced MSC. Scale bar = 20 µm. (I) Decreased MSC fraction in tumors from MMTV-Cre;PPARγf/f;Wnt1 MSC transduced with Angpt1 shRNAs or miR-15a lentivirus. Representative FACS analyses of MSC fractions in tumors from control (J), Angpt1 shRNAs (K), and miR-15a (L) lentivirus transduced MSC. (M) Quantitation of PCNA immunohistochemical staining in tumors from control, Angpt1 shRNAs, and miR-15a lentiviral transduced MSC. Representative images of PCNA expression in tumors from MMTV-Cre;PPARγf/f;Wnt1 MSC transduced with control (N), Angpt1 shRNAs (O), and miR-15a lentivirus (P). Sections were counterstained with hematoxylin. Scale bar = 10 µm. (Q) Quantitation of apoptotic cells in tumors from MMTV-Cre;PPARγf/f;Wnt1 control, Angpt1 shRNAs, and miR-15a transduced MSC. Representative TUNEL images of apoptotic cells in tumors from control (R), Angpt1 shRNAs (S), and miR-15a (T) lentivirus transduced MSC are shown. TUNEL positive cells are shown by dUTP-fluorescein labeling (green) and DAPI nuclear counterstain (blue). Merged images are shown at lower left. Scale bar = 5 µm.

PPARγ null mammary tumors expressed reduced miR-15a levels, suggesting that this microRNA may be a target gene for PPARγ. The canonical
PPAR\textsubscript{\gamma} response element is a direct repeat of the consensus sequence AGGTCA with a single nucleotide spacer (DR1 element). Sequence analysis of the miR-15a gene revealed a probable PPAR\textsubscript{\gamma} binding site in the 5' flanking region at -1593/-1573 bp (Fig. 5A). Chromatin immunoprecipitation using tumor derived CD24/CD49f MSC revealed PPAR\textsubscript{\gamma} binding to the DR1 region, but not in PPAR\textsubscript{\gamma} null cells (Fig. 5B). We cloned 1.8 kb of the miR-15a 5' flanking region into the pGL3 luciferase reporter vector. The miR-15a 5' flanking region induced luciferase reporter activity by 4 fold (Fig. 5C). Transient transfection of a mouse PPAR\textsubscript{\gamma} expression vector induced transcription of the miR-15a reporter construct by 40%, but not when the putative PPRE was mutated (P<0.008). These data indicate that PPAR\textsubscript{\gamma} activates expression of the miR-15a gene by binding to a DR1 element in the 5' flanking region.
**Fig. 5.** The 5’ flanking region of miR-15a gene has promoter activity and a DR1 element that is activated by PPARγ. (A) Sequence of the putative PPARγ response element (PPRE) located 1593 bp upstream of the transcriptional start (miR-15a wt). Site directed mutation in PPRE is shown in lowercase letters (miR-15a mut). (B) PPARγ binds to the miR-15a PPRE in genomic DNA. Chromatin immunoprecipitation of miR-15a PPRE from MMTV-Cre;PPARγ+/-;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 MSC was performed using
anti-PPARγ antibody. Control IgG was used as the negative immunoprecipitation control. Non-immunoprecipitated genomic DNA (input) was used as the amplification control. DNA size marker is shown at left. (C) PPARγ induces miR-15a promoter activity via the DR1 element. Relative luciferase activities in miR-15a promoter and site directed DR1 mutant are shown.

Our results indicate that PPARγ induces miR-15a expression which then targets Angpt1 to inhibit angiogenesis in Wnt1 mammary tumors. Loss of PPARγ expression inhibits miR-15a expression resulting in elevated Angpt1 levels and angiogenic mammary cancer phenotype. This increased angiogenesis directly correlates with MSC fraction in these tumors, suggesting that the angiogenic niche promotes stem cell expansion. To determine the effects of inhibiting angiogenesis on MSC expansion and mammary tumor phenotype, we treated MMTV-Cre;PPARγ+/-;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 mammary tumors with the targeted anti-angiogenesis chemotherapy drug sunitinib. Sunitinib is a receptor tyrosine kinase inhibitor targeting vascular endothelial growth factor receptor 2 (VEGFR2), platelet derived growth factor receptor b (PDGFB) and c-Kit (Widmer et al., 2014). Mammary tumor volume increased in both genotypes treated with sunitinib (Fig. 6A). Sunitinib treated tumors resembled poorly differentiated luminal adenocarcinomas characterized by reduction in basal cell numbers by histopathologic analysis (Fig. 6B,C). Sunitinib dramatically inhibited the angiogenic phenotype in MMTV-Cre;PPARγf/f;Wnt1 mammary tumors (P<0.01; Fig. 6D-F). Inhibition of the angiogenic phenotype by sunitinib
dramatically decreased the MSC fraction (by 95%; P<0.03) in MMTV-Cre;PPARγf/f;Wnt1 but not MMTV-Cre;PPARγ+/+;Wnt1 mammary tumors (Fig. 6G-I). Sunitinib treatment significantly decreased cell proliferation in MMTV-Cre;PPARγf/f;Wnt1 but not in MMTV-Cre;PPARγ+/+;Wnt1 mammary tumors (P<0.0001; Fig. 6J-L). Sunitinib treated MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 mammary tumors exhibited significant increases in TUNEL positive basal and luminal cells (P<2x10^-7; Fig. 6M-O), but no differences between genotypes were observed. These results indicate that disruption of the angiogenic niche results in depletion of the MSC population in MMTV-Cre;PPARγf/f;Wnt1 mammary tumors. However, tumor volume continued to increase following anti-angiogenic therapy.
Fig. 6. MSC from MMTV-Cre;PPARγf/f;Wnt1 mammary tumors are sensitive to anti-angiogenic chemotherapy. (A) Quantitation of change in tumor volume in MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 mammary tumors
treated with sunitinib chemotherapy. Histopathologic analysis of MMTV-Cre;PPARγ+/+;Wnt1 (B) and MMTV-Cre;PPARγf/f;Wnt1 (C) mammary tumors treated with sunitinib reveals poorly differentiated luminal adenocarcinomas as shown by H&E staining. Scale bar = 10 µm. (D) Decreased angiogenesis in sunitinib treated MMTV-Cre;PPARγf/f;Wnt1 mammary tumors. Representative CD31 immunofluorescence images of capillary endothelium in sunitinib treated MMTV-Cre;PPARγ+/+;Wnt1 (E) and MMTV-Cre;PPARγf/f;Wnt1 (F) tumors. Nuclei were counterstained with DAPI (blue). Scale bar = 20 µm. (G) Decreased MSC fraction in sunitinib treated MMTV-Cre;PPARγf/f;Wnt1 mammary tumors. Representative FACS analyses of MSC fractions from MMTV-Cre;PPARγ+/+;Wnt1 (H) and MMTV-Cre;PPARγf/f;Wnt1 (I) tumors are shown. (J) Reduced cell proliferation in sunitinib treated MMTV-Cre;PPARγf/f;Wnt1 mammary tumors is demonstrated by PCNA immunohistochemistry. Representative images of PCNA expression in sunitinib treated MMTV-Cre;PPARγ+/+;Wnt1 (K) and MMTV-Cre;PPARγf/f;Wnt1 (L) mammary tumors. Sections were counterstained with hematoxylin. Scale bar = 10 µm. (M) Increased apoptotic cells in sunitinib treated MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 tumors. Representative TUNEL images of apoptotic cells in MMTV-Cre;PPARγ+/+;Wnt1 (N) and MMTV-Cre;PPARγf/f;Wnt1 (O) tumors are shown. TUNEL positive cells are shown by dUTP-fluorescein labeling (green) and DAPI nuclear counterstain (blue). Merged images are shown at lower left. Scale bar = 5 µm.
The MSC population in angiogenic MMTV-Cre;PPARγ/f/f;Wnt1 tumors was significantly more sensitive to sunitinib monotherapy than control mammary cancers. However, no reduction in tumor volume was observed in either group. We hypothesized that sunitinib treatment might sensitize by normalizing tumor vasculature MMTV-Cre;PPARγ/f/f;Wnt1 tumors to conventional cytotoxic chemotherapy. We first examined the response of MMTV-Cre;PPARγ/f/f;Wnt1 and MMTV-Cre;PPARγ+/+;Wnt1 mammary tumors to cyclophosphamide, a first line chemotherapy drug used to treat human breast cancer. Cyclophosphamide treatment halted the growth of MMTV-Cre;PPARγ+/+;Wnt1 tumors (Fig. 7A). However the volume of MMTV-Cre;PPARγ/f/f;Wnt1 tumors continued to increase during cyclophosphamide treatment (mean increase 221.5 mm³; P<0.007). Mammary tumors were classified as poorly differentiated luminal adenocarcinomas with reduced basal cell numbers (Fig. 7B,C). Capillary density was significantly decreased in MMTV-Cre;PPARγ/f/f;Wnt1 tumors treated with cyclophosphamide (P<0.004; Fig. 7D-F). Cyclophosphamide treatment significantly reduced the MSC population (P<0.004) in MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγ/f/f;Wnt1 (P<0.001) mammary tumors when compared to control treated groups (Fig. 7G-I). Cyclophosphamide treatment significantly decreased cell proliferation in MMTV-Cre;PPARγ+/+;Wnt1 tumors (mean 13.3% vs. 23.8% PCNA positive cells in control treated cancers; P<0.02; Fig. 7J,K). Interestingly cell proliferation in MMTV-Cre;PPARγ/f/f;Wnt1 tumors was not significantly affected by cyclophosphamide treatment (Fig. 7J,L). Cyclophosphamide treatment induced significant increases in apoptotic luminal
and basal cells in both MMTV-Cre;PPARγ+/+;Wnt1 (mean 18.6%; P<0.03) and MMTV-Cre;PPARγff;Wnt1 (mean 8.3%; P<0.05) tumors (Fig. 7M-O). These results indicate that MMTV-Cre;PPARγff;Wnt1 mammary tumors are more resistant to cyclophosphamide monotherapy compared to control tumors.
**Fig. 7.** MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 mammary tumors are resistant to cyclophosphamide chemotherapy. (A) Quantitation of changes in tumor volume in cyclophosphamide treated MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 mammary tumors. Histopathologic analyses of cyclophosphamide treated MMTV-Cre;PPARγ+/+;Wnt1 (B) and MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 (C) mammary tumors indicates poorly differentiated luminal adenocarcinomas as shown by H&E staining. Scale bar = 10 µm. (D) Decreased angiogenesis in cyclophosphamide treated MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 mammary tumors. Representative CD31 immunofluorescence images of capillary endothelium in cyclophosphamide treated MMTV-Cre;PPARγ+/+;Wnt1 (E) and MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 (F) mammary tumors. Nuclei were counterstained with DAPI (blue). Scale bar = 20 µm. (G) Decreased MSC fraction in cyclophosphamide treated MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 mammary tumors. Representative flow cytometric analyses of MSC fractions in MMTV-Cre;PPARγ+/+;Wnt1 (H) and MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 (I) tumors is shown. (J) Decreased cell proliferation in cyclophosphamide treated MMTV-Cre;PPARγ+/+;Wnt1 but not MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 mammary tumors is demonstrated by PCNA immunohistochemistry. Representative PCNA immunohistochemical images in MMTV-Cre;PPARγ+/+;Wnt1 (K) and MMTV-Cre;PPARγ<sup>f/f</sup>;Wnt1 (L) mammary tumors. Sections were counterstained with hematoxylin. Scale bar = 10 µm. (M) Increased apoptotic cells in cyclophosphamide treated MMTV-
Representative TUNEL images of apoptotic cells in MMTV-Cre;PPARγ+/+;Wnt1 (N) and MMTV-Cre;PPARγf/f;Wnt1 (O) tumors is shown. TUNEL positive cells are shown by dUTP-fluorescein labeling (green) and DAPI nuclear counterstain (blue). Merged images are shown at lower left. Scale bar = 5 µm.

To determine the effects of cytotoxic chemotherapy following normalization of the tumor vasculature, we treated MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 mammary cancers with sunitinib followed by cyclophosphamide. While MMTV-Cre;PPARγf/f;Wnt1 tumors were resistant to both drugs as single agents, treatment with sunitinib followed by cyclophosphamide significantly inhibited tumor growth (P<0.0004; Fig. 8A). Tumors from both groups treated with sunitinib and cyclophosphamide resembled poorly differentiated luminal adenocarcinomas with reduced numbers of basal cells (Fig. 8B,C). Sunitinib and cyclophosphamide treatment significantly inhibited the angiogenic phenotype in MMTV-Cre;PPARγf/f;Wnt1 (P<0.002; Fig. 8D-F) but not MMTV-Cre;PPARγ+/+;Wnt1 mammary tumors. Sunitinib and cyclophosphamide treatment significantly decreased the MSC population in MMTV-Cre;PPARγf/f;Wnt1 (1% vs. 49%; P<0.05; Fig. 8G-I) but not in MMTV-Cre;PPARγ+/+;Wnt1 mammary tumors. Sunitinib and cyclophosphamide treatment dramatically decreased cell proliferation in MMTV-Cre;PPARγ+/+;Wnt1 (mean 13% vs. 24% in control treated tumors; P<0.04; Fig. 8J,K) and MMTV-Cre;PPARγf/f;Wnt1 (mean 27% vs. 34% in control treated
tumors; P<0.03; Fig. 8J,L) mammary tumors. Sunitinib and cyclophosphamide treatment significantly increased apoptotic basal and luminal cells in MMTV-Cre;PPARγ+/+;Wnt1 (42% vs. 0.1%; P<0.02; Fig. 8M,N) and MMTV-Cre;PPARγf/f;Wnt1 (38% vs. 0.2%; P<10^{-5}; Fig. 8M,O) mammary tumors. These results indicate that MMTV-Cre;PPARγf/f;Wnt1 mammary tumors are more sensitive to sunitinib and cyclophosphamide chemotherapy than control tumors.
Fig. 8. MSC from MMTV-Cre;PPARγfl/fl;Wnt1 mammary tumors are sensitive to sunitinib/cyclophosphamide chemotherapy. (A) Reduction in volume
of sunitinib/cyclophosphamide treated MMTV-Cre;PPARγ+/++;Wnt1 and MMTV-Cre;PPARγ/f/f;Wnt1 mammary tumors. Histopathologic analyses of sunitinib/cyclophosphamide treated MMTV-Cre;PPARγ+/++;Wnt1 (B) tumors and MMTV-Cre;PPARγ/f/f;Wnt1 (C) mammary tumors indicates poorly differentiated luminal adenocarcinomas as shown by H&E staining. Scale bar = 10 μm. (D) Decreased angiogenesis in sunitinib/cyclophosphamide treated MMTV-Cre;PPARγ/f/f;Wnt1 mammary tumors. Representative CD31 immunofluorescence images of capillary endothelium in sunitinib/cyclophosphamide treated MMTV-Cre;PPARγ+/++;Wnt1 (E) and MMTV-Cre;PPARγ/f/f;Wnt1 (F) mammary tumors. Nuclei were counterstained with DAPI (blue). Merged images are shown at lower left. Scale bar = 20 μm. (G) Decreased MSC fraction in sunitinib/cyclophosphamide treated MMTV-Cre;PPARγ/f/f;Wnt1 mammary tumors. Representative FACS analyses of MSC fractions in sunitinib/cyclophosphamide treated MMTV-Cre;PPARγ+/++;Wnt1 (H) tumors and MMTV-Cre;PPARγ/f/f;Wnt1 (I) mammary tumors are shown. (J) Decreased cell proliferation in sunitinib/cyclophosphamide treated MMTV-Cre;PPARγ+/++;Wnt1 and MMTV-Cre;PPARγ/f/f;Wnt1 mammary tumors as demonstrated by PCNA immunohistochemistry. Representative PCNA immunohistochemical images of sunitinib/cyclophosphamide treated MMTV-Cre;PPARγ+/++;Wnt1 (K) and MMTV-Cre;PPARγ/f/f;Wnt1 (L) mammary tumors. Sections were counterstained with hematoxylin. Scale bar = 10 μm. (M) Increased apoptotic cells in sunitinib/cyclophosphamide MMTV-
CRE;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 mammary tumors. Representative TUNEL images of apoptotic cells in tumors from MMTV-Cre;PPARγ+/+;Wnt1 (N) and MMTV-Cre;PPARγf/f;Wnt1 (O) mammary tumors are shown. TUNEL positive cells are shown by dUTP-fluorescein labeling (green) and DAPI nuclear counterstain (blue). Merged images are shown at lower left. Scale bar = 5 µm.

Despite chemotherapy mediated depletion of the MSC population, we observed increased tumor volume with sunitinib and cyclophosphamide monotherapies. Consistent with MSC depletion, we observed marked reduction in basal layer cells in chemotherapy treated tumors. This raises an important question as to which cells may be driving tumor progression during chemotherapy. CD61+ luminal progenitor cells were previously identified as tumorigenic and capable of regenerating both basal and luminal cells in the Wnt1 mammary tumor model (Vaillant et al., 2008). We examined the CD61+ cell fraction in control and chemotherapy treated MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 tumors using FACS. Control treated MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 tumors contained mean CD61+ cell fractions of 2% and 2.5% respectively (Fig. 9A,B). Sunitinib treatment resulted in dramatic expansion of the CD61+ cell population (63% in MMTV-Cre;PPARγ+/+;Wnt1 and 73% in MMTV-Cre;PPARγf/f;Wnt1 tumors; P<10^-7; Fig. 9C,D). Cyclophosphamide treatment increased the CD61+ cell fraction in MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 tumors to
66% and 63% respectively (Fig. 9E,F). Sunitinib and cyclophosphamide chemotherapy increased the CD61+ cell fraction in MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 tumors to 44% and 72% respectively (Fig. 9G,H). To confirm the failure of basal cell regeneration in chemotherapy treated tumors, we performed immunohistochemistry using the basal and luminal cell markers keratin 5 and keratin 18. We observed consistent reduction of keratin 5 expressing basal cells in chemotherapy treated MMTV-Cre;PPARγ+/+;Wnt1 (Fig. 9I,J) and MMTV-Cre;PPARγf/f;Wnt1 (Fig. 9K,L) tumors. In contrast, no significant changes in keratin 18 positive cells were observed due to chemotherapy treatment in MMTV-Cre;PPARγ+/+;Wnt1 (Fig. 9M,N) and MMTV-Cre;PPARγf/f;Wnt1 (Fig. 9O,P) tumors. To determine if loss of the angiogenic phenotype in MMTV-Cre;PPARγf/f;Wnt1 mammary tumors was due to basal cell specific Angpt1 expression, we examined this protein by immunofluorescence microscopy in control and chemotherapy treated tumors from both genotypes. Angpt1 protein was expressed in both basal and luminal cells in control treated tumors from both genotypes (Fig. 9R,T). Angpt1 expression was 2 fold higher in MMTV-Cre;PPARγf/f;Wnt1 tumors consistent with our western blot results. Chemotherapy treatment significantly decreased Angpt1 expression in MMTV-Cre;PPARγf/f;Wnt1 mammary tumors (-3 fold; P< 0.01; Fig. 9S,U) which correlated with reduced angiogenesis in these cancers. These results indicate that the CD61+ luminal progenitor population undergoes dramatic expansion in the context of chemotherapy induced MSC depletion. Chemotherapy treatment inhibited angiogenesis in MMTV-Cre;PPARγf/f;Wnt1 mammary tumors due to
failure of basal cell regeneration and reducing Angpt1 expression in the luminal population.

**Fig. 9.** CD61+ luminal progenitor cell expansion in chemotherapy treated MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγ/f/f;Wnt1 mammary tumors.
Representative flow cytometric analyses of CD61+ luminal progenitor cell fraction in control MMTV-Cre;PPARγ+/+;Wnt1 (A) and MMTV-Cre;PPARγf/f;Wnt1 (B), sunitinib treated MMTV-Cre;PPARγ+/+;Wnt1 (C) and MMTV-Cre;PPARγf/f;Wnt1 (D), cyclophosphamide treated MMTV-Cre;PPARγ+/+;Wnt1 (E) and MMTV-Cre;PPARγf/f;Wnt1 (F), sunitinib and cyclophosphamide treated MMTV-Cre;PPARγ+/+;Wnt1 (G) and MMTV-Cre;PPARγf/f;Wnt1 (H) mammary tumors. Reduced basal cell marker keratin 5 expression in MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 tumors treated with sunitinib/cyclophosphamide chemotherapy. Representative immunohistochemical images of keratin 5 expression in control (I) and sunitinib/cyclophosphamide treated (J) MMTV-Cre;PPARγ+/+;Wnt1 mammary tumors, and control (K) and sunitinib/cyclophosphamide treated (L) MMTV-Cre;PPARγf/f;Wnt1 mammary tumors. Representative immunohistochemical images of keratin 18 luminal marker expression in control (M) and sunitinib/cyclophosphamide treated (N) MMTV-Cre;PPARγ+/+;Wnt1 mammary tumors, and control (O) and sunitinib/cyclophosphamide treated (P) MMTV-Cre;PPARγf/f;Wnt1 mammary tumors. Scale bar = 10 µm. (Q) Decreased Angpt1 expression in MMTV-Cre;PPARγf/f;Wnt1 tumors treated with sunitinib/cyclophosphamide. Representative immunofluorescence images of Angpt1 expression (green) with DAPI nuclear counterstain (blue) in control (R) and sunitinib/cyclophosphamide treated (S) MMTV-Cre;PPARγ+/+;Wnt1 mammary tumors, and control (T) and
sunitinib/cyclophosphamide MMTV-Cre;PPARγf/f;Wnt1 (U) mammary tumors. Merged images are shown at lower left. Scale bar = 5 µm.

Our data indicate that chemotherapy treatment depletes the MSC fraction in Wnt1 tumors which correlates with CD61+ luminal progenitor cell expansion. These results indicate that CD61+ luminal progenitor cells may drive mammary tumor progression. To determine if chemotherapy-treated MSC and CD61+ luminal progenitor cells are tumorigenic, we transplanted these populations from MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 tumors to mammary fat pads of immunodeficient mice. Chemotherapy-treated MSC transplants from both genotypes failed to form tumors (data not shown). However, transplanted chemotherapy-treated CD61+ luminal progenitor cells from both genotypes consistently formed tumors. These cancers were classified as poorly differentiated luminal adenocarcinomas (Fig. 10A,B). Notably, basal layer cells failed to regenerate in these transplanted tumors. Strikingly, the angiogenic phenotype was suppressed in tumors derived from transplanted CD61+ luminal progenitor cells sorted from chemotherapy-treated MMTV-Cre;PPARγf/f;Wnt1 cancers as determined by CD31 expression (Fig. 10C-E). Angpt1 expression was significantly suppressed in tumors derived from transplanted CD61+ luminal progenitor cells sorted from chemotherapy-treated MMTV-Cre;PPARγf/f;Wnt1 tumors (P< 0.008; Fig. 10F-H). We confirmed failure of basal cell regeneration using keratin 5 immunohistochemistry on tumors derived from transplanted CD61+ luminal progenitor cells sorted from chemotherapy-treated MMTV-
Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 tumors (Fig. 10I,J). In contrast, luminal cells were regenerated in tumors derived from transplanted CD61+ luminal progenitor cells sorted from chemotherapy treated MMTV-Cre;PPARγ+/+;Wnt1 and MMTV-Cre;PPARγf/f;Wnt1 cancers as confirmed by keratin 18 immunohistochemistry (Fig. 10K,L). The PCNA positive cell fraction was not significantly different in MMTV-Cre;PPARγ+/+;Wnt1 cancers (15% vs. 23%; Fig. 10M,N), but remained suppressed in MMTV-Cre;PPARγf/f;Wnt1 (20% vs. 35%; P<0.00005; Fig. 10M,O) tumors derived from transplanted CD61+ luminal progenitor cells sorted from chemotherapy treated mammary tumors. The TUNEL+ cell fraction was low in transplanted tumors, and no significant differences were observed between genotypes (Fig. 10P-R). Consistent with failure of basal cells to regenerate, we observed suppression of the MSC fraction in transplanted tumors by FACS (0.5%; Fig. 10S). Similarly consistent with observed luminal histopathology, the CD61+ luminal progenitor population remained expanded in transplanted tumors (72%; Fig. 10T). These results indicate that the CD61+ luminal progenitor (but not MSC) population drives tumorigenesis in chemotherapy treated mammary tumors. However chemotherapy treatment results in persistent suppression of angiogenesis, cell proliferation, and basal cell regeneration.
**Fig. 10.** Chemotherapy treated CD61+ luminal progenitor cells are tumorigenic but unipotent and not angiogenic. Histopathologic analysis of tumors from CD61+ luminal progenitor cells from chemotherapy treated MMTV-Cre;PPARγ+/+;Wnt1 (A) and MMTV-Cre;PPARγf/f;Wnt1 (B) indicates poorly differentiated luminal adenocarcinomas as shown by H&E staining. Scale bar = 10 µm. (C) Reduced CD31 expression in mammary tumors derived from
transplanted CD61+ luminal progenitor cells from chemotherapy treated cancers of each genotype. Representative CD31 (red) immunofluorescence images of capillary endothelium in mammary tumors from CD61+ luminal progenitor cells sorted from chemotherapy treated MMTV-Cre;PPARγ+/+;Wnt1 (D) and MMTV-Cre;PPARγf/f;Wnt1 (E) mammary tumors. Nuclei were counterstained with DAPI (blue). Merged images are shown at lower left. Scale bar = 20 \( \mu \text{m} \). (F) Reduced Angpt1 expression in transplanted mammary tumors derived from CD61+ luminal progenitor cells sorted from chemotherapy treated MMTV-Cre;PPARγ+/+;Wnt1 (D) and MMTV-Cre;PPARγf/f;Wnt1 (E) mammary tumors. Representative immunofluorescence images of Angpt1 expression (green) and DAPI nuclear counterstain (blue) in transplanted mammary tumors from CD61+ luminal progenitor cells sorted from chemotherapy treated MMTV-Cre;PPARγ+/+;Wnt1 (G) and MMTV-Cre;PPARγf/f;Wnt1 (H) mammary tumors. Merged images are shown at lower left. Scale bar = 5 \( \mu \text{m} \). Lack of basal cell regeneration in tumors derived from transplanted CD61+ luminal progenitor cells sorted from chemotherapy treated MMTV-Cre;PPARγ+/+;Wnt1 (I) and MMTV-Cre;PPARγf/f;Wnt1 (J) mammary tumors demonstrated by keratin 5 immunohistochemistry. Scale bar = 10 \( \mu \text{m} \). Luminal cell regeneration in tumors derived from transplanted CD61+ luminal progenitor cells sorted from chemotherapy treated MMTV-Cre;PPARγ+/+;Wnt1 (K) and MMTV-Cre;PPARγf/f;Wnt1 (L) mammary tumors demonstrated by keratin 18 immunohistochemistry. Scale bar = 10 \( \mu \text{m} \). (M) Quantitation of cell proliferation in tumors derived from transplanted CD61+ luminal progenitor cells sorted from
chemotherapy treated tumors of both genotypes is demonstrated by PCNA immunohistochemistry. Representative images of PCNA expression in tumors derived from transplanted CD61+ luminal progenitor cells sorted from chemotherapy treated MMTV-Cre;PPARγ+/+;Wnt1 (N) and MMTV-Cre;PPARγf/f;Wnt1 (O) tumors. Scale bar = 10 µm. (P) Quantitation of apoptotic cells in tumors derived from transplanted CD61+ luminal progenitor cells sorted from chemotherapy treated cancers from both genotypes as shown by TUNEL analysis. Representative TUNEL images of apoptotic cells in tumors derived from transplanted CD61+ luminal progenitor cells sorted from chemotherapy treated MMTV-Cre;PPARγ+/+;Wnt1 (Q) and MMTV-Cre;PPARγf/f;Wnt1 (R) tumors are shown. TUNEL positive cells are shown by dUTP-fluorescein labeling (green) and DAPI nuclear counterstain (blue). Merged images are shown at lower left. Scale bar = 5 µm. (S) Representative flow cytometric analysis of the CD24+/CD49fhi tumor stem cell fraction in tumors derived from transplanted CD61+ luminal progenitor cells sorted from chemotherapy treated mammary tumors. (T) Representative flow cytometric analysis of the CD61+ luminal progenitor cell population in tumors derived from transplanted CD61+ luminal progenitor cells sorted from chemotherapy treated mammary tumors.
Fig. 11. Validated mechanism for PPARγ regulation of angiogenesis.

Decreased PPARγ expression decreases miR-15a expression that leads to an increase in Angpt1 expression promoting capillary stabilization and formation.
5. DISCUSSION

5.1 Discussion

Our study is the first to report a microRNA target gene for the tumor suppressor PPARγ. A genomic study of PPARγ target genes using chromatin immunoprecipitation followed by high throughput sequencing in adipocytes failed to identify the PPARγ binding site in the miR-15a gene (Nielsen et al., 2008). These results suggest that factors other than sequence similarity to the canonical PPARγ binding site are required for receptor binding in specific cell types (Schmidt et al., 2011). The miR-15a gene is evolutionarily conserved from bony fish to mammals (Chen et al., 2005). The miR-15 microRNA precursor seed family that is made of up six conserved miRNAs that shares seed sequence homology and are clustered on three different chromosomes. The members of the family include miR15-a and miR-15-b, miR-16-1, miR16-2, miR-497 and miR-195 (Peterson et al., 2009). Studies have shown the miR-15a family regulates a number of cellular pathways related to cell division (Liu Q et al., 2008) and angiogenesis (Karaa et al., 2009). Additionally, miR-15a has been shown to play a role in heart disease (Small et al., 2010), neurodegenerative diseases (Nelson et al., 2008) as well as neoplasia (Calin et al., 2006). miR-15a expression was decreased in human breast cancers and cell lines (Liu et al., 2011; Luo et al., 2013), although target genes were not identified. In ischemia, miR-15a has been shown to target FGF2 and VEGF mRNAs thereby mediating anti-angiogenic effects (Yin et al., 2012; Spinetti et al., 2013). miR-15a also was shown to inhibit angiogenesis in colon cancer xenografts (Dai et al., 2012). miR-15a expression
was significantly reduced in multiple myeloma cells and was shown to target VEGF, inhibit angiogenesis, and retard tumor growth (Sun et al., 2013). Other microRNAs have been shown to regulate angiogenesis in human breast cancer. miR-126 expression is downregulated in human breast cancer and targeted VEGF levels (Zhu et al., 2011). miR-148a expression is reduced in breast cancer and inhibits angiogenesis by targeting ERBB3 (Yu et al., 2011). Loss of PTEN in breast cancer stromal fibroblasts downregulates miR-320, thereby upregulating its target ETS2 which promotes tumor angiogenesis (Bronisz et al., 2012). miR-126 inhibits angiogenesis by targeting signaling pathways such as insulin-like growth factor (Png et al., 2012). miR-145 inhibits tumor angiogenesis by targeting N-RAS and VEGF in breast cancer (Zou et al., 2012). miR-98 inhibits tumor angiogenesis in breast cancer cells by targeting matrix metalloproteinase 11 and activin receptor like kinase 4 (Siragam et al., 2012). Expression of the transcription factor GATA3 is reduced in breast cancer; GATA3 inhibits breast cancer metastasis by inducing expression of miR-29b which regulates angiogenesis (Chou et al., 2013). miR-155 promotes tumor angiogenesis by targeting the von Hippel-Lindau tumor suppressor and is associated with triple negative breast cancer and poor prognosis (Kong et al., 2014). miR-542-3p inhibits angiogenesis in breast cancer xenografts by targeting angiopoietin-2 and is associated with poor prognosis in humans (He et al., 2014). These studies indicate that the breast cancer angiogenic niche is regulated by highly complex transcriptional and post-transcriptional mechanisms.
One of the novel findings of our study is the existence of a MSC angiogenic niche in mammary cancer. The angiogenic phenotype of MMTV-Cre;PPARγf/f;Wnt1 tumors is associated with MSC expansion and increased cellular proliferation. Inhibition of this phenotype by genetic and pharmacologic methods dramatically reduced the MSC fraction and cellular proliferation. This is in contrast to a previous study which indicated that tumor hypoxia resulting from anti-angiogenic therapy results in stem cell expansion (Conley et al., 2012). An important difference in our study is the angiogenic phenotype of MMTV-Cre;PPARγf/f;Wnt1 mammary tumors. Our results suggest that anti-angiogenic therapy would be more effective if targeted at tumors expressing the angiogenic phenotype.

Our results indicate that Angpt1 expression is critical to angiogenesis and MSC expansion. Angpt1 is a vascular stabilizing factor that is constitutively expressed in vascularized tissues (Thurston et al., 2000). Angpt1 mediated activation of its tyrosine kinase receptor Tie2 results in survival signals for endothelial cells, vascular maturation, and stability. Mice that lack Angpt1 or Tie2 develop normal primary vasculature, but fail to undergo normal vascular remodeling and ultimately die (Sato et al., 1995; Suri et al., 1996). Angpt1 is overexpressed in mouse mammary and human breast cancer specimens and cell lines (Hayes et al., 2000; Stratmann et al., 2001; Currie et al., 2001; Monsky et al., 2002; Caine et al., 2003; Caine et al., 2004; Reiss et al., 2009). Treatment of gastric cancer cells with PPARγ ligands inhibited Angpt1 expression and angiogenesis, although the mechanism was not determined (Fu et al., 2006).
Angpt1 expression was higher in estrogen receptor negative breast cancers and correlated with increased angiogenesis (Harfouche et al., 2011). BRCA1 mutant and triple negative breast cancers also expressed higher Angpt1 levels (Danza et al., 2013).

Our results showed that normal human breast epithelium demonstrated diffuse PPAR\textsubscript{\gamma} nuclear staining with minimal stromal capillaries. In contrast, human breast cancer specimens were predominantly negative for PPAR\textsubscript{\gamma} expression. These PPAR\textsubscript{\gamma} negative breast cancer specimens were highly angiogenic. Breast cancer samples that expressed PPAR\textsubscript{\gamma} did not exhibit the angiogenic phenotype. These human breast cancer specimens that expressed PPAR\textsubscript{\gamma} showed a different pattern of nuclear localization compared to normal breast epithelium. PPAR\textsubscript{\gamma} expression in these cancers was confined to the nuclear periphery. Previous studies indicated that the nuclear periphery sequesters transcription factors from their chromatin binding sites (Heessen and Fornerod, 2007). Transcription factor localization to the nuclear periphery restricts access to target genes, limits transactivation, and is associated with transcriptional repression (Steglich et al., 2013). These results suggest that the majority of human breast cancers lack PPAR\textsubscript{\gamma} mediated transcription due to loss of receptor expression. In those PPAR\textsubscript{\gamma} expressing breast cancers, altered nuclear localization may result in transcriptional repression of the angiogenic phenotype. Genomic analysis of short and long range PPAR\textsubscript{\gamma} target genes in breast cancer will provide important insight into these findings.
Previous studies have lead to the hypothesis that normalization of abnormal tumor vasculature leads to more efficient chemotherapy drug delivery (for review see Jain, 2005). These effects have been demonstrated in preclinical xenograft studies (Blansfield et al., 2008). Pretreatment with sunitinib followed by cytotoxic chemotherapy was previously shown to produce superior results compared to simultaneous administration (Zhang et al., 2011). Our CD31 immunofluorescence studies indicated that sunitinib or cyclophosphamide monotherapy significantly inhibited the angiogenic phenotype, depleted MSC and basal cells, reduced cellular proliferation, increased apoptosis, but did not decrease tumor volume in MMTV-Cre;PPARγf/f;Wnt1 mammary tumors. Treatment with sunitinib followed by cyclophosphamide was required to reduce tumor volume in MMTV-Cre;PPARγf/f;Wnt1 mammary tumors.

By contrast, sunitinib treatment of MMTV-Cre;PPARγ+/+;Wnt1 mammary tumors did not significantly inhibit angiogenesis, deplete MSCs, reduce proliferation, nor decrease tumor volume. Cyclophosphamide treatment of MMTV-Cre;PPARγ+/+;Wnt1 mammary tumors produced effects different from those of sunitinib by significantly depleting MSC, reducing proliferation, increasing apoptosis, and halting tumor growth. Treatment of MMTV-Cre;PPARγ+/+;Wnt1 mammary tumors with sunitinib followed by cyclophosphamide failed to deplete MSC but reduced proliferation and decreased tumor volume. These results indicate that normalization of the tumor vasculature conferred chemotherapeutic sensitivity in angiogenic mammary tumors. Tumors lacking the angiogenic phenotype were more sensitive to first
line cytotoxic chemotherapy such as cyclophosphamide. These results indicate that evaluation of angiogenesis in human breast cancers may improve clinical response to targeted chemotherapy. This can be accomplished by evaluating angiogenesis through CD31 immunohistochemistry or immunofluorescence imaging when tissue is biopsied.

Our data showed that the tumorigenic CD61+ luminal progenitor population dramatically expanded following sunitinib or cyclophosphamide chemotherapy in conjunction with increased tumor volume. These results contrast with previous preclinical studies using other cancer types in which anti-angiogenic therapy inhibited tumor growth (Zhang et al., 2009). BRCA1 deficient mammary glands, Her2/neu and phosphoinositide 3-kinase driven mammary tumors, and BRCA1 mutant human breast cancers exhibited expansion of luminal progenitor cells (Lim et al., 2009; Smart et al., 2011; Lo et al., 2012; Tikoo et al., 2012). However, our paper is the first report of luminal progenitor cell expansion in response to anti-proliferative therapy. In contrast, chemotherapy treated MSC failed to generate tumors following transplantation. Currently it is not clear whether tumor hypoxia resulting from anti-angiogenic therapy or drug resistance mechanisms result in luminal progenitor cell expansion. However this expanded luminal progenitor population was not capable of regenerating basal layer cells, indicating that chemotherapy treatment induced unipotency in the CD61+ tumor fraction. Angpt1 expression and the angiogenic phenotype was suppressed in mammary tumors derived from chemotherapy treated luminal progenitor cells, indicating that anti-proliferative
therapy has long term effects on tumor differentiation and the angiogenic phenotype. Future studies will determine the mechanisms responsible for these altered tumor phenotypes.
5.2 Limitations of the study

Early studies of PPARγ function demonstrated that activation of the receptor had anti-angiogenic effects in tumor models, although the target genes responsible for this phenotype were not characterized. Previous studies have examined PPARγ response elements during adipocyte differentiation (Nakachi et al., 2008), but cell type specific cofactors are likely to regulate receptor binding to these canonical sequences. We have not yet examined PPARγ binding sites on a genomic scale in breast cancer to determine additional target genes regulating the angiogenic phenotype.

Other microRNAs have been shown to regulate angiogenesis in human breast cancer. miR-126 expression is downregulated in human breast cancer and targeted VEGF levels (Zhu et al., 2011). miR-148a expression is reduced in breast cancer and inhibits angiogenesis by targeting ERBB3 (Yu et al., 2011). Loss of PTEN in breast cancer stromal fibroblasts downregulates miR-320, thereby upregulating its target ETS2 which promotes tumor angiogenesis (Bronisz et al., 2012). miR-126 inhibits angiogenesis by targeting signaling pathways such as insulin-like growth factor (Png et al., 2012). miR-145 inhibits tumor angiogenesis in breast cancer by targeting VEGF and N-RAS (Zou et al., 2012). miR-98 inhibits tumor angiogenesis in breast cancer cells by targeting activin receptor like kinase 4 and matrix metalloproteinase 11 (Siragam et al., 2012). Expression of the transcription factor GATA3 is reduced in breast cancer; GATA3 inhibits breast cancer metastasis by inducing expression of miR-29b which regulates angiogenesis (Chou et al., 2013). miR-155 promotes tumor
angiogenesis by targeting the von Hippel-Lindau tumor suppressor and is associated with poor prognosis and triple negative breast cancer (Kong et al., 2014). miR-542-3p inhibits angiogenesis in breast cancer xenografts by targeting angiopoietin-2 and is associated with poor prognosis in humans (He et al., 2014). We have not yet undertaken a comprehensive examination of microRNA expression in breast cancer and their regulation of the angiogenic phenotype.

Angpt1 is proposed to be a vascular stabilizing factor. Angpt1 induces blood vessel maturation through pericyte recruitment and prevention of plasma leakage (Holash et al., 1999; Thurston et al., 2000; Stoeltzing et al., 2003; Augustin et al., 2009). Angpt1 regulates angiogenesis through interaction with its receptor Tie2 on endothelial cells. The Angpt1 null mutation is embryonic lethal due to blood vessel dysfunction and myocardial defects (Suri et al., 1996), resembling the placental angiogenesis defects of the PPARγ knockout mouse (Barak et al., 1999). Since expression of pro-angiogenic factors such as VEGF and FGF2 is not significantly different in PPARγ null tumors compared to control cancers, the effects of Angpt1 in this model may be to slow capillary turnover by this stabilization mechanism resulting in gradual microvessel accumulation in the mammary gland (data not shown). This process of capillary stabilization and accumulation (which we refer to as statheropoiesis) continues during tumorigenesis resulting in an angiogenic MSC niche. While the angiogenic phenotype in our mammary tumor model is clearly controlled by Angpt1
expression, the mechanism by which this growth factor regulates angiogenesis is not yet clear.
5.3. FUTURE DIRECTIONS

Human breast cancer specimens that expressed PPARγ showed a different pattern of nuclear localization compared to normal breast epithelium. PPARγ expression in these cancers was confined to the nuclear periphery. Previous studies indicated that the nuclear periphery sequesters transcription factors from their chromatin binding sites (Heessen and Fornerod, 2007). Transcription factor localization to the nuclear periphery restricts access to target genes, limits transactivation, and is associated with transcriptional repression (Steglich et al., 2013). These results suggest that the majority of human breast cancers lack PPARγ mediated transcription due to loss of receptor expression. In those PPARγ expressing breast cancers, altered nuclear localization may result in transcriptional repression of the angiogenic phenotype. Genomic analysis of short and long range PPARγ target genes in breast cancer will provide important insight into these findings.

Tumorigenic CD61+ luminal progenitor cells dramatically expanded following sunitinib or cyclophosphamide chemotherapy in conjunction with increased tumor volume. These results contrast with previous preclinical studies using other cancer types in which anti-angiogenic therapy inhibited tumor growth (Zhang et al., 2009). BRCA1 deficient mammary glands, Her2/neu and phosphoinositide 3-kinase driven mammary tumors, and BRCA1 mutant human breast cancers exhibited expansion of luminal progenitor cells (Lim et al., 2009; Smart et al., 2011; Lo et al., 2012; Tikoo et al., 2012). However we report luminal progenitor cell expansion in response to anti-proliferative therapy.
Currently it is not clear whether tumor hypoxia resulting from anti-angiogenic therapy or drug resistance mechanisms result in luminal progenitor cell expansion. However this expanded luminal progenitor population was not capable of regenerating basal layer cells, indicating that chemotherapy treatment induced unipotency in the CD61+ tumor fraction. Angpt1 expression and the angiogenic phenotype was suppressed in mammary tumors derived from chemotherapy treated luminal progenitor cells, indicating that anti-proliferative therapy has long term effects on tumor differentiation and the angiogenic phenotype. Future studies will determine the mechanisms responsible for these altered tumor phenotypes.
6. CONCLUSION

Loss of PPARγ expression decreases tumor latency, expands the MSC population, and increases cell proliferation in mammary tumors. Loss of PPARγ expression does not affect in vitro clonogenicity and proliferation of tumor derived MSC. Loss of PPARγ expression correlates with increased angiogenesis in mouse and human mammary tumors. Angpt1 and miR-15a regulate the angiogenic phenotype PPARγ null mammary tumors. PPARγ binds a DR1 response element to activate the miR-15a gene.

Sunitinib treatment inhibits angiogenesis, MSC expansion, and proliferation in PPARγ null tumors. PPARγ null tumors are resistant to cyclophosphamide treatment. Cyclophosphamide fails to inhibit proliferation in PPARγ null tumors. PPARγ null mammary tumors are sensitive to sunitinib and cyclophosphamide chemotherapy. Sunitinib and cyclophosphamide treatment inhibits MSC expansion and proliferation in PPARγ null mammary tumors.

In response to chemotherapy treatment, the tumorigenic luminal progenitor cell but not the MSC population expands. These luminal progenitor cells become unipotent and failed to regenerate basal layer cells. Luminal progenitor cells expressed reduced levels of Angpt1.
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VITA

Kaitrin Kramer
Kbaloue2@uic.edu or kaitrinkramer2@gmail.com

EDUCATION

University of Michigan School of Dentistry
Doctor of Dental Surgery
Expected Graduation date: May 2016

University of Illinois at Chicago College of Dentistry
Ph.D. in Oral Sciences
Expected Graduation date: December 2014

University of Illinois at Urbana-Champaign
B.S. Molecular and Cellular Biology
Minor in Chemistry
Graduation date: May 2008

RESEARCH EXPERIENCE

Student Summer Research Program, College of Dentistry University of Illinois at Chicago (Summer 2008)
• Worked in a lab in the center of molecular biology of oral disease under Dr. David Crowe investigating the ability to convert somatic cells including oral epithelial cells into induced pluripotent stem cells.
• Practiced techniques in cell culture, PCR, gel electrophoresis and bacterial transformation.

Undergraduate Research Assistant, University of Illinois at Urbana-Champaign (Summer 2005-Spring 2008)
• Working in the neuroscience lab of Dr. William T. Greenough investigating the effects of Fragile X- Syndrome on adult neurogenesis in the hippocampus
• Practiced techniques in BrdU injections, immunohistochemistry, tissue sectioning, fluorescent microscopy and stereology techniques

Summer Undergraduate Internship Program, Biomedical Sciences at the University of Pennsylvania (Summer 2007)
• Worked as an undergraduate assistant in a radiation oncology lab under Dr. Gary Kao investigating the optimum parameters for xenotransplantation of human cancer cells into a zebrafish (Danio rerio) model.
• Attend weekly workshops through Biomedical Graduate Studies
• Practiced techniques in maintenance of zebrafish facility, zebrafish breeding, collection of zebrafish embryos, microinjection of tumor cells & RNA, fluorescent microscopy, cell culture and cell transfection.
ACCESS Summer Research Program, Weill Cornell Graduate School of Medical Sciences, Cornell University (Summer 2006)
• Worked as an undergraduate assistant in a cell & developmental biology lab under Dr. Yutaka Nibu, studying the effects of a C-terminal Binding Protein in *Drosophila Melanogaster*
• Attend workshops and journal clubs with other members of the program as well as workshops at Sloan Kettering.
• Practiced techniques in western blotting, immunostaining, confocal microscopy, biochemical fractionation and maintenance of Drosophila colony.

Howard Hughes Undergraduate Research Fellow, University of Illinois at Urbana-Champaign (Summer 2004-Summer 2005)
• Offers an opportunity for students currently enrolled in a biological sciences or chemistry curriculum to conduct research under the guidance of research faculty.
• Learning state-of-the-art techniques used in laboratory and field research

POSITIONS & EXTRACURRICULAR ACTIVITIES

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<th>Year</th>
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<td>2014-present</td>
<td><em>Committee Member</em>, Continuing Education Committee Michigan Dental Association</td>
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<td>2014-present</td>
<td><em>Tutor</em>, Guided study sessions University of Michigan School of Dentistry</td>
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<td>2013- present</td>
<td><em>Member</em>, The Honor Society of Phi Kappa Phi University of Michigan Chapter</td>
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<td>2012- present</td>
<td><em>Member</em>, American Dental Education Association University of Michigan Chapter</td>
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<tr>
<td>2012- present</td>
<td><em>Member</em>, American Student Dental Association University of Michigan Chapter</td>
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<td>2012- present</td>
<td><em>Faculty &amp; Alumni Co-Chair</em>, Delta Sigma Delta Alpha Chapter</td>
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<tr>
<td>2012-2013</td>
<td><em>Member</em>, American Association for Cancer Research</td>
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<td>2012-2013</td>
<td><em>Member</em>, American Society of Clinical Oncology</td>
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<tr>
<td>2011-2012</td>
<td><em>Board Member</em>, American Association of Dental Research Board of Directors</td>
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<td>2011-2012</td>
<td><em>President</em>, National Student Research Group of the American Association of Dental Research</td>
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<td><em>President-Elect</em>, National Student Research Group of the American Association of Dental Research</td>
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<td><em>Sergeant –At-Arms</em>, Dental Student Council at UIC Chapter</td>
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<td>2010-2011</td>
<td><em>Philanthropy Chair</em>, Delta Sigma Delta Rho Chapter</td>
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<td>2009-2012</td>
<td><em>Member</em>, Clinic and Research Day Planning Committee</td>
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<tr>
<td>2009-2010</td>
<td><em>Member at Large</em>, National Student Research Group of the American Association of Dental Research</td>
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2009-2010  
*President*, Student Research Group at UIC

2009-2010  
*Licensure Chair*, American Student Dental Association UIC Chapter

2009-2010  
*Recruitment Chair*, Delta Sigma Delta Rho Chapter

2008-2012  
*Member*, Chicago Dental Society

2008-2012  
*Member*, American Student Dental Association

2008-2012  
*Student Member*, American Association of Dental Research

2008-2009  
*Vice President*, Student Research Group at UIC

2008-2009  
*Legislative Chairperson*, American Student Dental Association UIC Chapter

2008  
*Fellow*, Student Summer Research Program College of Dentistry University of Illinois at Chicago

2007  
*Fellow*, Summer Undergraduate Internship Program, Biomedical Sciences at the University of Pennsylvania

2006  
*Fellow*, ACCESS Summer Research Program Fellow, Weill Cornell Graduate School Medical Sciences, Cornell University

2005  
*Fellow*, Howard Hughes Undergraduate Research Fellows, University of Illinois at Urbana-Champaign

2005-2008  
*Research Assistant*, University of Illinois at Urbana-Champaign

**ACADEMIC AND PROFESSIONAL HONORS**

**Awards**

- Michigan Periodontal Alumni Association Award 2014
- Fairman Louise Alumni Scholarship Award-The University of Michigan Alumni Council, 2013-present
- Phi Kappa Phi Honor Society- The University of Michigan Chapter, 2013-present
- Hinman Student Research Symposium- Best Clinical Oral Presentation 2011
- AADR/Johnson & Johnson Healthcare Products Hatton Awards Competition- Second Place North American Division Junior Category, 2011
- Hinman Award- University of Illinois at Chicago College of Dentistry, 2011
- Chancellor’s Student Service Award Volunteer Service- University of Illinois at Chicago, 2011
- Bloc Travel Grant IADR/AADR, 2011
- Annual Regional Meeting Delta Sigma Delta Research Presentation- First Place, 2010
- Bloc Travel Grant IADR/AADR, 2009
- Young Investigator Award Chicago Chapter AADR- First Place Basic Science, 2009
Presentations

Hinman Student Research Symposium Oral Presentation, 2011
AADR/Johnson & Johnson Healthcare Products Hatton Awards Competition Oral Presentation, 2011
IADR/Unilever Hatton Competition Oral Presentation North American Division, 2011
IADR/AADR Annual Session Poster Presentation, 2011
Clinic and Research Day at UIC Poster Presentation, 2011
Midwestern Regional Conference at the University of Minnesota Poster Presentation, 2011
Annual Regional Meeting Delta Sigma Delta Oral Presentation, 2010
AADR/IADR Annual Session Poster Presentation, 2009
Clinic and Research Day at UIC Poster Presentation, 2009
Midwestern Regional Conference at UIC Poster Presentation, 2009
Young Investigator Award Chicago Chapter AADR Oral Presentation, 2009
Leadership Alliance National Symposium Oral Presentation (University of Pennsylvania), 2007
Leadership Alliance National Symposium Poster Presentation (Cornell Weill Medical College), 2006

PUBLICATIONS

Papers


Abstracts


Awah FN, Baloue K, Crowe DL. Stem Cell Derived Biological Response Modifiers for Oral Mucositis Therapeutics. Abstract for poster presentation. 87th General Session & Exhibition of the International Association for Dental Research & the 38th Annual Meeting of the American Association for Dental Research.

Baloue K, Crowe DL. Master Switch of Oral Epithelial Differentiation. Abstract for poster and oral presentation. 89th General Session & Exhibition of the International Association for Dental Research & the 40th Annual Meeting of the American As