THE ADIPOCYTE AND ENDOTHELIAL CELL-SPECIFIC ROLE OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORγ IN BREAST TUMOURIGENESIS

by

Alexis Lynn Reid

A thesis submitted to the Graduate Program in Pharmacology and Toxicology

In conformity with the requirements for

the degree of Master’s of Science

Queen’s University

Kingston, Ontario, Canada

(December, 2012)

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Abstract

Peroxisome proliferator-activated receptor (PPARγ) plays a role in tumorigenesis. Previous studies with PPARγ(+/−) mice suggest PPARγ normally suppresses dimethylbenz[a]anthracene (DMBA)-induced breast, and other, tumor progression. Since many cell types associated with the mammary gland express PPARγ, each with unique signaling pathways, the present study aimed to define which tissues are required for PPARγ-dependent anti-tumor effects. Conditional adipocyte and endothelial cell-specific PPARγ knockout mice (PPARγ-A KO and PPARγ-E KO respectively) were used to evaluate whether PPARγ signaling normally acts to prevent DMBA-mediated breast tumour progression in a stromal cell-specific manner. Twelve week old PPARγ KO mice and their congenic wildtype (WT) controls were randomly assigned to one of two treatment groups. All mice were treated by gavage once/week for 6 weeks with 1 mg DMBA and maintained on a normal chow diet. At week 7, mice in each group were divided into those continuing normal chow, and those receiving a PPARγ ligand (ROSI, 4 mg/kg/day) supplemented diet for the duration of the 25 week study, and monitored weekly. Tumour and tissue samples were collected at necropsy, and portions of each were fixed and frozen for future analysis. In both PPARγ-A KOs and PPARγ-E KOs versus PPARγ-WT mice, malignant mammary tumor incidence was significantly higher and mammary tumor latency was decreased. DMBA+ROSI treatment reduced average mammary tumor volumes by 50%. Gene expression analyses of mammary glands by qRT-PCR and immunofluorescence indicated that untreated PPARγ-A KOs had significantly decreased BRCA1 expression in mammary stromal adipocytes. Compared to PPARγ-WT mice, serum leptin levels in PPARγ-A KOs were also significantly higher throughout the study. In the PPARγ-E KO mice, both treatment groups saw a significant increase in thymic tumour incidence, a finding not established before with the study of other
stromal cell knockout mice. These studies provide the first direct \textit{in vivo} evidence that PPARγ signalling in stromal adipocytes and endothelial cells attenuates DMBA-mediated breast tumourigenesis. This study supports a protective effect of activating PPARγ as a novel chemopreventive therapy for breast cancer.
Co-Authorship

The work presented in Chapter 2 was co-first authored, and is published in the journal of Carcinogenesis vol.33 no.7 pp.1412-1420, 2012. The work presented in Chapter 3 is a first authored manuscript in preparation for submission. Contributions of all co-authors are detailed in the respective sections. All other work was performed by myself.
Acknowledgements

I would like to thank the following individuals for their role in the completion of my thesis. My supervisor, Dr Chris Nicol, thank you for your patience in continuing to work with me for more than 5 years on this project. You made me feel as though I could finish, when I didn’t see it as a possibility. I am glad I carried on with my thesis when it would have been much easier to move on to new things and forget about it. I wish you much success with your research.

Our lab technicians Nichole Peterson, and Rachel Rubino. To Nikki thanks for teaching me everything I know about mice, and for being there on a day to day basis when I was the only student in the lab. Rachel thanks for all your help with breeding, necropsies and sending me any files that I needed after I had moved to Toronto.

My committee members Drs Louise Winn and Susan Cole. Thank you for your guidance, for help trouble shooting my problematic western blots, and for being patient with me.

Dr Tom Massey, for allowing me to take my first pharmacology course at Queen’s, Phar 811, when I was a graduate student at RMC, fresh out of chemical engineering and having never took a pharmacology class in my life!

Dr SenGupta, for taking the time out of his very busy schedule to analyze my tumour samples while taking the time to teach me along the way.

My dad and sister, Brett. Thanks for constantly asking me if I was “done that thesis yet”, for listening to my whining, for encouraging me to continue when it would have been much easier to quit and for always believing that I could get it done!
Table of Contents

Abstract ii
Co-Authorship iv
Acknowledgements v
Table of Contents vi
Abbreviations viii
List of Figures xi
List of Table xiii

Chapter 1 Introduction/Literature Review
  1.1 Etiology of Cancer 1
  1.2 Normal Mammary Gland Physiology 11
  1.3 Types of Breast Cancer 14
  1.4 Breast Cancer Risk Factors 21
    1.4.1 Lifestyle Risk Factors 21
    1.4.2 Environmental Risk Factors 23
    1.4.3 Genetic Risk Factors 25
  1.5 Peroxisome Proliferator-Activated Receptor (PPAR) 29
  1.6 The Role of PPARγ in Cancer 31
    1.6.1 In vitro Data Supporting an Anti-Cancer Role for PPARγ 33
    1.6.2 In vivo Data Supporting an Anti-Cancer Role for PPARγ 36
  1.7 PPARγ Mouse Models 37
  1.8 Hypothesis and Objectives 39

Chapter 2 Stromal Adipocyte PPARγ Protects Against Breast Tumorigenesis
  2.1 Abstract 41
  2.2 Introduction 42
  2.3 Materials and Methods 46
    2.3.1 Chemicals and reagents 46
    2.3.2 Animals 46
    2.3.3 In vivo tumourigenesis studies 47
    2.3.4 Pathology 47
    2.3.5 Mouse genotype and southern analysis 47
2.3.6 Immunoblot analysis 48
2.3.7 Analysis of gene expression by quantitative real-time PCR 48
2.3.8 Serum leptin ELISA 49
2.3.9 Immunofluorescent staining 50
2.3.10 Statistical analysis 50

2.4 Results

2.4.1 Confirmation of PPARγ recombination 51
2.4.2 PPARγ deletion in stromal adipocytes enhances breast tumourigenesis 51
2.4.3 Stromal adipocyte-specific PPARγ upregulates BRCA1 56
2.4.4 Stromal adipocyte-specific PPARγ expression decreases leptin secretion 59
2.4.5 PPARγ activity is dysregulated in tumourigenesis 62

2.5 Discussion 66

Chapter 3 The endothelial cell-specific role of PPARγ in DMBA-mediated Breast Tumourigenesis

3.1 Abstract 74
3.2 Introduction 76
3.3 Materials and methods 78

3.3.1 Chemicals and reagents 78
3.3.2 Animals 78
3.3.3 PPARγ mouse characterization and genotyping 79
3.3.4 In vivo tumourigenesis sturdies 79
3.3.5 Statistical analysis 80

3.4 Results 81

3.4.1 Endogenous PPARγ-E KO mouse phenotype 81
3.4.2 Mouse body mass 81
3.4.3 DMBA-mediated tumourigenesis 81

3.5 Discussion 95

Chapter 4 Conclusions and Future Directions 100

Bibliography 105
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>8-OH-2’dG</td>
<td>8-hydroxy-2’-deoxyguanosine</td>
</tr>
<tr>
<td>ADH</td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>ADH</td>
<td>Atypical ductal hyperplasia</td>
</tr>
<tr>
<td>ADRP</td>
<td>Adipose differentiation-related protein</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>BBC</td>
<td>Basal like breast cancer</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1, early onset</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>eLOX3</td>
<td>Epidermal-type lipoxygenase3</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extra cellular signal regulated kinases</td>
</tr>
<tr>
<td>FANCJ</td>
<td>Fanconi anemia group J</td>
</tr>
<tr>
<td>GFR</td>
<td>Growth factor receptor</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
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<td>HER-2</td>
<td>Human epidermal growth factor receptor 2</td>
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<td>HETE</td>
<td>Hydroxyeicosatetraenoic acid</td>
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<tr>
<td>HIA</td>
<td>5-hydroxy-indole acetate</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia inducible factor 1</td>
</tr>
<tr>
<td>IDC</td>
<td>Invasive ductal carcinoma</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin growth factor 1</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>Insulin growth factor binding protein 1</td>
</tr>
<tr>
<td>ILC</td>
<td>Invasive lobular carcinoma</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular carcinoma in situ</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>LNO₂</td>
<td>Nitrolinoleic acid</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>LO</td>
<td>Lipoygenase</td>
</tr>
<tr>
<td>LXRα</td>
<td>Liver X receptor α</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>mEH</td>
<td>Microsomal epoxide hydrolase</td>
</tr>
<tr>
<td>MIA</td>
<td>5-methoxy-indole acetate</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metaloprotease</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese super oxide dismutase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRP</td>
<td>Multi drug resistance-related protein</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acetyltransferases</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PALB2</td>
<td>Partner and localizer of BRCA2</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP-3</td>
<td>Phosphatidylinositol (3,4,5)-triphosphate</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptorγ</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 deleted on chromosome 10</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat Sarcoma Protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sitruin 1</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator of retinoid and thyroid hormone receptors</td>
</tr>
<tr>
<td>SNCG</td>
<td>Synuclien y gene</td>
</tr>
<tr>
<td>SRC</td>
<td>Steroid receptor coactivator</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>SULT</td>
<td>Sulfotransferases</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor β1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TPA</td>
<td>Tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferases</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XOR</td>
<td>Xanthine oxidoreductase</td>
</tr>
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</table>
List of Figures

Figure 1.1 Comparative representation of the human and mouse mammary glands 12
Figure 1.2 Progression of a normal mammary duct to invasive cancer 17
Figure 1.3 The proposed metabolic pathways of 7,12dimethylbenz[a]anthracene (DMBA)-mediated carcinogenesis 27
Figure 1.4 The mechanism of action of peroxisome proliferator-activated-Receptors (PPARs) 32
Figure 2.1 Characterization of adipocyte-specific PPARγA KO mice 53
Figure 2.2 In vivo effects of adipocyte-specific PPARγ deletion on DMBA-mediated breast tumourigenesis 54
Figure 2.3 In vivo effects of adipocyte-specific deletion on DMBA-mediated breast tumour onset and growth 57
Figure 2.4 In vivo effect of adipocyte-specific PPARγ deletion on mammary tumour pathology 58
Figure 2.5 Effects of adipocyte-specific deletion of PPARγ on gene expression in the mammary gland of untreated PPARγ-WT and PPARγ-A KO mice 60
Figure 2.6 In vivo effect of adipocyte-specific deletion of PPARγ on serum leptin and mammary tumour BRCA1 levels 63
Figure 2.7 In vivo effect of adipocyte-specific deletion of PPARγ on mammary tumour leptin receptor and ERα levels in PPARγ-WT and PPARγ-A KO mice 65
Figure 3.1 Body mass effect of DMBA treatment on endothelial cell-specific PPARγ-E KO and PPARγ-WT mice 82
Figure 3.2 Overall survival among DMBA-treated PPARγ-WT and PPARγ-E KO mice 84
Figure 3.3 Effect of endothelial-specific PPARγ deletion on DMBA-mediated total tumour incidences 85
Figure 3.4 Total tumour multiplicity among PPARγ-WT and PPARγ-E KO mice 87
Figure 3.5 Mammary tumour incidences among PPARγ-WT and PPARγ-E KO mice 88
Figure 3.6 Mammary tumour multiplicity PPARγ-WT and PPARγ-E KO mice 90
Figure 3.7 Mammary tumour latency PPARγ-WT and PPARγ-E KO mice 92
Figure 3.8 Mammary tumour volumes PPARγ-WT and PPARγ-E KO mice 93
Figure 3.9 Thymic tumour incidence PPARγ-WT and PPARγ-E KO mice 94
Figure 4.1 Summary of the protective effects of PPARγ expression in stromal adipocytes and endothelial cells during breast tumourigenesis 102
List of Tables

Table 1.1 Breast tumour subtypes 18
Chapter 1 Introduction and Literature Review

1.1 Etiology of Cancer

Cancer is the second leading cause of death after cardiovascular disease [1]. The Canadian Cancer Society estimates in 2012 that approximately 177,800 cases of cancer will be newly diagnosed and 75,000 of these diagnoses will result in death [1]. Breast cancer is the most commonly diagnosed type of cancer among women in Canada, and is the second leading cause of cancer-related deaths after lung cancer [1]. In 2012, 23,400 women were diagnosed with and 5,100 died from breast cancer [1]. It is expected that 1 in 9 women will develop breast cancer in their lifetime and 1 in 29 will succumb to metastatic complications from this disease [1]. In addition, among men who are susceptible to breast cancer, there is a similarly poor prognosis. The work that we are doing is broadly applicable. In 2012, it is estimated that 190 men will be diagnosed with this disease, and 55 dying as a result of breast tumour metastasis, suggesting that although men are less susceptible to this disease, those who are have a worse prognosis [1].

Determining populations that are at risk of developing cancer in their lifetime or who will successfully respond to available treatments, where they exist, is a continuing challenge due to the complex nature of this disease [2]. In 1775 the English physician and surgeon Percival Pott, reported the first possibility of an association between an environmental exposure and the development of cancer of the scrotum, in the case of chimney sweeps exposed to soot [3]. The role of genes in cancer was first proposed by Theodore Boveri in 1914 who postulated that chromosomes were the carriers of hereditary information and that cancer was due to problems with the chromosomes [4]. It was at this time that the first tumour viruses were identified in chickens by Ellerman and Bong in 1909 [4] and by Rous in 1911 [4], a revelation that would lead many years later to the discovery of the first oncogene [4]. The theories put forward by
these early scientific studies, supported by numerous others over the years, suggest carcinogenesis arises from a complex set of interactions between environmental and genetic factors.

Cancer is the consequence of the formation of neoplasms, fairly autonomous growths of tissue [5]. How cancer is initiated then progresses is still not fully understood. It is known that changes in DNA may result in cancer formation, and there are several hypotheses to explain how these changes can occur. One theory, referred to as the ‘one hit’ hypothesis, is that a single exposure to a carcinogen can induce the formation of a neoplasm regardless of the dose [6]. This is a good starting point, however it does not explain how this single alteration could be enough to trigger a neoplasm, given the frequency that people are exposed to carcinogens. This hypothesis was challenged by Knudson who, during studies of retinoblastoma, reported that cancer was likely caused by two mutational events [7]. Knudson also observed that the inherited form of retinoblastoma was associated with one mutation passed down from both a germinal and a somatic cell, while in the non-hereditary form of the disease, both mutations originated in somatic cells [7]. Thus, the two hit hypothesis accurately describes how mutations in both alleles of the single retinoblastoma gene, resulting in a loss or reduction of functional protein expression, may lead to cancer [8]. This also led to the classic term ‘tumour suppressor gene’, which applies to any gene whose normal function is to prevent some step in the carcinogenic pathway.

A proto-oncogene is a normal gene, which as the result of a genetic alteration, can transform into an oncogene. An oncogene is a gene that helps transform a normal cell into a tumour cell when it is mutated or expressed at high levels. Building on this information, cancer formation is generally associated with a dysregulation of oncogenes and/or tumour suppressor
genes [9]. However, as our understanding of carcinogenesis advances, these definitions are sometimes overly simplified. One such example, transforming growth factor β1 (TGF-β1) was originally reported to inhibit tumour growth by reducing the expression of proto-oncogenes such as c-myc and the platelet-derived growth factor-inducible KC and JE genes [10]. The status of TGF-β1 as a tumour suppressor came into question with the discovery that the metastatic ability of mammary adenocarcinoma cells in rats was enhanced when the cells were pre-treated with TGF-β1 [11]. Work done by Cui and colleagues (1996) [12] determined that TGF-β1 inhibited the formation of benign skin tumours, yet enhanced progression to invasive spindle carcinomas in transgenic mice. It is now accepted that depending on the stage of carcinogenesis examined, TGF-β1 is capable of serving as either a tumour suppressor or stimulator of tumour progression, invasion and metastasis.

In keeping with the notion that genes may play multiple roles in different stages or types of carcinogenesis, it was suggested by Hanahan and Weinberg [9] that the development of cancer is dependent on seven alterations in cell physiology that collaboratively dictate the growth of malignant tumours, namely: 1) self-sufficiency in growth signals; 2) insensitivity to antigrowth signals; 3) evasion of apoptosis; 4) limitless replicating potential; 5) sustained angiogenesis; 6) tissue invasion and metastasis; and 7) the ability to recruit normal cells to assist in the tumour acquiring the aforementioned hallmark traits, deemed the “tumour microenvironment”. Alterations can be further classified as essential milestones in either tumour initiation (numbers 1 and 2), promotion (numbers 3 and 4), or progression (numbers 5, 6 and 7). Changes that happen within any of these seven steps can lead to tumour formation, regardless of the source of these changes, such as genetic or environmental/lifestyle [9].
Tumourigenesis is a very complex process, which involves initiation, promotion and progression. Disruptions to the homeostatic balance between normal biological processes within a cell, whether inherited genetically or due to an environmental exposure, may overwhelm the mechanisms that regulate cellular growth, death and migration/invasion potential, eventually leading to tumour formation [13]. These disruptions may occur via many different mechanisms. Carcinogenesis may arise from DNA damage following exposure to several different classes of damaging agents, including radiation, heavy metals, and/or environmental chemicals. For example, exposure of human basal skin cells to UV radiation increases (C to T) mutations at dipyrimidine sites in one or both alleles of the p53 gene, leading to the development of basal cell carcinomas [14]. In addition, human exposure to heavy metals such as chromium, nickel and arsenic may cause the formation of DNA adducts, and changes in DNA methylation patterns and histone modifications [15]. Nickel can induce changes in DNA methylation as is observed in vivo in nickel-induced tumours of wild type C57BL/6 mice. Injecting nickel sulphide into mice results in 100% penetrance of malignant histiocytomas, and hypermethylation of the tumour suppressor p16’s promoter in all tumours [16]. Similarly, polycyclic aromatic hydrocarbons (PAHs), ubiquitous environmental chemicals, induce DNA damage by the formation of irreversible DNA adducts and/or the production of reactive oxygen species (ROS) [17]. ROS can react with the pyrimidine and purine bases of DNA resulting in the formation of different and frequently detrimental bases such as 8-hydroxy-2’-deoxyguanosine (8-OH-2’dG) [18]. Increased levels of ROS can be detoxified by cellular anti-oxidative pathways, including those regulated by manganese superoxide dismutase (MnSOD) [19]. MnSOD catalyzes the reduction of the superoxide anion into hydrogen peroxide and molecular oxygen [19]. Levels of MnSOD are
reportedly 2-3 fold lower in malignant breast tumours [19]. Although DNA damage can lead to cancer, the importance of DNA repair mechanisms in carcinogenesis must not be overlooked.

Due to the many protective pathways in a cell, exposures to environmental chemical carcinogens do not always lead to tumour formation [20]. Procarcinogenic PAHs require endogenous enzymatic bioactivation to assert their harmful effects [21]. One group of endogenous enzymes that assist in this bioactivation are the Cytochrome P450 enzymes (CYPs). CYPs are phase I enzymes that produce functional groups that may later serve as a site for conjugation catalyzed by the phase II enzymes such as UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), glutathione S-transferases (GSTs), and N-acetyltransferases (NATs) (best reviewed elsewhere) [22, 23]. There are more than 50 CYP isoforms, and it is the most important enzyme system involved in the biotransformation of endogenous and exogenous substances [23]. CYP families 1-3 have increased affinity for exogenous substances, like xenobiotics, and hence are not well conserved in evolution [23]. The most important CYP isoforms for the biotransformation of carcinogens are: CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2E1 and CYP3A4 [24]. The activity of these enzymes has great variability from person to person due in large part to genetic polymorphisms [25]. CYPs are highly inducible, serving as an adaptive response to past carcinogen exposure [23]. During CYP-catalyzed metabolism of PAHs, the formation of electrophiles that can covalently bind to macromolecules such as DNA and protein may result in cellular damage, which if unchecked, can ultimately lead to tumour initiation. [26]. CYP1A2 is involved in the metabolic activation of aryl and heterocyclic amines, is expressed highly in the liver [25]. It catalyzes the activation of PAH diols to reactive metabolites at a much slower rate than CYP1A1 and CYP1B1 [27]. The interindividual variability in CYP1A2 expression cannot be explained by genetic polymorphism, and it is
hypothesized that epigenetics may be playing a role [27]. CYP1B1 is expressed in the endoplasmic reticulum of extra-hepatic organs namely the uterus, breast, ovary, testis, prostate, and adrenal glands [23].

Defects in the bioactivation system of Phase I metabolism, through polymorphisms in either CYP1A1 or CYP1B1, can lead to increased risk of cancer development [28]. Four CYP1A1 polymorphisms were assessed for their implications in breast cancer risk: T3801C, T3205C (located within the 3’-noncoding region), A2455G and C2453A (with amino acid substitutions in exon 7 (Ile462Val and Thr461Asp, respectively) [29]. A subsequent meta-analysis revealed that there is a positive association between homozygous carriers of the A2455G (Ile462Val) allele and an increased risk for breast cancer amongst post-menopausal Caucasian women [29]. In contrast, despite its ability to metabolize estrogen to reactive catechols, no association between CYP1B1 polymorphisms (CYP1B1*1, CYP1B1*2, CYP1B1*3 and CYP1B1*4) and breast cancer risk was found [30]. However, participants with two copies of the CYP1B1*3 allele, and who had used menopausal hormones long term, had a 2-fold increased risk of breast cancer [30].

As a further cellular defence mechanism, the efficiency of detoxification pathways, for bioactivated carcinogens and their metabolites, plays an important role in determining whether chemical exposures will ultimately lead to tumour formation. Following detoxification, the ultimate carcinogen is typically converted from a lipophilic compound able to freely diffuse across membranes into a hydrophilic compound, such as following conjugation reactions catalyzed by UGTs and SULTs [31]. This results in a great increase in the hydrophilicity of the carcinogen and facilitates its excretion [31].
If exposure to a carcinogen or generation of carcinogenic metabolites overwhelm normal detoxification pathways, and damage DNA, tumour initiation may still be prevented through DNA repair pathways. The major forms of DNA repair are base excision, nucleotide excision, mismatch and double strand break repair [26]. These different pathways identify distinct types of DNA damage and either repair it, or guide the activation of cell death pathways if the damage is too severe. Base excision repair involves the removal of a single base by the enzyme DNA glycosylase; whereas, nucleotide excision repair typically removes damage formed by covalently bound DNA adducts, such as bioactivated PAHs [32, 33]. Mismatch repair differs in that it is triggered to assist normal nucleotides, if these nucleotides are incorrectly paired or not paired at all [34]. However, all three of these repair pathways involve respective enzymes that remove the altered bases, leaving a single stranded gap in the DNA. The correct DNA sequence is then restored with the aid of recruited DNA polymerases and ligases specific to each pathway [35]. The repair of DNA double-strand breaks involves a more complex approach, and is accomplished through either homologous recombination or non-homologous end-joining [18]. In homologous recombination, genetic information is exchanged between homologous regions of sister chromatids after DNA replication has occurred [18]. The complex which helps in the initiation of homologous recombination contains an exonuclease (MRE11A), a DNA double-strand break repair enzyme (Rad50), ATPase which catalyzes the conversion of ATP to ADP and a phosphate ion, and a protein (NBS1) which is critical for the positioning of the complex [18]. The complex serves as a flag of the break and recruits the ataxia telangiectasia mutated protein (ATM) which sends signals to cell-cycle checkpoint proteins and DNA repair enzymes to stop other replication and to focus on the repair of the strand break [18]. Non-homologous recombination, which is predominantly seen in higher eukaryotes, directly connects the two ends
of a DNA double strand break [18]. Non-homologous recombination is initiated by the binding of Ku-70/Ku-80 heterodimer to the DNA breaks, sheltering them from further damage and also aligning them for ligation to occur [18]. The Ku heterodimer recruits the protein kinase DNA protein kinase C which establishes a new complex that serves to recruit the necessary repair machinery [18].

If for any reason DNA damage that leads to expression changes of oncogenes or tumour suppressor genes is not repaired before cell proliferation occurs, then a tumour “stem” cell will arise. An example of a proto-oncogene is the small GTPase Ras, which has three forms (K-Ras, H-Ras and N-Ras) [36]. Mutations in Ras are detectable in 30% of all cancers and result in increased cell proliferation [37]. In contrast, the tumour suppressor gene phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is involved in the regulation of cell differentiation, adhesion and migration [38]. The discovery that Cowden disease, an autosomal dominant inherited disorder in which people have multiple tumour like growths and are at an increased risk for some cancers, is associated with germ-line PTEN mutations helped support the notion that it could be a tumour suppressor [39]. Among breast and prostate cancer patients, loss of even one copy of the PTEN gene, through immunohistochemical and chromosomal analyses, is associated with decreased overall survival [40, 41]. A loss or inactivation of the second copy of PTEN, referred to as a loss of heterozygosity (LOH), is also associated with poor overall patient survival in some cancers including prostate [41, 42]. Somatic PTEN mutations are also found in different types of cancers, such as endometrial cancer, where a PTEN LOH was noted in 48% of tumours [43].

The process of tumour formation progresses through the 3 stages of initiation, promotion and progression. Initiation occurs due to cellular transformation resulting from mutation or DNA
damage that was not properly repaired [44]. The change that induces tumour formation could be genetically inherited or due to environmental exposures. Promotion then acts to increase the growth [45] of these damaged cells into tumours. Promotion occurs because there is a disturbance in cell signal transduction which results in inflammation and reduced regulation of cell proliferation [45]. One important pathway associated with tumour promotion is the arachidonic acid metabolism pathway [46]. Specifically, the over expression of cyclooxygenase 2 (COX-2) which metabolizes arachidonic acid into downstream products, such as prostaglandin (PG)E2, that inhibit apoptosis and promote cell growth [45]. Progression can be defined as the point at which the genetic changes are karyotypic and irreversible [47]. The karyotypic changes result in invasion, anaplasia, metastatic spread and higher growth rates [47].

Epigenetic mechanisms are also known to play a role in tumourigenesis. Epigenetics is the alteration of a gene’s transcriptional activity without having a direct affect on the primary DNA sequence [48]. Examples of epigenetic alterations include changes in DNA promoter methylation and demethylation patterns, and modifications to histones such as methylation, acetylation and deacetylation [49]. As a result of epigenetic changes, disruption of normal expression patterns, including oncogenes and tumour suppressor genes, can lead to increased carcinogenesis. In tumour suppressor genes, increased DNA promoter methylation leads to a repressed chromatin state, thereby silencing gene transcription [48]. This occurs when DNA methyltransferases (DNMTs) guide the binding histone deacetylases (HDACs) and other methyl-CpG binding proteins to hypermethylated areas of the chromatin resulting in the formation of bulky complexes which prevent the binding of transcriptional machinery to the promoter [48]. The repression of tumour suppressor gene expression is also linked to methylation of histone H3 at lysines 9 or 27 and/or deacetylation at lysine 9, as well as methylation of histone H4 at lysine
20 and/or deacetylation at lysine 16 [48]. The epigenetics of carcinogenesis can be more closely examined by studying the epigenetics of tumour cells. Many tumour cells possess two prominent variations in DNA methylation patterns: 1) global hypomethylation and 2) regional hypermethylation of normally unmethylated CpG islands [48]. Global hypomethylation is observed in the early stages of neoplastic development and since this results in promoter accessibility, transcription can freely occur [48]. For example, the synuclien y (SNCG) gene, located on chromosome 10, is not expressed in normal mammary epithelial cells, yet is highly expressed in advanced stage breast carcinomas, in which hypomethylation of the CpG island located in exon 1 was also observed [50]. In contrast, hypermethylation of a promoter CpG island reduces transcription activity. If this occurs in a tumour suppressor gene, tumour formation may be enhanced [48]. For example, hypermethylation of the 5’ CpG island in the proximal promoter of SYK, a candidate tumour suppressor gene, is found in 30% of breast cancer cell lines [51].

Oxidative stress can also play a role in methylation status, as studies have shown that increased oxidative stress levels inhibit the binding of DNMTs to DNA, leading to hypomethylation [48]. Changes in gene expression levels of antioxidant enzymes, important in the detoxification of ROS, may also have a profound effect on carcinogenesis. It was reported that the levels of MnSOD are reduced in many different tumour types [52] and conversely when MnSOD levels are high, the tendency for tumours to progress to malignancy is lessened [53]. This suggests a relationship between epigenetics, ROS and carcinogenesis. Therefore, any imbalance in normal cellular pathways from exposure to environmental risk factors and leading to inherited genetic mutations can lead to carcinogenesis.
1.2 Normal Mammary Gland Physiology

The mammary gland is a dynamic tissue, capable of undergoing rapid changes to its structure and function. The mammary gland and its surrounding stroma are composed of many different cell types namely: epithelial, myoepithelial cells, adipocytes, endothelial cells, fibroblasts and macrophages [54]. The mammary gland also contains both stem and progenitor cells [55], either of which may be ultimate targets for transformation into tumour stem cells. Depending on the age, stage of development or pregnancy requirements, the cells of the mammary gland proliferate, differentiate, invade, migrate and apoptose, which may provide important clues about similar processes during tumourigenesis.

The human and mouse mammary gland follow a similar course of development, making the mouse a good tool for learning about breast cancer [56]. However significant differences also exist between the mammary glands of mice and humans. The functional portion of the human mammary gland is referred to as the terminal ductal lobular unit; whereas, in the mouse it is called the lobuloalveolar unit. There are stromal differences that exist between the two as well. The stoma in the human mammary gland is comprised mainly of dense fibroblasts, along with loose connective tissue, blood and lymphatic vessels, and adipocytes (Figure 1.1) [56]. In mice, the stroma consists mainly of adipocytes along with fibrous connective tissue, blood and lymphatic vessels [56]. Regardless of these differences, the human and mouse mammary glands are considered to be functionally similar [56].

The mouse has five sets of mammary glands located under the skin, outside of the fascia. The glands are distributed in both the thoracic and inguinal region, having three and two pairs, respectively [54]. The development of the mammary gland is controlled primarily by hormone signaling and occurs over 6 distinct stages: embryonic, prepubertal, puberty, pregnancy, lactation
**Figure: 1.1 Comparative representation of the human and mouse mammary glands.** Panel A highlights the human terminal ductal lobular unit, while Panel B highlights the mouse mammary gland. The human stroma is composed primarily of fibroblasts, fibrous connective tissue and blood and lymphatic vessels, with a small population of fat tissue. Conversely, the mouse mammary gland is predominantly composed of fat tissue, with a smaller population throughout of fibroblasts and fibrous connective tissue [56].
and involution [56]. The majority of mammary gland development occurs postnataley. In the embryonic stage, the penetration of the mesenchyme by an epithelial bud begins at embryonic day 10 or 11 [54]. The mammary fat pad is then invaded by the elementary ductal structure at embryonic day 17, which forms due to rapid proliferation of epithelial cells, and continues to grow until birth [54]. After the completion of the embryonic development, the mammary gland lays in an inactive state until the mouse reaches the age of 3 weeks postnatal [54]. At 3 weeks postnatal, referred to as the prepubertal stage, the ovaries begin secreting estrogen, progesterone and growth hormone resulting in the appearance of terminal end buds [54]. Rapid duct growth occurs during this stage as the primary ducts grow and branch into secondary and tertiary structures to fill the mammary fat pad, and form the mammary ductal tree [54]. This process is completed by 12 weeks of age in a virgin mouse. Pregnancy and lactation are times of vast mammary cell differentiation [57]. During pregnancy, there is extensive proliferation of ductal branches and the formation of the alveolar buds, resulting in the formation of the lobuloalveoli [57]. The lobuloalveoli consist of the secretory epithelial cells that produce milk for lactation, which are reported to form by transdifferentiation of nearby stromal adipocytes [58].

It is unclear whether this transdifferentiation process also occurs in humans. During involution, there is a rapid reversal of the changes that have occurred namely apoptosis of the mammary epithelial cells and adipogenesis that restores the prevalence of the adipocyte cells in the mammary gland [58]. In humans, mammary gland development begins on gestational day 35, with the proliferation of epithelial cells [56]. Epithelial buds sprout between week 10 and 12 of gestation and facilitate the formation of the epithelial stromal border [56]. At this time a
differentiation occurs and the mesenchyme can begin to be classified into fibroblasts, endothelial cells, and adipocytes [56]. At 6 months gestation, the structural basis of the mammary gland has been established [56]. At 9 months gestation, lactogenic hormones are released which triggers the epithelial cells to become functional [56]. From birth until puberty the mammary gland lies in a dormant state. At the onset of puberty, the ducts elongate resulting in the formation of the terminal end buds and the development of the alveolar buds [56]. Also at puberty, an increase in the amount of fibrous and fatty tissue is noted [56]. The primary ducts extend from the nipple to form the terminal ductal lobular unit (for milk production) [56]. There are 4 different stages for these lobules (known as type 1, 2, 3 and 4) depending on the physiological state of a woman, where a virgin state is type 1, a pregnant state is type 4, and a woman that has already given birth is type 2 and 3. These different stages highlight the dynamic and complex nature of the mammary gland, which provide many opportunities for alterations in normal function that could lead to breast tumourigenesis.

1.3 Types of Breast Cancer

Breast cancer death rates have declined over the past decade, due to improved targeted therapies and advances in early detection. In all age groups breast cancer death rates have been declining since the mid 1990s and for women 20-39 the rates have been declining since 1969 [59]. An important indicator of treatment success is long-term survival rates. In women aged 40-79 the 5 year survival rate is 88%, however the rates are lower for those diagnosed between 20 and 39 years of age (81%) and those diagnosed after the age of 80 (80%) [59]. Thus, there is still a need to not only improve our understanding of the processes involved in breast tumour progression, but also identify and appreciate the interactions between environmental and genetic
risk factors that contribute to susceptibility to this disease. This may allow for the identification of susceptible populations, and may help in the development of improved chemotherapeutic interventions for breast cancer.

There are many different types of breast tumours, each having distinguishing features and different prognoses. There are two main categories of breast cancer 1) \textit{in situ} and 2) invasive breast cancer (\textbf{Figure 1.2}) [13]. \textit{In situ} refers to cases in which the cells that are affected by the cancer remain isolated to the site of the original tumour and have not yet spread to the surrounding tissue [13]. \textit{In situ} tumour can be further classified into either ductal carcinoma \textit{in situ} (DCIS) or lobular carcinoma \textit{in situ} (LCIS) [13]. In DCIS, the more common form of \textit{in situ} breast cancer, the affected cells are localized to the milk duct [13]. DCIS results from atypical ductal hyperplasia (ADH), then can progress to invasive ductal carcinoma (IDC), and finally metastatic disease [60]. In LCIS, the affected cells are localized to the lobules of the breast; however, patients who are diagnosed with LCIS are 8-10 times more likely to develop an invasive, late onset breast cancer [61]. Unlike \textit{in situ} breast cancer, invasive breast cancer spreads from the primary site to into surrounding breast tissue or the rest of the body [13]. The different types of invasive tumours include invasive lobular carcinoma (ILC) and invasive ductal carcinoma (IDC) with prevalence rates of 10 and 70\% respectively [13].

At diagnosis, a portion of the patient’s breast tumour is sent for pathological assessment of Estrogen Receptor (ER), Progesterone Receptor (PR) and HER2/neu (HER) expression levels and TMN staging. The TMN system contains three main components: the extent, size and number of tumours; presence of lymph node involvement and metastasis to a distant site. Together, these indicators are clinical tools used by physicians to classify the severity of a
cancer, ensure the best course of treatment is initiated, and determine a patient’s prognosis. The main characteristics of the major breast tumour subtypes are summarized in Table 1.1.

Breast cancer is a very complex and molecularly heterogeneous disease. The advent of microarray technology has allowed researchers to learn of the molecular intricacies of breast cancer [13]. The two major molecular subtypes of tumour are basal and luminal [62]. Luminal A breast cancers are the most common subtype comprising 45% of cancers [62]. They are characterized as being low grade differentiated tumours that express hormone receptors resulting in there having a better prognosis and the ability to respond favorably to hormone therapy, although response to chemotherapy remains poor [62]. Approximately 15% of breast cancers are of the basal subtype and of poor prognosis due to being high grade, proliferative and hormone receptor negative [62]. The basal subtype is commonly referred to as triple negative because they are typically ER negative, PR negative and lack HER-2 overexpression [68]. Their poor prognosis is related to a lack of targeted therapies, depending on chemotherapy as the main form of treatment [62].

These subtypes can be further classified into ER positive luminal A and B, human epidermal growth factor (HER-2/neu also referred to as ERBB2)-overexpressing and basal [62] [67]. These classifications are made by analysis of the expression pattern of over 500 genes present in the tumour [62]. Luminal tumours express ER (ER positive) and are characterized by expression of many genes that are also expressed by breast luminal cells [13]. ER positive luminal A and B can be distinguished by which genes are involved in the tumour’s proliferation [67]. In luminal B tumours, the genes CCNB1, MKI67 and MYBL2 are more highly expressed [67]. Breast cancers that express high levels of the nuclear marker of cell proliferation Ki67 are associated with worse outcomes [69]. Proliferation status is an
Figure: 1.2. Progression of a normal mammary duct to invasive cancer. In the normal duct the lumen is lined with a layer of epithelial cells. When DCIS is present the duct of the breast is filled with proliferating cancer cells. In IDC the cancerous cells break through the basement membrane and invade the stroma surrounding the duct. MEC = myoepithelial cell, BM = basement membrane. Adapted from [13]
## Table 1.1: Breast Tumour Subtypes

<table>
<thead>
<tr>
<th>Breast Tumour Type</th>
<th>Frequency</th>
<th>Molecular Features</th>
<th>Prognosis</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>42-59%</td>
<td>ER+ and/or PR+, HER2-, low Ki67</td>
<td>Good, respond to hormone therapy [62]</td>
<td>Tamoxifen or Tamoxifen plus ovarian function suppression[63]</td>
</tr>
<tr>
<td>Luminal B</td>
<td>6-19%</td>
<td>ER+ and/or PR+, HER2+ (or HER2- with high Ki67)</td>
<td>Poorer disease-free survival Increased risk of early relapse [64]</td>
<td>Endocrine therapy and selective PI3K inhibitor [65]</td>
</tr>
<tr>
<td>Her2+</td>
<td>7-12%</td>
<td>ER-, PR-, HER2+ 1.1.1</td>
<td>More aggressive disease [66]</td>
<td>Monoclonal antibody targeting HER2 and adjuvant chemotherapy [67]</td>
</tr>
<tr>
<td>Triple negative/ Basal</td>
<td>14-20%</td>
<td>ER-, PR-, HER2-</td>
<td>Poor prognosis high risk of recurrence [68]</td>
<td>Chemotherapy (anthracyclines, platinum agents etc )[68]</td>
</tr>
</tbody>
</table>
important factor for distinction between luminal A and B breast cancer, and increased proliferation is associated with a poorer prognosis [67]. There is not currently an immunohistochemistry based assay that is able to distinguish between luminal A and B and Ki67 is a viable marker for distinction of luminal A and B molecular subtypes [67].

Luminal A tumours are treated with Tamoxifen only for postmenopausal patients or Tamoxifen plus ovarian function suppression for premenopausal women [63]. Luminal B tumours, although they express the estrogen receptor like luminal A tumours, have an increased risk of relapse with endocrine therapy [64]. It is hypothesized that luminal B tumours have increased signaling within the PI3K pathway, which is a mediator of growth-factor receptor (GFR) signaling pathways [65]. These results indicate that future therapies for luminal B breast cancer should target PI3K in this subset of tumours [65].

Conversely, Her-2/neu is over expressed in 25-30% of primary human breast cancers, and is associated with low levels of ER and ER related genes[19], and an increased risk of recurrence and mortality [70]. HER-2 gene amplification is an early event in the tumourigenesis process and amplification is seen in close to half of all ductal carcinomas in situ, which lack evidence of invasive disease, the molecular portrait of HER-2 over expressing tumours are maintained through progression of the breast cancer including involvement of distant metastasis [71]. HER-2 gene amplification in breast cancer however has been associated with an increase in cell proliferation, tumour invasiveness, and increased progression to distal metastasis, accelerated angiogenesis and reduced apoptosis [72]. When compared with HER-2 negative tumours, HER-2 positive tumours are more often of a higher histologic grade and lacking ER and PR [72]. Patients who are determined to have a HER-2 positive tumour receive as a first line therapy the
monoclonal antibody targeting HER-2 (trastuzumab) alone or in combination with endocrine and adjuvant chemotherapy [73].

Basal like breast cancer (BBC) comprises between 12.3-36.7% of breast cancer cases varying in different subgroups, the most common of which being African-Americans, and young premenopausal women [74]. BBC’s are characterized by a low expression of ER’s, PR’s and lack HER-2 overexpression this phenotype is referred to as triple negative [68]. BBC’s cluster with triple negative, but the two terms are not synonymous. Basal like is a molecular phenotype that results from the identification of cytokeratins in breast tumours that are normally found only in the basal cell layer of the mammary epithelium [68]. Although most triple negative tumours have a basal like expression profile and most BBC’s are triple negative both of these categories have up to 30% discord [68]. BBC’s have a poor prognosis and a high probability of recurrence [74]. Due to the lack of hormonal receptors, the primary method of therapy for BBC’s is chemotherapy, with tumours responding to anthracycline and anthracycline/taxane-based regimes, but they maintain a high risk of relapse [68].

Normal-like breast tumours are ER positive, having a high level of expression of genes of the basal epithelial cells and adipose tissue of the breast and has low expression of genes characteristic of the luminal epithelial cells [13]. Normal-like breast tumours vary in which treatments they respond to. It has been shown by [75], that normal-like breast tumours do not respond as well to preoperative chemotherapy with paclitaxel and doxorubicin as basal and Her-2 positive tumours. Despite advances in treatments, there still needs to be advances, and the discovery of new therapeutic targets.
1.4 Breast Cancer Risk Factors

There are many risk factors that contribute to breast cancer susceptibility. These risk factors can be classified into those within one’s control (i.e. lifestyle and environmental exposures) and those that are not (i.e. hereditary factors). The proportion of breast cancers attributed to environmental factors may be much greater than was previously believed. In a study of monozygotic twins, it was reported that only 27% of breast cancer had a genetic link, whereas the remaining 73% of breast cancer is caused by controllable factors [76].

1.4.1 Lifestyle Risk Factors

Many factors associated with breast cancer risk include age, age of menarche and menopause, number and timing of pregnancies, and use of hormone replacement therapy. The risk of developing breast cancer increases with age, where risk is usually low for women under the age of 30 and increased in women 65 and older. Young age at onset of menarche, less than 12 years old, and late onset of menopause, more than 54 years old, are both established risk factors for the development of breast cancer [77]. Pregnancies, particularly those which occur in women under 25, appear to provide some protection against breast cancer, as compared to nulliparous women or women who have their first full term pregnancy at a later age, who are at an increased risk for developing breast cancer [78]. There is no protective effect, with respect to breast cancer, from an early short term pregnancy ending in either spontaneous miscarriage or abortion [77]. Hormone replacement therapy, used quite extensively in the past for menopausal women, increases breast cancer risk due to the tumourigenic properties of these hormones [79].

Numerous other lifestyle factors are associated with an increased risk of acquiring breast cancer such as obesity, lack of physical activity and alcohol intake. The International Agency for Research on Cancer estimates that 25% of breast cancers worldwide are linked to having a
sedentary lifestyle and being obese [80]. With respect to physical activity, the mechanisms and impact on breast cancer differ according to menopausal status. In pre-menopausal women, high intensity physical exercise can result in a delayed onset of and reduced frequency of menstrual cycles, which are both associated with a decreased breast cancer risk [81]. This is not the case in menopausal women. Exercise increases the free circulating levels of the sex hormone binding globulin, a glycoprotein that competitively binds to estrogens and androgens, and therefore helps to decrease their circulating levels [81].

With regards to alcohol, it has become evident that the acetaldehyde, a product of the metabolism of alcohol, could be implicated in breast cancer. Alcohol is metabolized by the enzyme alcohol dehydrogenase (ADH). When ADH is highly active, it leads to an increase in levels of acetaldehyde [82]. One mutation of ADH associated with breast cancer risk is ADH1C*1 [82]. Women homozygous for the ADH1C*1 allele who have an average alcohol consumption of 1-2 drinks per day have a two-fold increase in breast cancer risk compared to women lacking this polymorphism [83]. ADH may also affect the levels of estrogen and estrogen receptors although the mechanism is not yet fully elucidated [82].

Obesity is positively correlated to an increased risk for breast cancer in postmenopausal women; however, the same result is not noted in premenopausal women causing people to question what the mechanism is behind the association of obesity and breast cancer. In pre-menopausal women, those who fell into the highest category for body mass index (BMI) had an 18% lower risk of breast cancer [84]. In younger women, obesity is associated with a greater frequency of anovulatory cycles and therefore lower levels of circulating hormones [85]. A study by Lahmann, 2005 [86] showed that a weight gain of greater than 20kg that occurs when a women is between 20 and 60 years of age results in a 52% increase in breast cancer incidence as
compared to women who maintained a stable weight throughout their adult life. After menopause, adipose tissue, not the ovaries, becomes the major source of oestradiol [87]. Consequently, obese post-menopausal women have higher levels of circulating sex hormones than non-obese women [87]. Compounding this is the effect of obesity related hyperinsulinaemia. Hyperinsulinaemia inhibits the hepatic secretion of sex-hormone binding globulin, which results in an increase in available estradiol and testosterone [85]. Obesity can also cause a decrease in insulin growth factor binding protein 1 (IGFBP-1), which results in an increased circulating level of insulin growth factor 1 (IGF-1) [88]. IGF-1 increases cell proliferation and inhibits apoptosis [88]. Thus, obesity may contribute to breast tumourigenesis through the exposure of the mammary epithelium to paracrine signals that are produced by excess adipose tissue.

1.4.2 Environmental Risk Factors

Exposures to environmental chemicals are also contributors to breast cancer risk. Polycyclic aromatic hydrocarbons (PAHs) are some of the most ubiquitous sources of environmental carcinogens, resulting from incomplete carbon combustion. An example of a common PAH is 7,12-dimethylbenz[a]anthracene (DMBA), which both initiates and promotes tumours [89]. DMBA is commonly found in car and industrial emissions, as a byproduct of the pulp and paper industry, chimney soot, charbroiled food and in cigarette smoke [90]. Given the ubiquitous sources of this carcinogen, it is difficult to accurately determine human exposure levels; however, it is estimated that smoking one cigarette exposes a person to an average of 40-100 ng of DMBA [91]. It is implicated in both genotoxic effects that involve direct damage to the DNA and in non-genotoxic effects, such a hypo/hyper-methylation [92]. DMBA is a lipophilic environmental carcinogen, which lends to its storage in human fat, which could lead to
a high level of accumulation and exposure in the mammary gland [93]. DMBA is a pro-
carcinogen that requires metabolic activation for the exertion of its carcinogenic effects [94]. In
the first pathway, CYP1B1 oxidizes DMBA to the 3,4-epoxide, which is subsequently
hydrolyzed by the enzyme microsomal epoxide hydrolase (mEH) to an intermediate metabolite,
DMBA-3,4-diol (Figure 1.3) [95]. CYP1A1 and CYP1B1 further oxidize the metabolite
resulting in the formation of the carcinogenic metabolite DMBA-3,4-diol-1,2-epoxide leading to
the formation of DNA adducts [95]. In the second pathway the formation of radical cations are
catalyzed by CYP peroxidases [21]. These radical cations have the ability to spontaneously
rearrange leading to the formation of unstable DNA adducts, which then undergo depurination to
become capable of carcinogenic initiation [21]. Finally, aldo-ketoreductases are also able to
activate DMBA-3,4-diol to a catechol which can lead to quinone formation and the generation of
ROS [21]. This processing leads to initiating events and potential carcinogenesis.

DMBA may also be converted to radical cations by CYP peroxidases. These cations are
capable of spontaneous rearrangement resulting in the formation of unstable DNA adducts,
which results in depurination and if not repaired can progress to carcinogenesis. The DMBA-3,4-
diol can also undergo oxidative metabolism by aldo-ketoreductases, resulting in the formation of
catechol metabolites [94]. These metabolites can undergo redox cycling to form the highly
reactive o-semiquinone and o-quinone radicals. The quinines are capable of forming stable DNA
adducts, leading to initiating events and they generate ROS such as H₂O₂, and O₂ which can also
cause oxidative damage to DNA, if not repaired. It is also thought that cyclooxygenase 2 (COX-
2) and 5 lipoxygenase (5-LO), two bioactivation enzymes that generate ROS and oxidative
stress, may contribute to the oxidative metabolism of DMBA. Transgenic mice over-expressing
COX-2 were notably resistant to tetradecanoylphorbol-13-acetate (TPA)-induced tumour
promotion; however, they had 3.5 times more tumours compared to wild type mice when they were treated with DMBA [96].

The authors hypothesized that COX-2 aids in the efficient metabolism of DMBA to its carcinogenic form [96]. COX and 5-LO are both involved in the regulation of pathways involving the metabolism of arachidonic acid to eicosanoids. For example, COX-2 mediates the rate-limiting step in the synthesis of PGE₂, a critical pro-inflammatory mediator linked to cancer. COX-2 and PGE₂ are highly expressed in breast cancer [97]. Arachidonic acid also increases the activity of the multi drug resistance-related protein (MRP-1) via PGE₂ in lung cancer cells, which makes it difficult to design effective therapies [98]. Interestingly, COX-2 is positively associated with many pro-tumourigenic pathways, such as cell proliferation, invasion, increased motility and the ability to evade apoptosis, which may be mediated through its downstream metabolite PGE₂ [97].

1.4.3 Genetic Risk Factors

Although some breast cancer risk factors may be controlled, inherited factors cannot. One of the most prevalent hereditary factors associated with increased breast cancer risk is a mutation in either one of the tumour suppressor genes BRCA1/2. BRCA1, discovered in 1990, is located on chromosome 17 at position 17q21 and encodes for a protein that is 1863 amino acids in length [100]. BRCA2 is located on chromosome 13 at position 13q12.3 and encodes for a protein that is 3418 amino acids in length [101]. BRCA1/2 have a very complex role in the development of breast cancer, as they affect and are affected by many other genes known to modify cancer susceptibility [102]. The BRCA tumour suppressor genes are involved in genetic stability, DNA repair, DNA damage response and cell cycle checkpoint control [103]. BRCA1-mediated DNA repair is facilitated through its involvement in homologous recombination and nucleotide
excision repair pathways [104]. The occurrence of \textit{BRCA1}/2 mutations varies across different populations. In more ethnically diverse populations, such as Canada, founder effects are not as prevalent [105]. In Sweden, amongst families that are high-risk for breast cancer, 34\% of people carry a deleterious mutation for \textit{BRCA1}, and 69\% of these mutations are single in nature [106].

The opposite observation has occurred in Sardinia where 32\% of high-risk families carry a \textit{BRCA2} mutation with 11\% carrying a single \textit{BRCA1} mutation [106]. Men are not immune to the effects of \textit{BRCA1}/2 status. It was shown by Tai (2007) [107], that men who carry either \textit{BRCA1} or \textit{BRCA2} mutations have a higher risk of developing breast carcinomas as they age. \textit{BRCA2} mutations result in the highest risk of developing cancer a risk of 6.8\% at age 70 [107]. Mutations in the \textit{BRCA1}/2 may interfere with binding partner interactions that aid in exerting downstream effects, such as \textit{PALB2} in the homologous recombination pathway [108], or preclude phosphorylation by \textit{CHEK2} which is required to stop mitosis after DNA damage [109]. Similarly, \textit{FANCJ}, the gene product of the known tumour suppressor gene \textit{FANCJ}, assists \textit{BRCA1} in double strand break repair [110].

Nevertheless, mutations in \textit{BRCA1}/2 are not the only factors that contribute to increased breast cancer risk. \textit{BRCA1}/2 mutations account for only 3-5\% of all breast cancer cases in the general population, 25\% among those who have a family history of breast cancer and 35-40\% of all hereditary breast cancers [111, 112]. In fact, sporadic breast cancers are the most prevalent accounting for 90-95\% of all breast cancer cases [106]. Interestingly, \textit{BRCA1} expression is nearly undetectable in many cases of sporadic breast cancer, leading to the suggestion that reduced \textit{BRCA1} expression and signaling, whether via mutations or other means, may enhance tumourigenesis [113].

Another important genetic factor associated with an increased risk of breast cancer is the
Figure 1.3: The proposed metabolic pathways of 7,12-dimethylbenz[a]anthracene (DMBA)-mediated carcinogenesis. Cytochrome P450 (CYP) peroxidises generate radical cations that can spontaneously rearrange, resulting in the formation of unstable DNA adducts, leading to depurination and possible carcinogenic initiation. CYP1B1 can also generate DMBA epoxides that can be hydrolyzed to DMBA-3,4-diol, a proximate carcinogen, by microsomal epoxide hydrolase (mEH). CYP1A1 or CYP1B1 can then further metabolize DMBA-3,4-diol to DMBA-3,4-diol-1,2-epoxide, the ultimate carcinogen that forms stable DNA adducts which initiate carcinogenesis. Aldo-ketoreductases are also able to bioactivate DMBA-3,4-diol to a catechol, that undergoes spontaneous rearrangement to form DMBA-semiquinone anion radical and –quinone redox cycling radicals and reactive oxygen species (ROS). These metabolites are capable of both initiating as well as promoting carcinogenesis. It is hypothesized that cyclooxygenases (COX) and lipooxygenases (LPO) may also act in the same manner as the aldo-ketoreductases, contributing to DMBA-mediated carcinogenesis. Adapted from [21, 99].
tumour suppressor gene *PTEN*. *PTEN*, located on human chromosome 10q23 coding for a 403 amino acid protein, undergoes a loss of heterozygosity in a variety of human tumours [114]. *PTEN* plays a role as a lipid phosphatase in suppressing growth [115]. Phosphatidylinositol (3,4,5)-triphosphate (PIP-3), an important molecule involved in cell growth signaling, is a substrate for PTEN [115]. The balance between PTEN and phosphoinositide 3-kinase (PI3K) determines PIP-3 levels at the plasma membrane [116]. Upon binding, PIP-3 activates Akt, a proto-oncogene with anti-apoptotic properties [117]. The PI3K/Akt pathway controls cell proliferation and survival, as well as invasiveness [117]. The balance between PTEN and PI3K determines levels of PIP-3 at plasma membranes which then regulates the Akt kinase [116]. PTEN functions to keep low levels of PIP-3 through the cleavage at the D3 phosphate, which in turn results in lowered levels of activated Akt [38]. *PTEN* is a critical tumour suppressor because it acts through many different mechanisms to achieve suppression. PTEN is not a member of an enzyme family, and therefore when PTEN is lost or experiences a reduction no other phosphatase can be a substitute in terms of function [117]. PTEN also interacts with the well established tumour suppressor p53 [118]. The PTEN promoter has a binding site for p53 and induction of p53 protein increases levels of PTEN, resulting in a positive feedback loop [118]. It is therefore hypothesized that cellular loss of one of the these tumour suppressor genes will cause decreased levels of the other, or in other words, one hit leads to reduced activity of two tumour suppressor genes [118]. The response to DNA damage is regulated by PTEN [117]. When there is a mutation or deletion in *PTEN* there is an increased activation of Akt and subsequent phosphorylation at Ser345 of Chk1, a DNA damage checkpoint kinase, leading to its ubiquitination [117]. This results in the failure to achieve DNA damage repair and an increase in the number of DNA strand breaks [117]. These findings make it clear that PTEN is involved in
tumour suppression in a multifactorial fashion and that a decrease in PTEN expression can increase susceptibility to breast cancer. Taken together, there are many well characterized risk factors, both genetic and environmental, that contribute to individual breast cancer susceptibility, albeit for a subset of all breast cancer cases. However, there are still many sporadic breast tumours for which the underlying causes remain elusive. Therefore having an increased understanding of all the risk factors involved in breast tumourigenesis and how they interact may enhance the ability to isolate susceptible populations as well as develop more effective and personalized therapies for breast cancer. Based on a growing body of evidence, understanding the emerging role of peroxisome proliferator-activated receptor (PPAR)γ during breast tumourigenesis may provide important clues to unravel this mystery.

1.5 Peroxisome Proliferator-Activated Receptor (PPAR)γ

PPARs, of which there are three isoforms PPARα, β, and γ, are members of the orphan nuclear receptor super family [143]. PPARα and PPARβ are best reviewed elsewhere [119, 120]. PPARγ is a transcription factor that regulates the expression of genes involved in glucose and lipid metabolism [121]. In the unliganded state, PPARγ forms heterodimers with the Retinoid X Receptor (RXR) and co-repressors such as the nuclear receptor corepressor protein (NCoR) and the silencing mediator of retinoid and thyroid hormone receptors (SMRT) (Figure 1.4) [122]. The heterodimeric complex then binds to peroxisome proliferator response elements (PPREs), within the promoter regions of target genes [123]. Although the unliganded complex does not activate transcription, it may prevent the activation of associated genes [123]. Once binding of a ligand occurs, either endogenous or exogenous, a conformational change results in release of co-repressors and recruitment of co-activators, such as steroid receptor coactivator-1 (SRC-1),
histone acetyltransferase p300 (CBP), followed by recruitment of the transcriptional complex required to upregulate gene expression [124].

PPARγ is expressed predominantly in adipocytes, both white and brown, as well as in the breast, prostate, colonic crypt cells, vascular cells including endothelial and vascular smooth muscle cells, and immunological cells such as macrophages [125]. The major functions of PPARγ are adipogenesis, glucose homeostasis, and lipid metabolism [126]. Naturally occurring ligands for PPARγ are suggested to include palmitic acid, oleic acid, linoleic acid, arachidonic acid [127]. The serotonin metabolites 5-methoxy-indole acetate (MIA) and 5-hydroxy-indole acetate (HIA) have recently been discovered to activate PPARγ. Endogenous PPARγ ligands also stimulate adipocyte differentiation [128]. Nitrolinoleic acid (LNO₂) is a ligand for PPARγ, and promotes adipocyte differentiation [129]. Among the largest group of synthetic PPARγ ligands, the thiazolidinedione (TZD) class of drugs are best known for their ability to improve insulin resistance and lower blood glucose levels in Type II diabetic patients [130]. Newly synthesized ligands for PPARγ include 5-arylidene-3-benzyl-thiazolidine-2,4-diones as a parent compound, along with eight derivatives tested with differing halides on their benzene ring; all had anti-inflammatory activity [131].

The human PPARγ gene was localized on chromosome 3 at position 3p25 [132] while the mouse PPARγ is located on chromosome 6 at position E3-F1 [133]. PPARγ genes in both the mouse and the human extend over approximately 100 kb of genomic DNA and produce four different mRNAs in humans [134]. In mice, only two subtypes of PPARγ mRNA (γ1 and γ2) have been detected [135]. Human PPARγ1 mRNA contains eight exons [136]. Relative to the protein domains exons A1 and A2 code for the N-terminal domain, exons 2 and 3 encode separate zinc finger-like motifs that allow for binding of the receptor to the promoter regions of target genes in
the C domain, exon 4 relates to the hinge region, and exons 5 and 6 translate into the ligand binding domain [135]. Human PPARγ2 exons are identical to human PPARγ1 except in the N-terminal domain the exon present is named B, and there are only 7 exons in total [135]. The four mRNAs are created as a result of differential splicing and alternative promoter usage [132]. However, although there are four different mRNAs, PPARγ1, PPARγ2, PPARγ3, and PPARγ4 which differ in their 5’ end due to alternative promoter usage and splicing [134]. Three mRNA, PPARγ1, PPARγ3 and PPARγ4, code for the same protein, resulting in the translation of two proteins, PPARγ1 (475 amino acids) and PPARγ2 (505 amino acids) [134]. The two isoforms PPARγ1 and PPARγ2 differ in their N-terminal amino acids (28 and 30, respectively) [137]. While both isoforms are expressed to some degree among all PPARγ–expressing cell types, PPARγ1 is expressed highly in most non-adipogenic cell types, while expression of PPARγ2 is higher among adipogenic cells [139].

1.6 The Role PPARγ in Cancer

The anti-proliferative effects of PPARγ are not limited to adipocyte differentiation. PPARγ expression is increased in several Carcinomas [123]. For example, PPARγ activation inhibits the proliferation of malignant cells from breast adenocarcinomas [140], prostate carcinoma [141], colorectal carcinoma [142], non-small cell lung cancer [143], and gastric carcinoma cells [144]. However, PPARγ expression may vary based on the type of cancer since there are reports of PPARγ expression decreasing as tissues progress from a benign to a malignant state [145], while somatic PPARγ loss of function mutations occur in sporadic colorectal carcinomas, and somatic translocation of PAX8 and PPARγ was noted in follicular thyroid carcinoma [146]. Several studies suggest that peroxisome proliferator-activated
**Figure: 1.4 The mechanism of action of peroxisome proliferator-activated receptors (PPARs).** PPAR:RXR heterodimer, in association with a co-repressor, binds to the PPRE and becomes activated by a ligand, resulting in the release of the co-repressor. In the presence of ligands, PPARs can induce either ligand-dependent transactivation or transrepression. Transactivation involves PPARs heterodimerization with the retinoid X receptors (RXRs) followed by recognition of specific PPAR response elements (PPREs) and interaction with coactivators. Transrepression involves interference with other signal transduction pathways, including NFκB, STAT, and AP1. NFκB-RE: NFκB response element; IFN-RE = “interferon-stimulated gene factor” responsive element; TRE = O-tetradecanoylphorbol 13-acetate-responsive element. Adapted from [138].
receptor (PPAR)\(\gamma\) down regulates the expression of carcinogen bioactivating enzymes, namely COX-2 [147] that can generate DMBA-mediated ROS and oxidative stress.

1.6.1 *In Vitro* Data Supporting an Anti-Cancer Role for PPAR\(\gamma\)

Several studies have been conducted using various cancer cell lines *in vitro* to elucidate the mechanism of PPAR\(\gamma\) in cancer. Research regarding the role of PPAR\(\gamma\) in colon cancer has been contradictory [148]. In studies using human colon cancer cell lines, fifteen of the nineteen cell lines analyzed expressed very high levels of PPAR\(\gamma\) when compared to human fat tissue, and this PPAR\(\gamma\) helped to induce differentiation of the colon cancer cells [149]. In addition, PPAR\(\gamma\) activation in the colorectal HT-29 cell line inhibited cell growth by suppressing the cell cycle and inducing apoptosis [150, 151]. Several studies have also successfully used both RXR and PPAR\(\gamma\) ligands, exploiting the PPRE present in the COX-2 promoter region, as a way to decrease COX-2 expression and increase cell death among colon cancer cell lines. Studies comparing PPAR\(\gamma\) expression levels in normal prostatic epithelial cells versus the prostate cancer cell line PC-3 show that normal cells do not express either isoforms of PPAR\(\gamma\) and were unaffected by PPAR\(\gamma\) ligands; whereas, PC-3 cells express PPAR\(\gamma\)1 and were sensitive to growth inhibition [152]. Expanding on this idea, Campbell et al, 2008 [173] demonstrated that gamma tocopherol caused growth arrest in the PC-3 cell line and that this growth arrest selected for the prostatic cancer cells and not normal prostate epithelial cells. Gamma tocopherol is not a direct PPAR\(\gamma\) ligand and therefore could not itself be causing the growth arrest [153]. It was noted that 15-S-HETE, an endogenous PPAR\(\gamma\) ligand, is upregulated in the PC-3 cells treated with gamma tocopherol, leading to the hypothesis that 15-S-HETE-mediated activation of PPAR\(\gamma\) led to the observed growth arrest [173].
Studies using both human ER+ (MCF-7) and ER- (MDA-MB-231) breast cancer cell lines have demonstrated that PPARγ ligands induce apoptosis, decrease G0-G1→ S phase cell cycle progression, decrease cyclin D1 production, and upregulate the expression of PTEN [154]. PTEN is a tumour suppressor gene that contains a PPARγ responsive element in its promoter region, and is transcriptionally upregulated by the PPARγ ligand rosiglitazone, suggesting a regulatory role of PPARγ in PTEN expression [155]. Furthermore, PPARγ also increases expression of BRCA1 through direct transcriptional activation by a PPRE present in its promoter region [156]. The induction of tumour suppressor genes could help reduce the progression of tumourigenesis.

The effect of PPARγ and its ligands on VEGF levels has yielded different results that vary by tissue type. In the lung adenocarcinoma cell line PC-14, TZDs increase the mRNA expression of VEGF-A, and when treated with a PPARγ antagonist, GW9662, the mRNA expression levels returned to baseline [143]. A similar result was obtained using vascular smooth muscle cells treated with troglitazone where VEGF levels were increased in a dose dependent manner [157]. Similarly, VEGF levels were increased by PPARγ ligands in bovine aortic endothelial cells [158]. In contrast, rosiglitazone-treated primary human endometrial cells had decreased VEGF secretion [159]. Further, rosiglitazone treatment of endothelial cells from human pulmonary valve leaflet tissue also antagonized VEGF-induced proliferation [160].

The protein hormone leptin, produced primarily in and secreted from adipocytes, is also of great interest with respect to PPARγ. Primary human monocyte-derived macrophages have decreased expression of PPARγ when treated with leptin [161]. This decrease in PPARγ could accelerate the development of atherosclerotic lesions. Based on results in hepG2 hepatoblastoma and Huh7 hepatocellular carcinoma cells, leptin also upregulates hepatocellular carcinoma
proliferation, via activation of the JAK/STAT-PI3K/AKT-ERK axis [162]. Zhou et al (2009) expanded on this knowledge to discover that leptin induced inhibition of PPARγ in primary rat hepatic stellate cells is mediated by the PI-3 K/AKT and ERK signaling pathways. In kidney and colonic epithelial cells, leptin expression also promotes the invasiveness of these premalignant cells [163].

A role for leptin breast cancer is also emerging. Leptin expression is induced by obesity, insulin, TNF-α and glucocorticoids and is inhibited by β-adrenergic agonists and TZDs [164]. Estrogen up-regulates leptin whereas testosterone down-regulates it [165]. Leptin receptors are expressed in the mRNA of human breast cancer cell lines and malignant breast tumours adding to the idea that leptin could have a direct effect on cell signaling within the breast tissue. This is supported by a report that tumourigenesis is associated with an increased level of leptin expression in mammary epithelial cells [166, 167] showed that normal mammary epithelial cells do not express a significant level of Ob-R; whereas, in carcinoma cells there was an overexpression of Ob-R in 92% of cases compared to no overexpression in the normal epithelium. In addition, leptin stimulates aromatase expression in MCF-7 cells, via mitogen activated protein kinase (MAPK) and STAT signals, suggesting a pivotal role in enhancing in situ estradiol production and promoting cell proliferation in an estrogen-dependent manner [168]. Furthermore, in MCF-7 cells there is a good correlation between high levels of leptin and moderate Her-2 levels, and high physiologic doses of leptin induce Her-2 tyrosine phosphorylation [169]. In endothelial cells, leptin induces migration via activation of the PI3K, Akt, eNOS and ERK1/2 signaling pathways, and promotes angiogenesis via the Akt pathway [170]. Interestingly, the PPARγ ligands troglitazone and ciglitazone inhibit endothelial cell migration by blocking leptin induced activation of Akt and eNOS [170]. Catalano et al, (2011) [171] demonstrated that the activation
of PPARγ, by rosiglitazone, reverses the leptin-mediated promotion of breast tumour growth in MCF-7 cells. MCF-7 cells, and the ERα negative breast cancer cells, BT20 adding further evidence to the mechanism of leptin and PPARγ in breast cancer is not tightly related to estrogen dependency[171]. Leptin and estrogen are not fully independent as leptin can positively modulate aromatase activity [171].

1.6.2 In vivo Data Supporting an Anti-Cancer Role for PPARγ

A tumour suppressor role for PPARγ in different types of cancers has also been demonstrated in vivo. In a carcinogen-induced colon cancer model using C57BL/6 mice, inhibition of PPARγ resulted in increased cell proliferation and expression of the c-myc and cyclin D1 genes in the colonic epithelium [148]. In hepatocellular carcinoma, the role of PPARγ in diethylnitrosamine induced tumours was examined utilizing PPARγ(+/-) heterozygous and wild type mice [172]. The loss of one allele of PPARγ resulted in a significant enhancement of hepatic carcinogenesis, and treatment with the PPARγ ligand rosiglitazone decreased hepatocellular carcinoma incidence only among wild type mice, indicating a role for PPARγ in tumour progression [172]. Similarly, the effect of PPARγ on gastric cancer in PPARγ(+/-) heterozygous and wild type mice. Mice were provided N-methyl-N-nitrosurea by drinking water to induce cancer, and then randomized to either receive troglitazone or serve as control [173]. The authors’ reported PPARγ(+/-) mice were more susceptible to developing gastric cancer, and wild type mice treated with troglitazone had a significantly reduced frequency of gastric cancer [173].

PPARγ ligands promote the regression and/or stasis of DMBA-mediated rat mammary tumours [174, 175]. In addition, using an MCF-7 xenograft model, PPARγ ligand PG-J2, a natural specific ligand of PPARγ, prevented the development of leptin-induced MCF-7
xenografts, attributed to a significant lowering of the plasma leptin levels [171]. It was also observed that the typical increases in cell to cell aggregation and proliferation following exposure to leptin were inhibited by the addition of the PPARγ ligand rosiglitazone [171].

The first direct *in vivo* evidence showed that DMBA-treated PPARγ(+/−) mice had a >3-fold increase in malignant and metastatic breast, and other tumours, compared with their wild-type controls, suggesting that PPARγ may normally play a role in suppressing DMBA-mediated breast tumour progression [176]. A subsequent study using transgenic mice expressing the Pax8PPARγ fusion protein showed an increased mammary epithelial expression of CD29hi/CD24neg, CD5+ and double positive CK14/CK18 “stem like” cells, enhanced susceptibility to DMBA-induced mammary carcinogenesis, and a decreased chemoprotective response to PPARγ ligand, GW7845 compared to control mice [177]. In addition, Pax8PPARγ transgenic mice also have reduced levels of PTEN and increased Ras, AKT and ERK activation [177]. The majority of the tumours that formed in the transgenic mice were ERα-positive ductal adenocarcinomas [177].

**1.7 PPARγ Mouse Models**

Having a global deficiency in PPARγ does not allow for the proper terminal differentiation of the trophoblast or adequate placental vascularization, resulting in death at approximately gestational day 10.5 [128]. Therefore, the Cre/LoxP system was a useful alternative. With the Cre/LoxP system, deletions can be induced in a tissue specific and/or temporal fashion [178]. In short, two different transgenic mice are used to generate the desired knockout mouse. The first strain has a critical region of the gene of interest flanked by two DNA loxP recognition sites, resulting in a ‘floxed’ mouse [178]. A loxP site is a 34 base pair DNA sequence, containing for directionality, an 8 base pair core and has on either side a 13 base pair
complementary sequence [178] The loxP sites are recognized by the 38 kDa bacterial enzyme Cre recombinase (Cre), allowing for the excision of the flanked region of the gene and a functional disruption [178]. This is utilized in vivo by crossing the ‘floxed’ mouse with a transgenic mouse expressing Cre under the control of a temporal or tissue specific promoter [178].

For the generation of PPARγ knockout mice, exon 2 was targeted because it codes for the DNA binding domain of the expressed protein, thereby limiting any possible transcriptional activity [179]. For my studies, three Cre/loxP generated mouse lines were used. The PPARγ(fl/fl);Cre− strain (or PPARγ−WT as they will be referred to from here on) were used as control mice. In the absence of Cre expression they are phenotypically identical to wild type (+/+) mouse. To selectively target PPARγ recombination in endothelial cells, PPARγ(fl/fl);Tie2-Cre+ (henceforth referred to as PPARγ E-KO) mice were used in which Cre expression is under the control of the angiopoietin receptor type 2 (Tie2) [180]. The Tie2 promoter is active from the initial endothelial cell formation during gestational day eight [181], and is detectable in all tissues examined, with highest expression in lung adult endothelial cells [180], making it a useful tool for the deletion of PPARγ in all endothelial cells. To selectively target PPARγ recombination in adipocytes, PPARγ(fl/fl);Ap2-Cre+ (henceforth referred to as PPARγ A-KO) mice were used, in which Cre expression is under the control of the adipocyte lipid binding protein 2 (Ap2) gene promoter [182]. These mice can be used to demonstrate the importance of adipocytes to tumourigenesis, and are a means by which to enhance the study of the role of stromal cells in breast cancer. These mouse models are currently the best available to assess the in vivo endothelial and adipocyte cell-specific roles of PPARγ in DMBA-mediated breast tumourigenesis.
1.8 Hypotheses and Objectives

Collectively, the literature to date suggests PPARγ expression and signaling is important in stopping the growth and spread of breast tumours. However, the cell types and protective PPARγ-dependent mechanisms involved remain to be clarified.

To complement ongoing research in the lab, I hypothesized that in a mammary stromal cell-specific manner DMBA-mediated breast tumour progression is:

1) Enhanced by loss of PPARγ expression
2) Decreased by activation of PPARγ signaling

My objectives were to test these hypotheses by:

1. Evaluating the *in vivo* importance of PPARγ expression within adipocytes and endothelial cells using mouse knockout models during DMBA-mediated tumourigenesis

2. Examining whether activation of PPARγ signaling in these models stops breast tumourigenesis
Chapter 2 Stromal Adipocyte PPARγ Protects Against Breast Tumorigenesis

Authors:

* Equally contributing first authors

Contributions
I completed the mouse animal studies, performed the immunoblots, mouse genotyping
The co-authors contributions are listed below
GSK: RNA expression levels, protein expression levels, aided with necropsies, graphing and statistical analyses
AJA: aided with necropsies
MAD: Immunofluoresence imaging
RER and NTP: performed colony genotyping and breeding, aided with necropsies and the collection and processing during mouse studies. Performed leptin ELISA
MS, SKS: pathological classification of samples
CJBN: Supervised study, editing of manuscript
2.1 Abstract

Peroxisome proliferator-activated receptor (PPAR)γ regulates the expression of genes essential for fat storage, primarily through its activity in adipocytes. It also has a role in carcinogenesis. PPARγ normally stops the in vivo progression of 7,12-dimethylbenz[a]anthracene (DMBA)-mediated breast tumors as revealed with PPARγ haploinsufficient mice. Since many cell types associated with the mammary gland express PPARγ, each with unique signal patterns, the present study aimed to define which tissues are required for PPARγ-dependent anti-tumor effects. Accordingly, adipocyte-specific PPARγ knockout (PPARγ-A KO) mice and their wild-type (PPARγ-WT) controls were generated, and treated with DMBA for six weeks to initiate breast tumorigenesis. On week 7, mice were randomized to continue on normal chow diet or one supplemented with rosiglitazone (ROSI), and followed for 25 weeks for tumor outcomes. In PPARγ-A KOs versus PPARγ-WT mice, malignant mammary tumor incidence was significantly higher and mammary tumor latency was decreased. DMBA+ROSI treatment reduced average mammary tumor volumes by 50%. Gene expression analyses of mammary glands by qRT-PCR and immunofluorescence indicated that untreated PPARγ-A KOs had significantly decreased BRCA1 expression in mammary stromal adipocytes. Compared to PPARγ-WT mice, serum leptin levels in PPARγ-A KOs were also significantly higher throughout the study. Together, these data are the first to suggest that in vivo PPARγ expression in mammary stromal adipocytes attenuates breast tumorigenesis through BRCA1 upregulation and decreased leptin secretion. This study supports a protective effect of activating PPARγ as a novel chemopreventive therapy for breast cancer.
2.2 Introduction

Currently, one out of every eight North American women is diagnosed with breast cancer during her lifetime, and it is estimated that one in 35 will ultimately succumb to the disease [1]. In 2011, the American Cancer Society estimates that 230,480 American women will be diagnosed with breast cancer and 39,520 will die from disease progression, with equally poor prognosis among the smaller number of men who are also susceptible [183]. Improving our understanding of the interactions between genetic and environmental risk factors will enhance the therapeutic options and outcomes for breast cancer patients.

Several genetic factors are known to contribute to breast cancer including the functional loss of tumor suppressor genes, such as BRCA1 [184] and PTEN [185], as well as the over-expression of oncogenes, such as HER2 [186]. The candidate tumor suppressor gene peroxisome proliferator-activated receptor (PPAR)γ was discovered in the early 1990s [187]. It is a member of the nuclear receptor superfamily, and encodes two isoforms, PPARγ1 and PPARγ2, derived from alternative splicing. PPARγ is predominantly expressed in adipose tissue and is found at lower levels in epithelial cells of the colon, breast, bladder, prostate, liver, heart, and lymphoid tissues, and macrophage cell types [188-190]. PPARγ1 is preferentially expressed in non-adipogenic cell types, whereas PPARγ2 is expressed in cells committed to the adipocyte lineage, although both isoforms are often detectable in all PPARγ-expressing cells.

PPARγ heterodimerizes with the retinoid X receptor (RXR)α and the complex recognizes direct-repeat (DR)-1 motifs, referred to as peroxisome proliferator response elements (PPREs), in the promoters of target genes. In the unactivated state, this PPARγ;RXRα complex associates
with co-repressors, and may rest on PPREs, physically blocking transcriptional machinery or other transcription factors from transcribing target genes and, indirectly influencing gene transcription [187]. In contrast, when activating ligands bind, the PPARγ;RXRα complex undergoes a conformational change that allows it to release co-repressors and bind co-activators, resulting in direct transcriptional upregulation of gene expression [191]. PPARγ activating ligands include synthetic thiazolidinediones (TZD) such as the gold standard activator rosiglitazone (ROSI) [192], used widely for >10 years to treat and prevent Type II diabetes [193], and many natural fatty acids and fatty acid metabolites, such as linoleic acid [194].

PPARγ ligands are also reported to exert anti-breast tumorigenic properties in vitro and induce tumor growth arrest or shrinkage in rat models in vivo [140, 174, 175, 195, 196]. In addition, PPARγ agonists have been used in combination with chemotherapeutic agents such as cisplatin, to synergistically reduce breast tumour growth and nephrotoxicity in a DMBA-treated mouse model [197]. Despite the numerous in vitro-based PPARγ-dependent pathways proposed to explain these findings, more work is needed to clarify the cell-specific contributions of activating PPARγ signalling relevant to inhibiting breast tumourigenesis in vivo.

PPARγ signalling is well characterized for regulating gene expression important for maintaining normal fat and glucose metabolism [191]. In vitro assays have also identified several putative genetic targets of PPARγ that may be relevant to its role in cancer. For example, PPARγ activation reduces leptin secretion [198] and increases expression of BRCA1 through direct transcriptional activation via a PPRE in its promoter region [156]. In vivo evidence revealed that PPARγ was directly protective against breast tumorigenesis [176]. PPARγ(-/-) mice die in utero
around gestation day 10.5 so a PPARγ\(^{(+/-)}\) haploinsufficient mouse model was used to show that PPARγ normally protects against DMBA mediated breast, ovarian and skin tumorigenesis. In this experiment, PPARγ\(^{(+/-)}\) mice exhibited significantly increased mammary tumour multiplicity, and more interestingly, tumour progression to malignant and metastatic states was also significantly increased compared to PPARγ-WT controls [176], thus suggesting that normal PPARγ signalling stops breast tumorigenesis. Given that PPARγ is expressed in multiple epithelial and stromal mammary gland associated cell types [123], further in vivo studies are required to dissect the cell-type specific contributions of PPARγ expression and signaling that stops the growth and spread of breast tumors.

As discussed earlier, PPARγ is most highly expressed in adipocytes [123]. PPARγ is required for adipogenesis; forced expression of PPARγ induces NIH3T3L1 cells and mouse embryo fibroblasts to differentiate into adipocytes [199]. While the vast majority of breast tumours that present in the clinic are of epithelial origin, stromal cells, particularly adipocytes, have a profound effect on tumour progression [200]. Adipose tissue secretes several adipokines including protumorigenic leptin and aromatase, the latter catalyzing the rate-limiting step in estrogen biosynthesis, as well as the anti-tumorigenic adiponectin. The influence of these adipokines is believed to contribute to the increased susceptibility of obese individuals to breast tumor development [201]. Given the prevalence of PPARγ in adipose tissue and its demonstrated role in adipokine secretion, the role of adipocyte-specific PPARγ signalling in breast tumourigenesis was evaluated.
7,12-dimethylbenz(a)anthracene (DMBA) is a polycyclic aromatic hydrocarbon (PAH) widely used in the lab to both initiate and promote a variety of chemical-induced cancer models, including animal models of hormone-dependent breast cancer [202, 203]. DMBA is likely the most extensively characterized procarcinogen representative of other PAHs found in the environment. It requires metabolic activation to electrophiles that can lead to DNA adducts, gene mutations and oxidative damage [202, 203]. Due to the fact that DMBA is a lipophilic procarcinogen, accumulation of parent and/or proximal carcinogenic metabolites within adipocytes may also extend exposure times for surrounding tissues.

In the present study, in vivo studies using adipocyte-specific PPARγ knockout (PPARγ-A KO) mice, where PPARγ expression and signaling is disrupted in mammary stromal adipocytes, show enhanced susceptibility to DMBA-mediated breast tumorigenesis. This suggests the presence of stromal-epithelial crosstalk mediated by PPARγ, and is supported by findings of an increased survival advantage conveyed by loss of PPARγ in mammary stromal adipocytes associated with decreased BRCA1 levels, and enhanced circulating leptin.
2.3 Materials and Methods

2.3.1 Chemicals and reagents

7,12-dimethylbenz[a]anthracene (DMBA), 10% phosphate-buffered formalin, glycerol, ammonium persulfate were purchased from Sigma (St Louis, MO). Tris, SDS were purchased from Fisher (Whitby, ON). iScript cDNA synthesis kit, iQ SYBR Green Supermix, and acrylamide were purchased from Bio-Rad (Hercules, CA). Trizol and RNAse away were obtained from Invitrogen (Carlsbad, CA). Rosiglitazone (Avandia) was purchased from GlaxoSmithKline (Mississauga, ON). Primary and secondary antibodies to detect PPARγ, BRCA1 and β-actin were obtained from Santa Cruz Biotechnology (San Diego, CA).

2.3.2 Animals

All mice were treated in accordance with protocols approved by the Queens University Animal Care Committee, and housed in microisolator cages to ensure pathogen-free status. The cages were maintained on a 12h light/dark cycle, with food and water provided *ad libitum*. PPARγ-WT and PPARγ-A KO females were generated from crosses of previously described PPARγ-floxed mice [179] with transgenic mice expressing Cre recombinase (Cre) under the control of the adipocyte lipid-binding promoter 2 (Ap2) [204] generously provided by Dr. Barbara Kahn. The mice used in this study are of mixed C57Bl/6N;Sv129/N;FVB/NCr background and were bred using sibling matings for at least 15 generations prior to the start of the study.
2.3.3 *In vivo tumourigenesis studies*

*In vivo* mammary tumorigenesis was generated as previously described [176]. Briefly, 8-12 week-old female mice were gavaged p.o. with 0.1 ml (total 1 mg) DMBA (dissolved in corn oil) once a week for 6 weeks. On week 7, mice were randomized to continue on a normal chow diet or one supplemented with the gold standard PPARγ activator, rosiglitazone (4mg/kg/day). Gross/clinical examinations of mice were performed biweekly, for a total of 25 weeks, to monitor body weights, tumor progression and overall health. Mice were humanely killed by cervical dislocation either at the end of the study or earlier if they lost 15% body weight, showed signs of distress or presented with tumours >2cm in diameter. All mice were necropsied, and following recording of macro- and microscopic assessments of total tumor burdens, samples were measured with callipers and weighed. Normal and tumour tissues were then divided and either fixed in 10% phosphate-buffered formalin and embedded in paraffin, or snap frozen in liquid nitrogen and stored at -80°C for future analyses.

2.3.4 *Pathology*

Formalin fixed, paraffin embedded tumour sections were cut into 5 µm sections and stained with hematoxylin and eosin. These samples were then subjected to a blind review for classification and stage by our collaborating pathologists.

2.3.5 *Mouse Genotyping and Southern Analysis*

Homozygous PPARγ floxed mice expressing the Ap2Cre⁺ transgene were identified using PCR screening as previously described [176]. Genomic DNA was isolated from several PPARγ expressing tissues, including white adipose (WAT) from epididymal (Epi) and inguinal
(Ing) sites as well as brown adipose (BAT), and skeletal muscle, from male and female 
Pparg<sup>fl/fl</sup>;Ap2-Cre<sup>+</sup> (PPARγ-A KO) and congenic Pparg<sup>fl/fl</sup>;Cre<sup>+</sup> (PPARγ-WT) mice and 
digested overnight using BamH1. Samples were then assessed, using our 3'-probe, by Southern 
blot hybridization to detect the extent of Cre-mediated recombination of floxed PPARγ alleles as 
described elsewhere [179].

2.3.6 Immunoblot analysis

Nuclear protein preparations of frozen whole mammary glands including fat pads from 8-
week-old PPARγ-WT and PPARγ-A KO virgin females were prepared according to 
manufacturer’s protocols (BioRad, Hercules, CA) and quantitated for protein concentration using 
the BCA protein assay (Pierce Chemical Co., Rockford, Ill.). Equal amounts of protein for each 
sample were then separated on SDS-PAGE and transferred onto nitrocellulose membranes. Blots 
were then incubated with primary antibodies for PPARγ (sc-7273; 1:500) and Histone H1 (sc-
10806; 1:1000), followed respectively by horseradish peroxidase conjugated goat α-mouse (sc-
2005) and goat α-rabbit (sc-2004) secondary antibodies (1:10,000) purchased from Santa Cruz 
Biotechnology (Santa Cruz, CA). Immunoreactive protein detection was performed using the 
enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ). Relative 
protein levels were quantified using ImageJ analysis software.

2.3.7 Analysis of gene expression by quantitative real-time PCR

Total RNA was isolated from untreated tissues as well as tumours from PPARγ-WT and 
PPARγ-A KO mice according to the TRIZOL method. Spectrophotometric analysis was 
performed to monitor RNA purity and concentration. RNA samples were then converted to
cDNA using the iScript cDNA synthesis kit (Bio-Rad). cDNA was quantified similar to RNA samples, and combined with iQ SYBR Green mix, autoclaved water and the necessary primers to evaluate expression of four PPARγ targets: PPARγ1, PPARγ2, BRCA1 and PTEN. Primers were purchased from Invitrogen (San Diego, CA), mPPARγ1F; CCT GAC GGG TCT CGG TTG, mPPARγ1R; TGT CCT GAA TAT CAG TGG TTC ACC, mPPARγ2F; TGG GTG AAA CTC TGG GAG ATT C, mPPARγ2R; AAT TTC TTG TGA AGT GCT CAT AGG C, mBRCA1F; GCA GCT GTG TGG GGC TTC CGT G, mBRCA1R; GTT GCT GTC TTC TGT CCA GGC GC, mPTENF; TGA CAG CCA TCA TCA AAG AGA, mPTENR; CTG CAG TTA AAT TTG GCG GT, mGAPDH F; TCA TGA CCA CAG TGG ATG CC, mGAPDH R; GGA GTT GCT GTT GAG AAC ATG CGA CTT GTC GC and IDT (Skokie, IL), mEsr1(ERα)F; GCA CAG GAT GCT AGC CTT GTC TC, mEsr1(ERα)R; AAT TGT CAC CAG CTT GCA GGT TC [205], mLepR(leptin receptor)F; CAT ACT CGG TCA CTC ACA ATG CT, mLepR(leptin receptor)R; GCC AAT GAG AAC ATG CGA CTT CT [206]. The Bio-Rad thermocycler was programmed to perform a 5 min 95°C hot-start followed by 50 cycles of 95°C for 15s, 65°C for 15s and 72°C for 30s.

2.3.8 Serum Leptin ELISA

Blood collected from mice pre-treatment, at week 13 and upon necropsy was spun down to obtain serum, flash frozen in liquid nitrogen and stored in -80°C. Plates and standards to detect mouse/rat serum leptin were set up according to manufacturer’s (Cayman Chemical) instructions and read at 450nm.
2.3.9 Immunofluorescent Staining

Formalin fixed, paraffin embedded tissue blocks were sectioned into 5µm slices, mounted on slides, and incubated at 55°C overnight. Samples were deparaffinized and rehydrated by washing in: xylene, 4 min; xylene, 4 min; xylene, 4 min; 100% ethanol, briefly; 85% ethanol, briefly; 70% ethanol, briefly; and ddH₂O, 4 min.Slides were placed in 1:10 sodium citrate buffer solution at 95°C for 20 min, then trypsined with 1×trypsin (Sigma) for 20 min at 37°C. After washing, the slides were placed in Triton X/TBS buffer solution, followed by a 30 min incubation period in 5% BSA block in TBS. After washing, primary antibodies (Santa Cruz) for PPARγ (sc-7196, 1:500) and β-casein (sc-166520, 1:500) were applied in a 5% BSA solution for 60 min at room temperature. Slides were rinsed with TBS, and then incubated in FITC (Santa Cruz, 1:500) and Alexa Fluor 594 (Invitrogen, 1:500) fluorescent-conjugated secondary antibodies in 5% BSA for 15 min at room temperature. After a final rinsing regimen, tissues were cover-slipped with mounting media containing DAPI stain (Vectashield). Fluorescence was detected and quantitated using an Olympus BX51 fluorescent microscope, and QCapture Pro (Version 5) and ImagePro Plus (Version 6) software.

2.3.10 Statistical analysis

All values are expressed as the mean±SE or mean±SD as indicated. Statistical significance for multiple group comparisons was determined using a two-way or one-way ANOVA as appropriate followed by Bonferroni post hoc analyses. Overall and tumor free survival was analyzed using a Log Rank test, and proportions were assessed using Chi-Square analysis. Significance was accepted for p values <0.05.
2.4 Results

2.4.1 Confirmation of PPARγ recombination

The PPARγ-A KOs generated here showed some lipodystrophy at a young age, reflected in lower adipose tissue and body weights compared to PPARγ-WT mice (5.0 Appendix, Supplemental Figure 1) consistent with other adipocyte targeted PPARγ KO models [207, 208]. PPARγ-A KOs had decreased WAT and BAT weights as a percentage of total body weight, as well as some hepatomegaly although other tissues examined, such as spleen and skeletal muscle, were unaffected (5.0 Appendix Supplemental Figure 1). To confirm the efficacy of Ap2Cre+ transgene in this floxed mouse model, a southern blot was performed. About 70-90% PPARγ recombination occurred in all adipose tissue stores examined, with the highest levels in brown adipose, in both sexes (Figure 2.1.A). Western analysis of untreated whole mammary gland tissue from 8-week-old virgin females from each genotype revealed that less than 2% relative PPARγ protein expression remained in female PPARγ-A KO mice compared to congenic PPARγ-WT controls (Figure 2.1B). Untreated PPARγ-A KO mice had no significant spontaneous increase in lethality or tumorigenesis when followed for up to 15 months (data not shown).

2.4.2 PPARγ deletion in stromal adipocytes enhances breast tumourigenesis

Tumorigenic studies were initiated in female virgin mice from each genotype by treating with 1mg DMBA once/week for six weeks. At week 7, mice were randomized into continuing on a normal chow diet (DMBA Only: PPARγ-WT, n=25 and PPARγ-A KO, n=29) or one supplemented with ROSI (DMBA+ROSI: PPARγ-WT, n=34 and PPARγ-A KO, n=35) and
followed for 25 weeks for tumour outcomes. Two PPARγ-A KO mice being treated with DMBA were found dead in their cage on weeks 10 and 12 respectively, due to unrelated causes, and were excluded from further analyses. Disruption of both PPARγ alleles in adipocytes did not significantly affect overall survival of mice treated with DMBA alone or DMBA+ROSI compared to PPARγ-WT controls. Mice from both genotypes treated with DMBA reached 50% survival at week 16 (Figure 2.2 A and B). There was a trend towards increased overall survival in PPARγ-WT mice treated with DMBA+ROSI (median survival week 21) compared to similarly treated PPARγ-A KO mice (median survival week 18), although this difference was not statistically significant.

Total tumor incidence was not significantly different for DMBA-treated PPARγ-WTs (84%) compared to similarly treated PPARγ-A KO (100%) mice. The pattern of DMBA-mediated tumor types were consistent with those previously associated with administration of this chemical carcinogen, and comprised mainly mammary tumours, as well as ovarian, uterine, skin tumours and lymphomas. Co-treatment with ROSI did not significantly alter the types or total tumor incidences observed at necropsy among DMBA+ROSI-treated PPARγ-WT (91%) or PPARγ-A KO (100%) mice. In contrast, PPARγ expression in adipocytes showed a trend towards reduced overall mammary tumor incidence with DMBA-treated PPARγ-WT controls compared to PPARγ-A KO mice (respective mean: 32% versus 48%, p<0.08). More interestingly, PPARγ-A KO mice had a >2-fold significantly increased incidence of malignant mammary tumours versus PPARγ-WT mice (mean±SD respectively: 44±10% vs 20±9%, p<0.01). In DMBA+ROSI co-treatment studies, activation of PPARγ signalling significantly
Figure 2.1 Characterization of adipocyte-specific PPARγ-A KO mice. (A) Southern blot analysis of BamH1 digested genomic DNA from indicated tissues of untreated PPARγ-WT and PPARγ-A-KO mice. WAT, white adipose tissue; Epi, epididymal; Ing, inguinal; BAT, brown adipose tissue; Muscle, skeletal muscle; FL/FL Control, positive control DNA for homozygous floxed PPARγ alleles; FL/Null Control, positive control DNA for recombined floxed and null PPARγ alleles. (B) Nuclear PPARγ protein expression in whole mammary glands from 8-week-old virgin female PPARγ-A-KO and PPARγ-WT mice. (C) Quantitation of PPARγ expression. Relative densitometry levels of PPARγ to Histone H1 protein among PPARγ-A KO and PPARγ-WT mice, as determined by western blot, were evaluated using Image J analysis software.
Figure 2.2: *In vivo* effects of adipocyte-specific PPARγ deletion on DMBA-mediated breast tumorigenesis. Female PPARγ-WT and PPARγ-A KO mice were treated and assessed as described (Methods section 2.3.3) (A and B) Overall survival. Overall survival was expressed as the percentage of mice per genotype per treatment group surviving in a given week. Solid lines, PPARγ-WT and dashed lines PPARγ-A KO mice; n = number of mice. (C) Mammary Tumour Incidences. The incidence of total mammary tumours (overall) were expressed as the percentage of mice with any mammary tumours out of the total number of mice within a given genotype and treatment group, and expressed as a percentage ± standard deviation (SD). n = number of mice, ***, significantly different compared with respective PPARγ-WT controls, P<0.001. (D) Mammary tumours per mouse. The total number of mammary tumours (overall), as well as malignant or benign mammary tumour subtypes, per mouse for a given genotype and treatment group are expressed as a mean ± SD. n = number of mice *, significantly different compared with respective PPARγ-WT controls, P<0.005.
reduced DMBA-mediated mammary tumour incidence among PPARγ-WTs more so than in PPARγ-A KO mice (mean±SD: 29±8% vs. 46±8% respectively, p<0.05). In addition, DMBA+ROSI-treated PPARγ-A KO mice had significantly more malignant mammary tumours than similarly treated PPARγ-WT mice (mean±SD, respectively: 40±8% vs. 26±8%, p<0.01) (Figure 2.2.C).

We next evaluated the role of adipocyte-specific PPARγ expression on mammary tumor multiplicity (Figure 2.2.D). Endogenous PPARγ expression significantly decreased total mammary tumours/mouse by ~60% among DMBA Only-treated PPARγ-WTs compared to PPARγ-A KO mice (respective mean±SD: 0.44±0.15 versus 0.70±0.17 tumours/mouse, p<0.05). Interestingly, compared to DMBA-treated PPARγ-WT mice, mean malignant mammary multiplicity was significantly increased 2.5-fold among PPARγ-A KO mice, (mean±SD, respectively 0.59±0.14 versus 0.24±0.10 tumours/mouse, p<0.05). This pattern was consistent in mice co-treated with ROSI as mean malignant multiplicity was also significantly increased nearly 2-fold in PPARγ-A KOs compared to PPARγ-WT mice (mean±SD, respectively 0.65±0.15 versus 0.38±0.13 tumours/mouse, p<0.05).

Beyond reductions in mammary tumour incidence, PPARγ signalling delayed mammary tumour onset (Figure 2.3 A,B). Latency was not affected in mice treated with DMBA regardless of genotype. In contrast, there was significantly increased latency among DMBA+ROSI-treated PPARγ-WTs compared to PPARγ-A KO mice (p=0.05). Notably, adipocyte PPARγ signalling did not affect mammary tumour volume (Figure 2.3.C). Co-treatment studies with DMBA+ROSI reduced mammary tumor volume. PPARγ-WTs developed confirmed mammary
tumours with an average volume ~ 3-fold larger than the average mammary tumours in PPARγ-WT mice treated with DMBA+ROSI (respective volume ± SD, 1.84±1.75cm³ versus 0.77±0.74cm³, p<0.05). PPARγ-A KO mice also benefited from ROSI treatment, with average necropsy mammary tumours of nearly half the size of the average mammary tumours in DMBA-treated PPARγ-A KO mice, (respectively 0.56±0.71cm³ versus 0.95±1.25cm³, p<0.05).

Mammary tumours were classified by our collaborating pathologists as benign or malignant, and subtyped as adenocarcinomas, squamous cell carcinomas, spindle cell carcinomas or small cell carcinomas (Figure 2.4). PPARγ-A KO mice presented with significantly more adenocarcinomas (p<0.05), the most common type of mammary tumour found in the clinic, compared to PPARγ-WT mice treated with or without ROSI co-treatment (Figure 2.4E).

2.4.3 Stromal adipocyte-specific PPARγ Upregulates BRCA1

QRT-PCR was performed to evaluate breast tumour relevant targets (PPARγ1, PPARγ2, BRCA1, PTEN) using RNA isolated from (n=3) untreated normal mammary gland samples from PPARγ-WT and PPARγ-A KO mice (Figure 2.5A). GAPDH was used as an internal standard with which to compare changes in target transcription levels and all data is presented relative to GAPDH expression. Relative PPARγ1 and PPARγ2 transcript levels were significantly decreased in untreated PPARγ-A KO compared to PPARγ-WT mice by five and twenty-fold (mean target gene transcriptional expression ±SE: 22.2±11% for PPARγ1, and 4.4±2% for PPARγ2, p<0.05). BRCA1 expression was also significantly reduced by twenty-fold to 5.8±2% in PPARγ-A KO mammary glands compared with PPARγ-WT control mammary glands (p<0.05). Comparably, PTEN transcription was not affected by PPARγ signalling in adipocytes.
Figure 2.3: *In vivo* effects of adipocyte-specific PPARγ deletion on DMBA-mediated breast tumour onset and growth. Female PPARγ-WT and PPARγ-A KO mice were treated and assessed as described (Materials section 2.2.4). (A and B) Mammary tumour latency. Latency of mammary tumours are expressed as the percentage of mice with palpable mammary tumours within a given genotype and treatment group. Solid lines, PPARγ-WT and dashed lines PPARγ-A KO mice; *n* = number of mice. Mammary tumour latency is significantly different between DMBA + ROSI treated PPARγ-WT and PPARγ-A KO mice, *p* = 0.05. (C) Mammary tumour volumes. Mammary tumours were measured at necropsy, and volumes calculated using the standard formula (*L* *W*²/2) and expressed as mean cubic centimetres for each treatment group. Open circles and squares, mammary tumours from DMBA only treated PPARγ-WT and PPARγ-A KO mice, respectively; Closed circles and squares, mammary tumours from DMBA + ROSI treated PPARγ-WT and PPARγ-A KO mice, respectively *n* = number of mice per group.
Figure 2.4. *In vivo* effect of adipocyte-specific PPARγ deletion on mammary tumour pathology. Formalin fixed, paraffin-embedded mammary tumours from each treatment group were sectioned and stained to determine origin and subtype, and analyzed in a blinded fashion by pathologists as described (Materials section 2.2.4). Representative images of (A) adenocarcinoma, (B) squamous cell carcinoma, (C) spindle cell carcinoma, (D) papilloma subtypes are shown. (E) Mammary tumour subtype incidence. The incidence of each mammary tumour subtype observed within a given treatment group is expressed as a mean percentage ± SD. *n*, number of mice per group. * significantly different compared with respective mammary tumour subtypes in PPARγ-WT controls *p* <0.05.
In order to evaluate BRCA1 expression changes resulting from loss of adipocyte-specific PPARγ, immunofluorescent (IF) imaging for PPARγ and BRCA1 expression was performed and quantitated (Figure 2.5B) on (n=3) untreated mammary glands from PPARγ-WT (respectively Figures 2.5C and E) and PPARγ-A KO (respectively Figures 2.5D and F) mice. IF analysis indicated that loss of PPARγ expression was specific to mammary stromal adipocytes in PPARγ-A KO mice (Figure 5C-D). Images from PPARγ-WT glands indicate that BRCA1 is expressed in epithelial (pan-cytokeratin positive) cells as well as stromal (pan-cytokeratin negative) cells. Bright field images confirm that these stromal cells are predominantly adipocytes (data not shown). Images from PPARγ-A KO mammary glands show that BRCA1 expression is no longer present in adipocytes while it is retained in epithelial cells.

2.4.4 Stromal Adipocyte-specific PPARγ Expression Decreases Leptin Secretion

Adipocyte-specific PPARγ signalling decreased pre-study serum leptin secretion in untreated PPARγ-WT mice compared to PPARγ-A KO mice (respective mean ±SE: 22.3±2ng/ml versus 27.9±5ng/ml, p<0.05) (Figure 2.6A). Similarly, serum leptin levels measured throughout the tumorigenic study followed the same pattern with DMBA Only-treated PPARγ-A KO mice having higher serum leptin levels than PPARγ-WT mice at mid-study (respectively, 26.9±4.4 vs. 15.6±3.3 ng/ml, p<0.05) and necropsy (respectively, 10.0±0.8 vs. 8.0±0.6 ng/ml, p<0.05). Co-treatment with DMBA+ROSI did not significantly alter serum leptin levels among any of the groups examined.
Figure 2.5. Effects of adipocyte-specific deletion of PPARγ on gene expression in the mammary gland of untreated PPARγ-WT and PPARγ-A KO mice. (A) Mammary gland RNA expression levels for PPARγ1, PPARγ2, PTEN and BRCA1 in untreated PPARγ-WT and PPARγ-A KO mice. Total RNA from untreated whole mammary glands from each genotype were extracted, converted to cDNA templates and assessed by qualitative real-time polymerase chain reaction as described. Values for each target gene were performed in triplicate three independent, and expressed as a percentage fold expression change ± SE relative to untreated PPARγ-WT samples. Black bars, PPARγ-WT; white bars PPARγ-A KO. (B) Mammary gland protein expression of PPARγ and BRCA1 in PPARγ-WT and PPARγ-A KO mice. Formalin fixed, paraffin-embedded untreated whole mammary glands from each genotype were sectioned, and target protein expression was visualized using IF and quantified using ImagePro 6.0 analysis software as described. Protein levels were measured in triplicate on three independent samples and expressed as mean integrated optical density + SE. Black bars, PPARγ-WT; white bars PPARγ-A KO. * significantly different from PPARγ-WT controls, \( P < 0.05 \). (C_F) Mammary epithelial versus stromal cell expression of PPARγ and BRCA1 protein. Representative IF imaging comparing sections of untreated mammary glands from PPARγ-WT (C and E) and PPARγ-A KO (D and F) mice. (C and D) PPARγ (green) is expressed in stromal adipocytes of PPARγ-WT but not PPARγ-A KO mammary glands. (E and F) BRCA1 expression is no longer present in PPARγ-A KO adipocytes, while it is retained in pan-cytokeratin (red) expressing epithelial cells and in stromal adipocytes of the PPARγ-WT mammary glands. DAPI nuclear staining (blue). Scale bar, 10 µm.
2.4.5 PPARγ Activity is Dysregulated in Tumourigenesis

Representative tumours from PPARγ-WT and PPARγ-A KO mice treated with DMBA or DMBA+ROSI were evaluated for expression changes in PPARγ target genes described previously (Figure 2.6B-E) as well as leptin receptor and estrogen receptor (ER)α (Figure 2.7A,B). A trend was observed towards increased PPARγ1, PPARγ2 and PTEN transcription in tumours from PPARγ-WT, but not from PPARγ-A KO, mice treated with DMBA+ROSI compared to respective DMBA alone controls, although this pattern was not statistically significant. BRCA1 mRNA expression varied between tumours collected from mice in the same group as well as between groups, but favoured reduced expression in all but one sample from PPARγ-A KO mice compared to PPARγ-WT controls. Leptin receptor RNA levels within mammary tumors were similarly evaluated, and expressed to the same extent irrespective of genotype or treatment group (Figure 2.7A). More interestingly, mammary tumors from PPARγ-A KO mice in either treatment group expressed ~3-fold more ERα levels compared to their respectively treated PPARγ-WT controls (p<0.05) (Figure 2.7B). DMBA+ROSI treatment significantly reduced ERα levels in both genotypes (p<0.05) (Figure 2.7B).
A  Leptin

Concentration (ng/mL)

N/T  Mid-Study  End-Study

- PPARγ-WT DMBA, n=9
- PPARγ-A KO DMBA, n=12
- PPARγ-WT DMBA + ROSI, n=9
- PPARγ-A KO DMBA + ROSI, n=12

B  PPARγ1

Fold Expression (%)

C  BRCA1

Fold Expression (%)

D  PPARγ2

Fold Expression (%)

E  PTEN

Fold Expression (%)

PPARγ-WT  PPARγ-A KO
Figure 2.6. *In vivo* effect of adipocyte-specific deletion of PPARγ on serum leptin and mammary tumour BRCA1 levels in treated PPARγ-WT and PPARγ-A KO mice. (A) Serum leptin levels. Serum leptin levels were obtained pre (N/T), mid and end-study from PPARγ-WT and PPARγ-A KO mice in each group and assessed as described. Analysis was performed in triplicate on indicated independent samples from each group, and expressed as a mean + SE. n, number of mice. *, significantly different compared with respective PPARγ-WT treated controls, $P < 0.05$. (B_E) Mammary adenocarcinoma RNA expression levels for PPARγ1, PPARγ2, PTEN and BRCA1 in treated PPARγ-WT and PPARγ-A KO mice. Quantitative real-time polymerase chain reaction analysis of target gene expression was performed as described. Values for each target gene were performed in triplicate three independent times, and expressed as percentage fold expression + SE relative to untreated PPARγ-WT samples. *, significantly different compared with respective PPARγ-WT controls, $P < 0.05$. 
Figure 2.7 *In vivo* effect of adipocyte-specific deletion of PPARγ on mammary tumour leptin receptor and ERα levels in treated PPARγ-WT and PPARγ-A KO mice. (A and B) Mammary adenocarcinoma RNA expression levels for leptin receptor and ERα in treated PPARγ-WT and PPARγ-A KO mice. Quantitative real-time polymerase chain reaction analysis of target gene expression was performed as described in the Materials and methods. Values for each target gene were performed in triplicate on four tumours from each group, three independent times, and expressed as a percentage fold expression + SE relative to untreated PPARγ-WT samples. *, Significantly different compared with respective PPARγ-WT controls, $P < 0.05$; ++, significantly different compared with respective DMBA only controls, $P < 0.01$. 

**A**

**B**

![Graphs showing expression levels of leptin receptor and estrogen receptor-1](image)
2.5 Discussion

We generated PPARγ-A KO mice that are as viable as their congenic PPARγ-WT littermates, and had no significant spontaneous increase in lethality or breast tumorigenesis by 15 months of age (data not shown). The present study provides the first direct in vivo evidence that PPARγ signalling in stromal adipocytes attenuates DMBA-mediated breast tumorigenesis. This is consistent with other studies suggesting that PPARγ plays a protective role in the progression of tumorigenesis [209]. Analysis of the PPARγ-A KO mouse model revealed the Ap2Cre+ transgene produced between 70-90% PPARγ recombination depending on the adipose store examined, with BAT representing highest recombination levels. This translated into significant reduction in PPARγ protein expression in whole mammary glands including their fat pads, which was subsequently revealed by IF analysis as specific to mammary stromal adipocytes. Gross body and adipose store weights, were decreased in PPARγ-A KO mice which is consistent with other adipocyte targeted PPARγ KO mouse models [207]. However, disruption of PPARγ in the present model, subsequent to adipogenesis, did not alter serum glucose levels (data not shown), which may have otherwise produced a diabetic phenotype and confounded the tumorigenic findings.

We and others have reported on the role of PPARγ expression within mammary epithelial cells in DMBA-mediated breast tumorigenesis [147, 177]. Here, the role of PPARγ in mammary stromal adipocytes, as well as the effect of PPARγ activation, during breast tumourigenesis was also clarified. PPARγ-A KO mice were twice as likely to develop DMBA-mediated malignant mammary tumours and there was a trend towards increased overall mammary tumour incidence
compared to identically treated PPARγ-WT control mice. In light of the other statistically significant data between these groups, this trend is probably a real biologic effect and would be significant with greater numbers of treated mice. These data strongly suggest that PPARγ signalling within adipocytes inhibits the onset of mammary tumours. While changes in tumor incidence did not correlate with an effect on overall survival during the course of this study, a longer duration of observation may have revealed that other measures of breast tumorigenesis were affected. PPARγ-A KO mice developed significantly (p<0.001) more adenocarcinomas, the type of breast tumour that presents most frequently in the clinic [1].

Molecular tissue evaluation has elucidated several important PPARγ targets. Specifically, Pignatelli et. al. found that endogenous PPARγ ligands increase expression of the critical tumor suppressor, BRCA1, in MCF-7 breast cancer cells and used site directed mutagenesis to find a PPRE in the BRCA1 promoter [156]. Patel and colleagues demonstrated that multiple ligands of PPARγ increase expression of another tumour suppressor gene, PTEN, in MCF-7 cells and found two PPREs within the PTEN promoter [210]. PPARγ is also capable of autoregulation [191]. However, to date, no one has examined the cell-specific transcription and expression of these PPARγ targets in breast tumours.

The present study revealed that normal PPARγ activity in adipocytes upregulates expression of PPARγ1, PPARγ2 and BRCA1 expression within these cells. Even though BRCA1 is most well-known for its role in DNA damage repair, adipocyte-specific BRCA1 expression downregulates aromatase, preventing the conversion of androgens to estrogen [211]. In addition to providing a molecular rationale for the protective effects of PPARγ in adipocytes, the
association with BRCA1 also suggests another predictive biomarker for PPARγ-activating therapy. If, in fact, the protective effects of PPARγ are dependent on functional BRCA1, clinicians could use BRCA1 to predict response. Other mechanisms have been proposed to increase BRCA1 expression including autoregulation and response to DNA damage [212]. This may explain the varying levels of BRCA1 transcription in the tumours, since each tumor was subdivided into fixed and frozen samples. However, the role of tumor heterogeneity as a contributing factor cannot be eliminated. Nevertheless, given the important role played by BRCA1 in familial and sporadic breast cancer, continued clarification of BRCA1 regulatory mechanisms would seem appropriate.

He et al. previously reported that their adipocyte-targeted PPARγ KO mice have reduced circulating leptin [208]. However, their mice were not stressed with chemical carcinogens, and serum analysis was done with 14 month old mice, nearly 8 months after our mice completed tumorigenic study. These differences in study parameters may explain the suggested discrepancy between leptin levels observed in our respective untreated mice. Nevertheless, in support of our findings, Catalano et. al. published in vitro and in vivo evidence that PPARγ ligands inhibited leptin-induced upregulation of leptin and the leptin receptor [171] while our manuscript was in preparation. Leptin enhances mammary tumourigenesis by stimulating cell growth and survival in addition to increasing the effects of estrogen on mammary epithelial cells [213]. Here we refine the model by showing that PPARγ within adipocytes affects leptin secretion in vivo. The present data also suggests that PPARγ in adipocytes does not directly regulate PTEN transcription, however, other factors are involved in PTEN regulation including autoregulation
and expression through the stabilization of p53[214]. This is the first direct \textit{in vivo} evidence that endogenous PPARγ signalling within adipocytes prevents against DMBA-mediated breast tumour progression.

In addition to suggesting that PPARγ signalling within adipocytes confers a protective \textit{in vivo} role in mammary tumourigenesis, the present study indicates that PPARγ activation with ROSI is also protective. Others have shown that activating PPARγ with various natural and synthetic ligands protects against tumorigenesis [215]. A short pilot study was performed using ROSI on human patients diagnosed with breast cancer. After a few weeks, the ROSI treatment did not affect Ki67 levels, a marker of proliferation, however it did increase serum adiponectin [216]. In order to justify further clinical trials using PPARγ-activating mono- or combination-therapy, over longer timelines, more pre-clinical work needs to be done to elucidate the protective mechanisms and identify which patients and breast tumour subtypes are most likely to respond to therapy.

Here strong evidence is provided that activation of PPARγ with ROSI protects against DMBA-mediated breast tumorigenesis, and that this protection is dependent on the presence of PPARγ in adipocytes. For more than a decade, ROSI has been used clinically to treat and prevent type II diabetes [193], a disease which is associated with several cancers including breast [217]. ROSI co-treatment significantly increases mammary tumor latency in PPARγ-WT, but not PPARγ-A KO, mice. This implies that PPARγ activation slows or attenuates mammary tumour growth in an adipocyte-specific manner. In contrast, ROSI co-treatment decreases mammary tumor volume, evaluated at necropsy, in both mouse genotypes. Given that all mammary tumors
irrespective of genotype, are of epithelial cell origin, which are not affected by the targeted PPARγ deletion approach used here, activation of PPARγ signalling would be expected to exert similar effects on the mammary tumors in both genotypes. Taken together, ROSI co-treatment provides significant direct and indirect protective effects on DMBA-mediated mammary tumor growth in our model.

PPARγ activation also increases PPARγ1, PPARγ2 and PTEN transcription in tumours from PPARγ-WTs but not PPARγ-A KO mice. Importantly, this suggests that the protective effect of PPARγ activation requires the presence of PPARγ in adipocytes. The variability between samples is likely due to the inherent heterogeneity within tumours. One mammary tumor from a PPARγ-WT mouse treated with DMBA+ROSI exhibited unusually high levels of BRCA1 transcription. Interestingly, this tumor was confirmed to be more de-differentiated and aggressive compared to the others in its matched group. It also cannot be excluded that there may be tumor-specific translational inhibition preventing expression of BRCA1 protein, thus causing mRNA accumulation.

Mammary tumors from all groups expressed the leptin receptor suggesting that those from PPARγ-A KO mice would be responsive to the significantly elevated serum leptin levels observed in those animals. As described above, it was independently reported that PPARγ activation counteracted leptin stimulatory effects on breast tumourigenesis in vitro and in vivo [171]. Here we show more directly that loss of PPARγ increases mammary stromal adipocyte-specific secretion of leptin, and PPARγ activation with ROSI decreases serum leptin levels contributing to the protective effect against mammary tumourigenesis. Leptin has multiple
biological actions including the regulation of endothelial cell proliferation and apoptosis through which it can promote angiogenesis [218-220]. It was also reported that high levels of circulating leptin, similar to those found in obese individuals, can drive breast tumorigenesis [221]. The data here suggests that serum leptin levels may be useful as a biomarker for at least a subset of breast cancer patients who may warrant combination therapies, including PPARγ activators, to aid in the management of the growth and spread of breast tumours.

More strikingly, ERα levels were almost 3-fold higher among mammary tumours from PPARγ-A KO mice compared to their respectively treated PPARγ-WT controls, and while significantly reduced by co-treatment with ROSI, this fold difference was maintained between genotypes. This suggests that mammary tumors from PPARγ-A KO mice are especially capable of responding to estrogen signalling. Ghosh et al (2007) reported that BRCA1 signalling in adipocytes reduces expression of aromatase which limits estrogen synthesis [211]. Taken together, in our PPARγ-A KO model with reduced BRCA1 expression, aromatase is likely increased, enhancing estrogen secretion which, alone or in combination with increased leptin secretion, may drive DMBA-mediated growth and progression of ERα expressing mammary tumours. Ongoing studies are further examining the enhanced susceptibility of PPARγ-A KO mice to DMBA-mediated breast tumorigenesis, and how the expression and activated signalling of PPARγ within other mammary-associated cell types interact in vivo, but is beyond the scope of this paper.

Ultimately, the long term aims of these studies are to reduce breast cancer related deaths by not only elucidating how genetic and environmental risk factors interact in vivo, but also
discerning the genetic signature of patients who may benefit from PPARγ-targeted therapy. The data here provides the first direct evidence that mammary stromal adipocyte-specific PPARγ signalling locally maintains BRCA1 expression and systemically reduces leptin levels. It also provides further evidence that PPARγ activating drugs, alone or in combination therapy, may be efficacious in treating at least a subset of breast cancer patients.
Chapter 3 The Endothelial Cell-specific Role of PPARγ in DMBA-mediated Breast Tumourigenesis

Authors:
Reid, Alexis L., Rubino, Rachel E., Peterson, Nichole T., Schneider, Mark, SenGupta, Sandip K., Lebrun, David P., Hurlbut, David and Nicol, Christopher J.B.

Contributions
I preformed the mouse studies, graphs, statistical analysis, immunoblots, genotyping, necropsies

The following co-authors contributions are listed below

RER and NTP: performed colony genotyping and breeding, aided with necropsies and the collection and processing during mouse studies. Performed leptin ELISA

MS, SKS, DPL, DH: pathological classification of samples

CJBN: Supervised study, editing of manuscript

All other work was performed by myself
3.1 Abstract

Peroxisome proliferator-activated receptor (PPAR)γ plays a role in tumourigenesis. Previous studies with PPARγ(+/−) mice suggest PPARγ normally suppresses dimethylbenz[a]anthracene (DMBA)-induced breast, and other, tumour progression. Since many cell types associated with the mammary gland express PPARγ, each with unique signal patterns, the present study aimed to define which tissues are required for PPARγ-dependent anti-tumor effects. Conditional endothelial cell-specific PPARγ knockout mice (PPARγ-E KO) were used to evaluate whether PPARγ signaling normally acts to prevent DMBA-mediated breast tumour progression in a stromal cell-specific manner. Twelve week old PPARγ KO mice and their congenic wildtype (WT) controls were randomly assigned to one of two treatment groups. All mice were treated by gavage once/week for 6 weeks with 1 mg DMBA and maintained on a normal chow diet. At week 7, mice in each group were divided into those continuing normal chow, and those receiving a PPARγ ligand (rosiglitazone, 4mg/kg/day) supplemented diet for the duration of the 25 week study, and monitored weekly. Tumour and tissue samples were collected at necropsy, and portions of each were fixed and frozen for future analysis. These studies provides the first direct in vivo evidence that PPARγ signalling in stromal endothelial cells attenuates DMBA-mediated breast tumourigenesis. In PPARγ-E KOs versus PPARγ-WT mice, malignant mammary tumor incidence was significantly higher and mammary tumor latency was decreased. DMBA+ROSI treatment reduced average mammary tumor volumes by 50%. In the PPARγ-E-KO mice, both treatment groups saw a significant increase in thymic tumour incidence, a finding not established before with the study of other stromal cell knock mice, which may
confounded the progression of mammary tumours. More work is now needed to elucidate the mechanisms by which loss of endothelial cell-specific PPARγ results in enhanced DMBA-mediated thymic tumourigenesis, and in particular, whether PPARγ dependent signaling alterations in thymic immune cells affect mammary tumour progression. This study provides the first direct in vivo evidence that loss of PPARγ in stromal endothelial cells increases DMBA-mediated breast tumourigenesis.
3.2 Introduction

Breast cancer is a very prominent disease in our society. Currently, one out of every nine Canadian women is diagnosed with breast cancer over the course of her lifetime, and 1 in 29 will eventually succumb to complications of the disease [1]. In 2012, it is estimated that 22,700 women and 200 men will be diagnosed with breast cancer. More disturbing is that 5,100 women and 55 men are estimated to die from disease progression, suggesting that outcomes after breast cancer diagnosis are equally poor among susceptible individuals [1]. Much progress has been made in breast cancer survival statistics since the late 1980’s, through improved screening technology and programs. In order to make further progress with this complex disease it is imperative to improve our understanding of the impact between environmental and genetic risk factors, to improve therapeutic options and outcomes for breast cancer patients.

Peroxisome proliferator-activated receptor (PPAR)γ has an emerging role as a breast tumour suppressor. Discovered in the early 1990’s, it is a member of the nuclear receptor superfamily [123]. PPARγ is predominantly expressed in the adipose tissue, but is also found at lower levels in other tissues and cell types including the breast, and endothelial cells [188, 189, 190]. PPARs form heterodimers with retinoid X receptor (RXR)α which facilitates the regulation of transcriptional targets [122]. The heterodimeric complex then binds to peroxisome proliferator response elements (PPREs), within the promoter regions of target genes [123]. In the unliganded state, this PPARγ:RXRα complex associates with co-repressors which physically block transcriptional machinery, or other transcription factors, from transcribing target genes, and indirectly influences gene transcription [222]. Following endogenous or exogenous ligand
binding, a conformational change occurs resulting in the release of co-repressors and recruitment of co-activators, leading to direct transcriptional upregulation or transrepression depending on the gene target [124, 138]. Ligand that activate PPARγ include synthetic thiazolidinediones such as the gold standard activator rosiglitazone (ROSI), used widely for >10 years to treat and prevent Type II diabetes [193].

Previously, a PPARγ(+/−) haploinsufficient mouse model was used to show that the global loss of even one PPARγ allele significantly increased DMBA-mediated malignant and metastatic breast tumours, suggesting PPARγ normally stops breast tumour progression [176]. More recently, in vivo studies using adipocyte-specific PPARγ knockout (PPAR-A KO) mice provided the first direct in vivo evidence that PPARγ signaling in mammary stromal adipocytes attenuates DMBA-mediated breast tumourigenesis, and confirmed the presence of PPARγ-mediated stromal-epithelial crosstalk [223]. Since there are many different epithelial and stromal PPARγ cell types associated with the mammary gland, more work is needed to help dissect the cell-type specific contributions of PPARγ expression and signaling during arrest of the growth and spread of breast tumours.

Endothelial cells serve as key players during tumour progression, both during angiogenic responses as required for tumour growth, and as barriers through which tumour cells must migrate during metastasis to distal sites [224, 225]. Previous work shows PPARγ ligands reduce differentiation and proliferation of human umbilical vein endothelial cells in vitro, and inhibit VEGF-induced angiogenesis in vivo [177]. Accordingly, it was hypothesized that loss of endothelial cell (E)-specific PPARγ would enhance susceptibility of breast tumourigenesis. Here
it is shown that PPARγ E-knockout mice are more susceptible to DMBA-mediated breast, and in particular thymic, tumourigenesis compared to their controls. In addition, activation of E-specific PPARγ-dependent signaling is chemoprotective. These findings provide the first in vivo evidence of the critical importance of PPARγ signaling in mammary stromal endothelial cells during tumourigenesis, and add further support for the use of PPARγ activating drugs to treat breast cancer patients.

3.3 Materials and Methods

3.3.1 Chemicals and Reagents

DMBA, 10% phosphate-buffered formalin, glycerol and ammonium persulphate were purchased from Sigma (St Louis, MO). Tris and SDS were purchased from Fisher (Whitby, ON). Rosiglitazone (Avandia) was purchased from GlaxoSmithKline (Mississauga, ON). Primary and secondary antibodies to detect PPARγ, GAPDH, and β-actin were obtained from Santa Cruz Biotechnology (San Diego, CA).

3.3.2 Animals

All mice were housed and treated in accordance with animal protocols following the guidelines set by the Canadian Council for Animal Care (CCAC), and approved by the Queen’s University Animal Care Committee (UACC). Mice were housed in microisolator racks under 12 hour light/dark cycle with food and water provided ad libitum. Endothelial cell-specific \( PARγ^{0/-};Tie2-Cre^+ \) (PPARγ E-KO) mice and their congenic controls are on a mixed C57B1/6N;SV/129;FVB/N background, and were generated as previously described [226].
### 3.3.3 PPARγ Mouse Characterization and Genotyping

Characterization of PPARγE-KO mice and controls was previously reported [226]. Genotyping of all animals was performed using a PCR analysis under conditions previously described [176]. Briefly, DNA was isolated from tail clips of 10 day old weanlings and incubated with a mastermix containing optimized final concentrations of PCR Buffer, primers, MgCl$_2$, dNTPs, ddH$_2$O and Taq polymerase. Samples then underwent amplification in a thermocycler (Bio-Rad), and were then electrophoresed on a 2% agarose gel containing ethidium bromide for detection of amplified DNA bands under UV light.

### 3.3.4 In vivo tumourigenesis studies

Eight to 11 week old virgin female PPARγ−WT and PPARγ E-KO mice were entered into study similar to that previously described [223]. Briefly, mice in each group were weighed, and bled via submandibular route to collect non-fasted pre-study blood samples. Pre-study serum was then isolated and frozen in liquid N$_2$ for future analysis. One week later, all mice were treated with 1mg of DMBA (10mg/ml stock dissolved in corn oil) by oral gavage once a week for 6 weeks. On week 7, mice were randomized into two groups, those receiving only DMBA and maintained on a normal chow diet (DMBA Only: PPARγ WT, n=28; PPARγ E-KO, n=31) and those provided a rosiglitazone (ROSI)-supplemented (4 mg/kg/day) normal chow diet (DMBA+ROSI:PPARγ WT, n=24; PPARγ E-KO, n=32). All mice were then monitored weekly up to 25 weeks for changes in body weight, food intake, as well as the number and size of any tumours. Non-fasted serum samples were also obtained mid-study (week 13) and prior to necropsy (week 25) as described above. Mice were sacrificed by cervical dislocation at the end
of the 25 weeks or earlier if they had tumours greater than 20mm*20mm or displayed signs of weight loss, lethargy, or distress. Portions of tissue and tumour samples collected at necropsy were snap-frozen using liquid N\textsubscript{2} and stored at -80°C, or fixed in formalin. H&E stained slides of all tumours, prepared from formalin fixed, paraffin embedded samples, were assessed for pathological classification and confirmed in a blinded fashion by collaborating pathologists Drs. Schneider, SenGupta, Hurlbut and Lebrun (KGH, Kingston, ON).

3.3.5 Statistical Analysis

Graph-Pad Prism (Version 6.0) software was used for all statistical analyses. Statistical differences between treatment groups and genotypes were assessed using a Two-way Analysis of Variance (ANOVA), followed by Bonferroni post hoc tests for group comparisons. Survival was analyzed using a Log Rank test. A p value <0.05 was accepted as statistically significant.
3.4 Results

3.4.1 Endogenous PPARγ E-KO mouse phenotype

To determine the mammary stromal endothelial cell role of PPARγ during breast tumourigenesis, PPARγ E-KO mice and their controls were generated and characterized previously [226]. PPARγ deletion was ubiquitously observed in both sexes and all vascularized tissues examined from PPARγ E-KO, but not PPARγ−WT mice, confirming the targeting specificity of the Tie2 promoter as previously reported [180]. PPARγ E-KO mice have normal reproductive fertility and litter sizes, and are normal with respect to heart rate and blood pressure, except when stressed in a manner mimicking Type II diabetes [226]. Long term (> 1 year) assessment of PPARγ E-KO mice also confirms the absence of any enhanced spontaneous tumour incidences arising from loss of endothelial specific PPARγ expression.

3.4.2 Mouse body mass

PPARγ E-KO mice body mass was tracked throughout the course of the study to assess whether the mice were being adversely affected by treatments. A 15% decrease in body mass can be indicative of distress or tumour development. There were no significant differences in body mass noted between the PPARγ E-KO and PPARγ WT mice in either the DMBA Only or DMBA+ROSI treatment groups (Figure 3.1).

3.4.3 DMBA-Mediated Tumourigenesis

Compared to PPARγ-WT controls, endothelial cell loss of both PPARγ alleles did not significantly alter overall survival of DMBA Only- or DMBA+ROSI-treated mice
Figure 3.1. Body mass effect of DMBA treatment on endothelial cell-specific PPARγ knockout (PPARγ-E KO) and congeneric wild type (PPARγ-WT) mice. Mice were treated as discussed in Methods section 2.2.4. A, Body mass for DMBA Only-treated PPARγ-WT (n=25) and PPARγ-E KO (n=32) mice; B, Body mass for DMBA+ROSI-treated PPARγ-WT (n=34) and PPARγ-E KO (n=31) mice. Values are expressed in grams (g) and represent mean ± SD.
DMBA Only-treated PPARγ-WT and PPARγ-E KO mice reached a similar 50% survival by 17 and 19 weeks respectively (Figure 3.2A). Compared to respective DMBA Only groups, there was a trend towards increased overall survival among ROSI co-treated PPARγ-WTs (median survival 22 weeks) but not among DMBA+ROSI-treated PPARγ E-KO mice (median survival 19 weeks), although this difference was not statistically significant (Figure 3.2B).

Total tumour incidence was not statistically different for DMBA Only-treated PPARγ-WTs (84%) compared with similarly treated PPARγ-E KO (75%) mice ROSI treated PPARγ-WT (91%) or PPARγ-E KO (87%) mice. (Figure 3.3). A trend towards increased overall tumour incidence was noted among DMBA Only-treated PPARγ-E KOs versus PPARγ-WT mice (mean ± SD: 83 ± 8% vs. 75 ± 10%). Co-treatment with ROSI did not significantly alter the incidence of total benign tumours in PPARγ-WT and PPARγ-E KO mice respectively (mean ± SD: 32% ± 8% versus 26% ± 8%). A significant increase in malignant overall tumours was noted in the PPARγ-E KO mice when compared to PPARγ-WT mice in the DMBA only group (mean ± SD: 63 ± 9% versus 32 ± 10%, P < 0.05). In the DMBA only group a trend towards reduced metastasis was noted in the PPARγ-WT mice versus PPARγ-E KO (mean ± SD; 20 ± 8% versus 58± 9%, P > 0.05). Co-treatment with ROSI had a greater protective effect in PPARγ-WT mice, resulting in a significant reduction in malignant overall tumours as compared to PPARγ-E KO mice (mean ± SD; 32 ± 8%, versus 74 ± 8% P < 0.001). (Figure 3.3).

When the data for all DMBA Only-initiated tumours was assessed, the total number of tumours per mouse (overall tumour multiplicity) was significantly increased 2–fold among
Figure 3.2. Overall survival among DMBA-treated PPARγ-WT and PPARγ-E KO mice. Overall survival was expressed as the percentage of mice per genotype per treatment group surviving in a given week. A, DMBA Only treated mice; B, DMBA+ROSI-treated mice. Solid lines, PPARγ-WT and dashed lines, PPARγ-E KO mice; n = number of mice. Female PPARγ-WT and PPARγ-E KO mice were treated and assessed as described in Methods (Section 3.2.4).
Figure 3.3. Effect of endothelial–specific PPARγ deletion on DMBA-mediated total tumour incidences. The incidence of total tumours (overall) were expressed as the percentage of mice with any tumours out of the total number of mice within a given genotype and treatment group. Mean malignant and benign tumour incidences were similarly calculated based on the number of each within a given genotype and treatment group, and expressed as a percentage ± standard deviation (SD). \( n \) = number of mice. *, significantly different compared with respective PPARγ-WT controls, \( p < 0.05 \), *** significantly different compared with respective PPARγ-WT controls, \( p < 0.001 \).
PPARγ-E KOs compared to PPARγ-WT mice (mean ± SD: 2.4 ± 0.4 vs. 1.2 ± 0.3, p< 0.01) (Figure 3.4). Similarly, compared to genotypic PPARγ-WT controls, DMBA+ROSI-treated PPARγ-E KOs showed a trend toward increased total overall tumour multiplicity (PPARγ-E KO vs. PPARγ-WTs, mean ± SD: 2.1 ± 0.2 vs. 1.6 ± 0.4 respectively) and total malignant tumour multiplicity (mean ± SD: 1.9±0.2 vs. 1.2 ± 0.4, respectively), although these differences were not statistically significant. There were no significant DMBA Only and DMBA+ROSI treatment differences in total tumour multiplicity for any genotype (Figure 3.4).

When mammary tumour data was assessed, DMBA Only-treated PPARγ-E KOs had a modest trend toward increased overall mammary tumour incidences compared to similarly treated PPARγ-WT mice (mean ± SD: 38 ± 9% vs. 32 ± 8% respectively) (Figure 3.5). Interestingly PPARγ-E KO mice had a 2 fold increased incidence of benign mammary tumours versus PPARγ-WT mice (mean ± SD: 31 ± 8% vs. 16 ± 6%,). The similar trend was not noticed with malignant mammary tumours, with the PPARγ-WT mice displaying an increased incidence (mean ± SD: 20 ± 8% vs. 16 ± 6%). In DMBA + ROSI co-treatment studies, activation of PPARγ signaling reduced overall DMBA-mediated mammary tumour incidence amongst PPARγ-WTs more so than PPARγ-E KO mice (mean ± SD: 32 ± 8% vs. 39 ± 9%). The same trend was noted with respect to malignant mammary tumours with the PPARγ-WT having a lower tumour incidence (mean ± SD: 12 ± 6% vs. 19 ± 7%).

Interestingly, the opposite was observed with benign mammary tumour incidence. PPARγ-WT mice had a almost 2 fold increased incidence of mammary tumours versus PPARγ-E KO mice (mean ± SD: 29 ± 8% vs. 16.1 ± 7%). A trend towards increased
Figure 3.4. Total tumour multiplicity among PPARγ-WT and PPARγ-E KO mice. The total number of tumours (overall), as well as malignant or benign tumour subtypes, per mouse for a given genotype and treatment group are expressed as a mean + SD. n, number of mice; ***, significantly different compared with respective PPARγ-WT controls, p< 0.001.
Figure 3.5. Mammary tumour incidences among PPARγ-WT and PPARγ-E KO mice. The incidence of total mammary tumours (overall) were expressed as the percentage of mice with any mammary tumours out of the total number of mice within a given genotype and treatment group. Mean malignant and benign mammary tumour incidences were similarly calculated based on the number of each within a given genotype and treatment group, and expressed as a percentage ± standard deviation (SD). n = number of mice
metastasis in PPARγ-E KO mice was observed (mean ± SD: 19 ± 7% vs. 12 ± 5%) (Figure 3.5).

We next evaluated the role of endothelial-specific PPARγ expression on mammary tumour multiplicity. Endogenous PPARγ expression significantly decreased total mammary tumours per mouse by ~58% among DMBA Only-treated PPARγ-WT compared with PPARγ-E KO mice (respective mean ± SD: 0.4 ± 0.2 vs. 1.0 ± 0.3, p< 0.05) (Figure 3.6A). A similar result was noted with benign mammary tumours where PPARγ-WT had a significant decrease of ~81% as compared to PPARγ-E KO mice (mean ± SD: 0.2 ± 0.1 versus 0.8 ± 0.3, p< 0.05). This effect was not observed for malignant mammary tumours per mouse (PPARγ-WT vs. PPARγ-E KO, mean ± SD: 0.2 ± 0.1 vs. 0.2 ± 0.1, respectively). Metastasis with respect to multiplicity was near equal in PPARγ-E KO’s as compared to the PPARγ-WT mice (mean ± SD: 1.1 ± 0.2 vs. 1.0 ± 0.3). In mice co-treated with ROSI, overall mammary tumour multiplicity was not different between PPARγ-WT and PPARγ-E KO mice (mean ± SD: 0.6± 0.2 versus 0.5 ± 0.1) (Figure 3.6B). Benign mammary tumour multiplicity was slightly increased among DMBA+ROSI-treated PPARγ-WT versus PPARγ-E KO mice (mean ± SD: 0.4 ± 0.1 versus 0.2 ± 0.1). In contrast, there was a trend toward decreased malignant mammary tumours per mouse in ROSI co-treated PPARγ-WT as compared to PPARγ-E KO mice (mean ± SD: 0.1 ± 0.06 vs. 0.2 ± 0.07). A slight increase in metastasis with respect to multiplicity was observed in the PPARγ-E KO’s as compared to the PPARγ-WT mice (mean ± SD: 0.8 ± 0.2 vs. 0.7 ± 0.2).

Mammary tumour incidence is not the only parameter for which to assess the impact of PPARγ signaling. Mammary tumour onset, in the form of latency, is also a critical measure.
Figure 3.6. Mammary tumour multiplicity among PPARγ-WT and PPARγ-E KO mice. The total number of mammary tumours (overall) as well as malignant or benign mammary tumour subtypes, per mouse for a given genotype and treatment group are expressed as a mean ± SD. n, number of mice; *,+significantly different compared with respective PPARγ-WT controls, p<0.05.
of tumour progression. Mammary tumour latency was not significantly affected in mice treated with DMBA, or DMBA + ROSI, regardless of genotype (Figure 3.7).

Next, mammary tumour volumes were assessed for changes resulting from endothelial cell-specific loss of PPARγ expression (Figure 3.8). DMBA-treated PPARγ-WTs had a significant 4-fold increase in mammary tumour volumes than similarly treated PPARγ-E KO mice (respective mean ± SD, 1.8 ± 1.75 cm$^3$ versus 0.4 ± 0.1 cm$^3$, $p< 0.001$). DMBA+ROSI treatment resulted in a significant 2-fold decrease in mammary tumour volumes among PPARγ-WT mice compared to respective DMBA-treated controls (respectively mean ± SD 1.84 ± 1.75 cm$^3$ versus 0.8 ± 0.2 cm$^3$, $p< 0.05$). Mean mammary tumours volumes among PPARγ-E KO mice were not affected by co-treatment with ROSI compared to respective DMBA Only controls (respective mean ± SD 0.4 ± 0.1 cm$^3$ versus 0.4 ± 0.1 cm$^3$) (Figure 3.8).

Interestingly, the incidence of thymic tumours in DMBA Only-treated PPARγ-E-KO mice was significantly increased >5-fold when compared to similarly treated PPARγ-WT mice (mean ± SD: 66 ± 9% versus 12 ± 7%, $p< 0.0001$). A more striking trend was noted in the DMBA+ROSI treated mice where thymic tumour incidences were significantly increased >8-fold (mean ± SD: PPARγ-E KO 74 ± 8% versus PPARγ-WT 9 ± 5%, $P < 0.0001$) (Figure 3.9).
Figure 3.7: Mammary tumour latency among PPARγ-WT and PPARγ-E KO mice. Latency of mammary tumours are expressed as the percentage of mice with palpable mammary tumours within a given genotype and treatment group. Solid lines, PPARγ-WT and dashed lines, PPARγ-E KO mice; n, number of mice.
Figure 3.8. Mammary tumour volumes among PPARγ-WT and PPARγ-E KO mice. Mammary tumours were measured at necropsy, and volumes calculated using the standard formula \( V = \frac{L \times W^2}{2} \) and expressed as mean cubic centimeter for each treatment group. Open circles and squares, mammary tumours from DMBA Only-treated PPARγ-WT and PPARγ-E KO mice, respectively; Closed circles and squares, mammary tumours from DMBA + ROSI-treated PPARγ-WT and PPARγ-E KO mice respectively. \( n \) = number of mice per group *, significantly different compared with respective PPARγ-WT controls, \( p < 0.05 \), ***, significantly different compared with respective PPARγ-WT controls, \( P < 0.001 \)
Figure 3.9. Thymic Tumour Incidences among PPARγ-WT and PPARγ-E KO mice. The incidence of total thymic tumours were expressed as the percentage of mice with thymic tumours out of the total number of mice within a given genotype and treatment group, and expressed as a percentage + standard deviation (SD). n. number of mice; ***, significantly different compared with respective PPARγ-WT controls, P<0.001.
3.5 Discussion

The *in vivo* role of PPARγ in endothelial cells was first investigated following generation of the first strain of PPARγ E-KO mice [226]. When treated with either a normal diet or salt loading, PPARγ E-KO mice were indistinguishable from wild-type controls with respect to vascular function [226]. However, PPARγ E-KO mice had significantly increased hypertension following diabetic induction by western-style high fat (HF) diet treatment [226]. More importantly, co-treatment with the PPARγ ligand rosiglitazone improved serum insulin levels but not HF-mediated hypertension in PPARγ-E KO mice [226]. This provided the first evidence that cell specific PPARγ expression in endothelial cells is important for mediating the antihypertensive effects of the ligand. In another study [227] a different strain of PPARγ-E KO mice were used to evaluate whether the loss of PPARγ in the pulmonary arteries is the cause or effect of pulmonary arterial hypertension. Since levels of PPARγ are reduced in arteries of patients experiencing pulmonary arterial hypertension [228]. The authors confirmed that loss of endothelial PPARγ results in the development of pulmonary arterial hypertension, although the mechanism by which this occurs is yet to be elucidated [227]. This confirms cell specific expression of PPARγ exerts different effects depending on the stress conditions. Dysregulation of the angiogenic process is an important factor in the progression of cancers, leading to the theory that PPARγ expression in the endothelial cells is involved in regulation of tumour progression [229].

The role of PPARγ in cancer is currently the topic of much research. Many *in vitro* studies have been conducted using various cancer cell lines to elucidate the mechanism of
PPARγ in breast cancer. PPARγ expression is increased in several epithelial cancer cells [123]. For example, PPARγ activation inhibits the proliferation of malignant cells from breast adenocarcinomas [140], prostate carcinoma [141], colorectal carcinoma [142], non-small cell lung cancer [143], and gastric carcinoma cells [144]. PPARγ ligands are also reported to exert anti-breast tumourigenic properties in vitro and induce tumour growth arrest or shrinkage in rat models in vivo ([140, 174, 175, 195, 196]).

In addition, PPARγ activation reduces leptin secretion [198] and increases expression of BRCA1 through direct transcriptional activation via a PPRE in its promoter region [156]. To further complicate the idea of cross-talk between stromal-epithelial cells in breast cancer it has been demonstrated that leptin signaling promotes the growth of mammary tumours and increases expression of VEGF in vivo and this effect is more marked in vivo than in vitro, suggesting a potentiating effect of cellular cross-talk [230].

An extension of cellular cross talk is the role of stromal cells in cancer. We, and other labs, have previously reported on the role of cell specific PPARγ expression within mammary epithelial cells and mammary stromal adipocyte cells during DMBA-mediated breast tumourigenesis [177, 223]. Here, the stromal influence of PPARγ within endothelial cells during DMBA-mediated breast tumourigenesis, using endothelial-specific PPARγ knockout mice was assessed. Co-treatment of PPARγ-WT mice with ROSI, resulted in a trend towards increased benign and decreased malignant mammary tumours with respect to both incidence and multiplicity [171, 223, 231]. In contrast, increases in DMBA-mediated malignant mammary tumours and mammary tumour multiplicity were observed among PPARγ-E KOs versus wild
type control mice irrespective of treatment group [223]. This suggests PPARγ activation results in a decrease in the growth and spread of mammary tumours in vivo [171, 176, 223, 232]. Given the increases in mammary tumours among the PPARγ-E KO mice provides evidence supporting the hypothesis that loss of PPARγ in endothelial cells enhances the susceptibility to DMBA-mediated breast tumourigenesis. It must be noted that these changes in tumour incidence and multiplicity did not correlate with an effect on overall survival during the course of the study. However a longer duration of observation may have revealed that other measures of breast tumourigenesis such as survival and latency were affected. Nevertheless, these date provide further evidence that PPARγ signaling in stromal endothelial cells influences mammary tumourigenesis.

Consistent with previous reports, PPARγ activation resulted in a significant decrease in mammary tumour volumes in the PPARγ-WT mice [140, 223]. However, among the DMBA-Only treated mice there was a significant decrease in mammary tumour volumes in PPARγ-E KO mice compared to PPARγ-WTs, suggesting that loss of endothelial cell-specific PPARγ expression in these mice enhances early tumour formation but prevents further tumourigenic growth. Since angiogenesis contributes to tumour growth [233], further evaluation of changes in angiogenic factors that are reportedly PPARγ downstream targets, such as VEGF or VEGF receptors, following endothelial cell-specific loss of PPARγ may prove to be a fruitful area of research.

Interestingly, PPARγ-E KO mice in both treatment groups a significantly increased incidences of DMBA-mediated thymic tumours. The role of immune response in growth and
spread of tumours is well known [234-236]. It has also been documented that aging results in increased adipogenesis, and consequently, reduction in T cell production in the thymus [237, 238]. PPARγ activation, with ROSI, results in an acceleration of thymic aging, reduction in naïve T cells, increased adipogenesis of the thymus and decreased thymopoiesis [239]. Further evidence that PPARγ is linked to loss of thymopoiesis was provided by a study analyzing ghrelin and ghrelin receptor deficient mice, which developed accelerated thymic involution and expressed higher levels of PPARγ in their thymic stromal cells [240]. Conversely, inhibition of PPARγ in thymic stromal cells prevented the adipogenic transformation of the thymus, suggesting a role for PPARγ antagonists in maintenance of thymic integrity [240]. In endothelial cells, activation of PPARγ by ciglitazone and troglitazone results in the inhibition of IFN-γ induced major histocompatibility complex (MHC) mediated T cell activation [241]. These findings suggest an immune-modulatory role for PPARγ. Similar to the thymic stromal cells undergoing transdifferentiation into adipocytes, it was shown that mammary secretory epithelial cells transdifferentiate into adipocytes during mammary gland involution in a direct and reversible fashion, supporting the notion of transdifferentiation of stromal cells [242]. More work is now needed to elucidate the mechanisms by which loss of endothelial cell-specific PPARγ results in enhanced DMBA-mediated thymic tumourigenesis, and in particular, whether PPARγ dependent signaling alterations in thymic immune cells affect mammary tumour progression.

This study provides the first direct in vivo evidence that loss of PPARγ in stromal endothelial cells increases DMBA-mediated breast tumourigenesis. In addition, PPARγ-E KO mice had enhanced susceptibility to DMBA-mediated thymic tumours which may have
confounded mammary progression. More work is needed to examine targets downstream of PPARγ, such as VEGF, as tumour growth requires angiogenesis [233]. Further study must also assess how decreased endothelial-specific PPARγ expression influences late stage tumour growth and spread. Further work must be done in other mammary-associated cell types, and then when the totality of mammary cell types have been studied, how they interact with each other should be assessed in vivo. The eventual goal of these studies is to reduce breast cancer-related deaths through the elucidation of genetic mechanisms in vivo.
Chapter 4: Conclusions and Future Directions

These studies aimed to elucidate the role of PPARγ in the stromal cells of the mammary gland and their effect on tumourigenesis. PPARγ is well established to play a role in carcinogenesis. Previous studies with PPARγ(+/-) mice suggest that PPARγ normally suppresses DMBA-induced breast, and other, tumour progression; however, the mechanisms remain unknown [176]. In this study conditional adipocyte and endothelial cell-specific PPARγ knockout mice (PPARγ-A KO and PPARγ-E KO respectively) were used to evaluate whether PPARγ signaling normally acts to prevent DMBA-mediated breast tumour progression in a stromal cell-specific manner. It was shown that there is clearly a stromal effect of PPARγ on mammary tumourigenesis. In the PPARγA KO mice, the first direct in vivo evidence that PPARγ signaling in stromal adipocytes attenuates DMBAmediate breast tumourigenesis was shown. PPARγ-A KO mice were twice as likely to develop DMBA-mediated malignant mammary tumours and there was a trend towards increased overall mammary tumour incidence when compared with identically treated PPARγ-WT control mice. Interestingly it was discovered that ERα levels were almost 3-fold higher among mammary tumours from PPARγ-A KO mice compared with their PPARγ-WT controls, suggesting the mammary tumours from PPARγ-A KO mice are even more capable of responding to estrogen signaling. The PPARγ-A KO mice also had a reduction in BRCA1 expression, which enhances aromatase, further enhancing estrogen signaling. This study provided the first direct evidence that mammary stromal adipocyte-specific PPARγ signaling locally maintains BRCA1 expression and systematically reduces leptin levels. In the PPARγ-E KO mice, there was increase in mammary tumour multiplicity and significant
reduction in mammary tumour volume. This reduction in volume was attributed to a significant increase in thymic tumours in the PPARγ-E KO mice. It is hypothesized that the changes in immune response due to the thymic tumours could have been responsible for the blunting of mammary tumour progression. Overall survival and tumour latency were not overtly affected by the presence of the thymic tumours, however there may have been changes noted in these parameters if the study was of longer duration. Further studies need to be undertaken to assess the impact on downstream targets of PPARγ, such as VEGF. These two studies clearly highlight that there is stromal-epithelial cross talk at play with respect to PPARγ in DMBA-mediated breast tumourigenesis.

PPARγ-E KO mice require further characterization of changes in PPARγ-dependent target genes expressions that are either up or down regulated (such as BRCA1 or PTEN). One way to further determine the role of PPARγ in the endothelial cells on breast tumourigenesis would be to utilize a spontaneous model of mammary carcinogenesis to reduce the potential impact of thymic tumour formation on breast tumour progression in PPARγ-E KO mice. In a future study, crossing PPARγ-E KO mice with either of the well established MMTV-Her2/neu or MMTV-PyT mouse models that spontaneously develop mammary tumours much more quickly would aid in assessing if the same stromal/epithelial cross talk exists [243-245].

This research has concluded that there quite likely is a cell-specific effect of PPARγ signaling in breast tumourigenesis (Figure 4.1). It was established that the presence of PPARγ expression and signaling in adipocytes significantly reduced mammary tumour incidence and multiplicity, and increased both mammary tumour volumes and the amount of time required for
Figure 4.1 Summary of the protective effects of PPARγ expression in stromal adipocytes and endothelial cells during breast tumourigenesis. The critical role of PPARγ expression and signaling during DMBA-mediated breast tumourigenesis within mammary stromal adipocytes and endothelial cells is shown above.
tumours to become palpable. Mechanistically, activation of PPARγ signaling resulted in a reduction in serum leptin levels and an increase in BRCA1 RNA expression. Similarly, PPARγ expression in endothelial cells also impacts on breast tumour progression. In the presence of PPARγ, total tumour incidences and multiplicity were reduced, and benign mammary tumour multiplicity was also decreased. Loss of PPARγ expression in endothelial cells also increased thymic tumour incidences, and may have impacted on breast tumour progression. More studies are required to determine why thymic tumours arose, and how this may influence breast tumourigenesis in a PPARγ-dependent manner. The knowledge gained from these studies advances our understanding of both stromal-epithelial and environmental-genetic interactions in breast cancer.

To get this research more amenable to the clinical setting it would be useful to examine samples using a tumour array from breast cancer patients. These samples could be assessed for changes in stromal cell expression of PPARγ. This data could then be correlated to clinical outcomes and tumour grade. Sub populations could also be examined to determine if there are other confounding factors that increase the role of PPARγ in mammary tumourigenesis. Potential areas of investigation would be obesity, high fat diet and exposure to environmental carcinogens.

Taken together, this thesis provides insight into how stromal PPARγ may be involved in breast tumour progression. It was shown, in vivo, that both adipocyte and endothelial special PPARγ play a role in breast tumourigenesis. The ultimate goal of this study is to reduce breast cancer-related deaths through better understanding of the interactions between genetic and environmental risk factors. When these mechanisms are known, they may provide the basis for
developing future biomarkers of breast tumour susceptibility. This works also adds support for
the use of PPARγ activating drugs, alone or in combination therapy, to reduce deaths among at
least a subset of breast cancer patients.
Bibliography


106


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116


Appendix A
Supplemental Figure 1: Effects of adipocyte-specific PPARγ deletion on tissue and body weights of untreated mice. Morphological assessments at necropsy of untreated 12 week old A) normal PPARγ-WT compared to B) lipodystrophic PPARγ A-KO mice. Representative resected livers and spleens from C) PPARγ-WT and D) PPARγ A-KO mice. E) Tissue weights as a percentage of body weight (BW) expressed as a mean+SD. WATe, epididymal white adipose tissue; WATi, inguinal white adipose tissue; BAT, brown adipose tissue; Skel Mus, skeletal muscle. F) Mean BW for male (M) and female (F) PPARγ-WT and PPARγ A-KO mice expressed as a mean+SD. n=4 for each group. ***, significantly different from PPARγ-WT male mice, p<0.01; *, significantly different from PPARγ-WT female mice, p<0.05.