SOCIAL ISOLATION STRESS, OBESITY, AND BREAST CANCER RISK IN MICE

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By

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ABSTRACT

Social isolation is a potent psychosocial stressor and is associated with obesity and increased breast cancer risk and mortality. In this thesis, I investigated the combined effects of social isolation stress and obesity on insulin resistance, mammary tumorigenesis, and the mechanisms involved.

First, I studied the effects of social isolation stress in combination with an obesity inducing diet (OID) on mammary tumorigenesis induced by medroxyprogesterone acetate (MPA) and 7,12-dimethylbenz(a)anthracene (DMBA) in female C57BL/6 mice. Social isolation, with or without OID, increased food intake and body weight, and caused impaired insulin and glucose tolerance. Serum NPY levels were increased only in socially isolated OID fed mice, whilst adiponectin levels were reduced both by OID and social isolation. These changes may have contributed to insulin resistance. Mammary tumorigenesis was significantly higher in the socially isolated OID fed mice than in group-housed obese or control mice. Consistent with a previous study indicating that social isolation does not increase mammary tumorigenesis in p53 knockout mice, we found an increase in p53 mRNA expression in socially-isolated mice that exhibited increased mammary cancer risk. Apoptosis was not altered in these mice, but cell proliferation was significantly increased. Social isolation increased the expression of p53-induced autophagy-linked genes and other genes indicative of autophagy in the mammary gland. In summary, we found that obesity
potentiates the effects of social isolation on mammary carcinogenesis, but primarily social isolation induced autophagy and cell proliferation but not apoptosis.

Next, histone deacetylase inhibitor (HDACi) and DNA methyltransferase inhibitor (DNMTi) were administered in drinking water to group-housed or socially-isolated OID fed mice. Insulin resistance and body weight gain were reversed with these inhibitors. However, HDACi/DNMTi treatment did not reverse mammary tumorigenesis and neither did they reverse up-regulation of p53 or alter the expression of cell cycle and cell death genes. Taken together, these data suggest that insulin resistance in obese mice may result from epigenetically-induced changes and therefore DNMTi/HDACi drugs may be useful in preventing weight gain and improving insulin sensitivity, but the increase in mammary tumorigenesis in socially-isolated obese mice is not caused by an increase in DNMT and HDAC activity.
ACKNOWLEDGEMENTS

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I would like to dedicate the whole of this work to my father, Dale M. Sumis. He was diagnosed with cancer when I was a teenager, and while it has been over ten years now since he passed away, his absence in my life will never be filled or forgotten. I miss you.

In loving memory,
Allison M. Sumis
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<tbody>
<tr>
<td>ABSI:</td>
<td>A Body Shape Index</td>
</tr>
<tr>
<td>ACACA:</td>
<td>Acetyl-CoA carboxylase alpha</td>
</tr>
<tr>
<td>ACLY:</td>
<td>ATP citrate lyase</td>
</tr>
<tr>
<td>ACTH:</td>
<td>Adrenocorticotrophic hormone</td>
</tr>
<tr>
<td>AMPK:</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA:</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APP:</td>
<td>Aminopeptidase P</td>
</tr>
<tr>
<td>AR:</td>
<td>Adrenoreceptors</td>
</tr>
<tr>
<td>ATM:</td>
<td>Ataxia-telangiectasia</td>
</tr>
<tr>
<td>ATR:</td>
<td>Ataxia-telangiectasia and Rad3-related</td>
</tr>
<tr>
<td>AUC:</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BDNF:</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BRCA1:</td>
<td>Breast cancer 1</td>
</tr>
<tr>
<td>BRCA2:</td>
<td>Breast cancer 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCNY</td>
<td>City College in New York</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CDH1</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre Recombinase</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz(a)anthracene</td>
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<tr>
<td>DMNT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DMNTi</td>
<td>DNA methyltransferase inhibitor</td>
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<tr>
<td>DPPIV</td>
<td>Dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>DRAM1</td>
<td>DNA damage regulated autophagy modulator 1</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>EPI drugs</td>
<td>HDAC/DNMT inhibitors (inhibitors of EPIgenetic regulators)</td>
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<tr>
<td>ERα</td>
<td>Estrogen receptor alpha</td>
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<tr>
<td>ERRB2</td>
<td>Receptor tyrosine-protein kinase erbB-2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>G-C</td>
<td>Group-housed, control diet</td>
</tr>
<tr>
<td>GEM</td>
<td>Genetically engineered mice</td>
</tr>
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<td>G-OID</td>
<td>Group-housed, control diet</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<td>HDACi</td>
<td>Histone deacetylase inhibitor</td>
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<tr>
<td>HeLa</td>
<td>Cervical cancer cell line</td>
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<tr>
<td>HK2</td>
<td>Hexokinase 2</td>
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<td>HRP</td>
<td>Horse radish peroxidase</td>
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<td>HPA</td>
<td>Hypothalamus pituitary axis</td>
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<td>Hyperparathyroidism 1</td>
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<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>I-C</td>
<td>Isolated, control diet</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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I-OID: Isolated, obesity-inducing diet
JNK1: c-Jun N-terminal kinase
LC3: Microtubule associated proteins 1A/1B light chain 3A
LEP: Human leptin
LEPR: Human leptin receptor
MAPK: Mitogen-activated protein kinase
MCF10: Michigan Cancer Foundation 10 mammary epithelial cell line
MCF-7: Michigan Cancer Foundation-7 breast cancer cell line
MCP-1: Monocyte chemoattractant protein 1 (CCL2)
MDM2: Mouse double minute 2 homolog
MMP: Matrix metalloproteinases
MMTV-LTR: Mammary tumor virus long terminal repeat
MNU: N-methyl-N-nitrosourea
MPA: Medroxyprogesterone acetate
NAMPT: Nicotinamide phosphoribosyltransferase, visfatin
NFκB: Nuclear factor kappa-light-chain-enhancer of activated B
NPY: Neuropeptide Y
OB: Mouse gene for leptin
<table>
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<td>OBR</td>
<td>Mouse leptin receptor</td>
</tr>
<tr>
<td>OID</td>
<td>Obesity-inducing diet</td>
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<tr>
<td>p62</td>
<td>Nucleoporin p62</td>
</tr>
<tr>
<td>PBEF</td>
<td>Pre-B-cell colony-enhancing factor, visfatin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDK4</td>
<td>Pyruvate dehydrogenase lipoamide kinase isozyme 4</td>
</tr>
<tr>
<td>PgR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Nuclear peroxisome proliferator-activated receptor γ</td>
</tr>
<tr>
<td>PPYR1</td>
<td>NPY4R</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time reverse transcription-PCR</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma tumor suppressor protein</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio-Immunoprecipitation Assay buffer</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SAS</td>
<td>Sympo-adrenomedullary system</td>
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<tr>
<td>SEM</td>
<td>Standard error of means</td>
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<table>
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<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>STAT:</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBST:</td>
<td>Tris-buffered saline with tween 20</td>
</tr>
<tr>
<td>TEB:</td>
<td>Terminal end bud</td>
</tr>
<tr>
<td>TDLU1:</td>
<td>Terminal ductal lobular unit 1</td>
</tr>
<tr>
<td>TdT:</td>
<td>Terminal deoxynucleotidyl transferase enzyme</td>
</tr>
<tr>
<td>TNF-α:</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Tp53/p53:</td>
<td>Cellular tumor antigen p53</td>
</tr>
<tr>
<td>TSG:</td>
<td>Tumor suppressor gene</td>
</tr>
<tr>
<td>VEGFR:</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VD3</td>
<td>Vitamin D3</td>
</tr>
<tr>
<td>WAP:</td>
<td>Whey acidic protein</td>
</tr>
<tr>
<td>Y1R-Y6R:</td>
<td>Neuropeptide Y Receptor 1-6 (excluding Y3R)</td>
</tr>
</tbody>
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CHAPTER 1  INTRODUCTION

1.1 Breast cancer statistics

Breast cancer is only second to skin cancer as the most common cancer diagnosis in women, and aside from lung cancer, breast cancer accounts for more cancer deaths in women than any other cancer [1]. While breast cancer incidence and mortality rates have been decreasing in recent decades due to treatment options such as the wide-spread use of tamoxifen and aromatase inhibitors, earlier detection via mammogram, and a decline in the use of hormone replacement treatment after menopause [2], a recent study has shown that breast cancer incidence may no longer be declining [3]. More than 230,000 new invasive breast cancer cases are expected to be diagnosed in 2014, and 40,000 women will die from breast cancer. With this plateau in breast cancer incidence reduction in addition to a woman’s lifetime risk to get breast cancer being one in eight, it is clear that breast cancer is a foremost concern in women’s health [1].

1.2 Breast cancer risk factors

Both genetic inheritance and environmental factors play a role in breast cancer risk. However, only a small percentage, 5 to 10 percent, of breast cancers are inherited. While a large percentage of these cannot be attributed to a specific gene mutation and may be due to other factors such as epigenetic patterning, some gene mutations are well known to be heritable and directly linked to breast cancer risk. In contrast to spontaneous cancers, inherited breast cancers generally account for early cases which are diagnosed before age 40 [4]. For example, mutations in BRCA1 or
*BRCA2*, which are responsible for 20-25% of heritable breast cancers, increase risk for breast cancer by 80% [5-7]. *BRCA1* and *BRCA2* are also linked to increased ovarian cancer risk [8]. Other recognized breast cancer-linked gene mutations, though accounting for only about 1% of familial breast cancers [7], include tumor protein p53 *TP53* (Li-Fraumeni syndrome) [9] and phosphatase and tensin homolog *PTEN* (Cowden’s syndrome) [10]. Breast cancer risk doubles by having one first degree relative and increases to five-fold with two or more first degree relatives [11].

Non-genetic risk factors for breast cancer have been studied thoroughly and include age, menstrual and menopause history, parity, hormone therapy, geographic location, race, and lifestyle factors. Age increases breast cancer risk more than any other factor. The older a woman is, the higher the risk that she has for breast cancer. Before age 30, the risk for breast cancer is only 0.44%. By age 50, risk has increased to 2.38%, and by age 60, to 3.56%. However, after age 80 breast cancer incidence no longer increases and may even start to decrease [12]. The addition of other risk factors will alter these risk percentages.

Lifetime exposure to estrogen is extremely important in determining breast cancer risk. Therefore, early start of menstruation (before age 12) and late menopause (after age 55), which prolong estrogen exposure to breast tissue, increase a woman’s risk to breast cancer by 20% and 30%, respectively [13-16]. High levels of estrogen in the blood of post-menopausal women are also associated with an increased risk of breast cancer [17]. Further, when compared to women who go through natural menopause, women who have an oophorectomy have lower risk of
breast cancer [18]. However, although estrogen levels are several fold higher in pregnant than non-pregnant women, early pregnancy (before age 20) and having more than five pregnancies are protective against breast cancer [19,20]. Conversely, nulliparity and first pregnancy after age 30, and even more if after age 35, increase breast cancer risk [14]. The protective effects of early pregnancy may reflect the ability of estrogens to induce differentiation of normal mammary epithelial cells and elimination of targets for future malignant transformation.

Likewise, hormone therapy increases breast cancer risk. The association of the usage of birth control pills to breast cancer risk has been extensively studied, with the consensus reporting that there is a slight increase in breast cancer risk with the use of these medications. This elevation in risk retreats after 10 years of disuse [21]. Hormone replacement therapy composed of both estrogens and progestins, used to treat hot flashes and other adverse effects of menopause, significantly increases breast cancer risk [22].

Breast cancer risk varies by geographical location. Western Europe, Northern America, and Australia all have higher breast cancer incidence than other parts of the world such as Asia or Africa [23]. However, moving from Asia to the United States increases second generation’s risk for breast cancer to that of an American woman; thus, proving that breast cancer risk is linked to environmental factors [24,25]. Even in the United States, breast cancer incidence and mortality varies by region, with the Northeast having the highest rate of incidence and the Midwest and South with the highest rate of mortality [26]. Women of Ashkenazi Jewish decent have a slightly higher breast cancer incidence. This may be due to a higher rate of \emph{BRCA1} and \emph{BRCA2}
mutations, as Ashkenazi Jews have a BRCA1 mutation rate of 1 in 40, compared to 1 in 400 for the general populace [27-29]. While white women have the highest incidence of post-menopausal breast cancer, women of African American decent have the highest rate of premenopausal breast cancer and breast cancer mortality.

Life style factors are important in breast cancer risk determination. Alcohol consumption, dietary choices, physical activity, obesity, and stress all confer a change in breast cancer risk. Moderate to heavy alcohol intake is correlated to breast cancer risk. The more a woman imbibes, the more her breast cancer risk increases. In fact, drinking 2-3 alcoholic beverages a day increases breast cancer risk by 20% [30]. This increase is partially due to alcohol changing the metabolization of estrogen, resulting in an increase in serum estrogen levels [31,32]. Further, alcohol lowers the levels of the vitamin folic acid in the blood. Folic acid is involved in DNA repair; therefore, low levels of folic acid due to alcohol intake, especially in women with preexisting low levels of folic acid, can increase breast cancer risk [32]. Some alcohol metabolites also directly induce DNA damage.

Dietary choices are extensively studied in relation to breast cancer risk. Studies on fruit and vegetable intake have shown contradictory data [33-36]; however, a meta-analysis from 15 studies on the effect on breast cancer risk from eating fruits and vegetables found that high intake in fruits or fruits and vegetables, but not vegetables alone, had a slight protective effect against breast cancer [37]. Another meta-analysis from 20 studies showed that high vegetable intake had a modest protective effect from estrogen receptor negative breast cancer [38]. Studies on dietary
fat intake and its relation to breast cancer risk are also contradictory, with most studies showing no correlation [39-43]. However, the type of fat may be important [39,40].

Regular physical activity reduces breast cancer risk by 10-20% [44,45]. One reason for this is that physical activity lowers levels of serum estrogens [46,47]. Additionally, when combined with caloric restriction, physical activity promotes weight loss. As obesity is causally linked to breast cancer risk [48-50], physical activity can lower breast cancer through reversing or preventing obesity. Physical activity also decreases risk for breast cancer recurrence [51,52].

Obesity and stress as risk factors for breast cancer will be comprehensively discussed further on in this chapter, sections 1.5 to 1.9 and 1.10 to 1.13, respectively.

1.3 Modeling breast cancer for research

While the study of humans is the ideal model to research breast cancer risk and prevention, the availability of suitable participants and costs and the invasiveness of some studies can cause restrictions. Because of this, other models are needed for research. Many researchers use human breast cancer cell lines; however, cell lines have many draw backs, especially when studying breast cancer prevention. Importantly, while cell lines are exceptional for studying the intricacies of biological pathways such as apoptosis, cell cycle, or migration and the study of drug discovery and effectiveness, cell lines do not take into account cellular microenvironments or the physiological effects of the entire body. Additionally, cell lines acquire genetic mutations and
epigenetic modifications over time, resulting in genetically different cell lines from study to study [53,54].

Cell lines are commonly used as xenografts in immune-deficient mice to study tumor growth, metastasis, the effects of specific changes in pathways, and drug efficacy. While this allows for the study of human cancer cell lines in an \textit{in vivo} setting, there are many drawbacks to this type of model. The main limitation to this model is that the mice have a compromised immune system, which does not align with normal tumor growth in the human body as the immune system plays a critical role in tumor development. Moreover, oftentimes the xenografts are injected into the flanks of the mice instead of the mammary fat pads which eliminates the correct microenvironment for the tumor cells. Introduction of robust tumor cells into the mammary fat pad also does not simulate real initiation of a tumor. Xenograph metastases favor the lungs, while human breast cancers more commonly metastasize to the bone, brain, and liver [55]. Primary tumors from a human breast cancer patient have been used as xenografts in immuno-deficient mice. Unfortunately, the availability of primary tumor samples for transplantation, the usage of immune system compromised mice, and a low success rate of transplantation make this method implausible as a standard for breast cancer modeling [54,56].

To bypass some of the limitations of the xenograft model, genetically engineered mice (GEM) that either overexpress oncogenes or decrease tumor suppressor expression can be used to model breast cancer. Some models use promoters such as mouse mammary tumor virus long terminal repeat (MMTV-LTR) or whey acidic protein (Wap) to overexpress oncogenes such as \textit{Myc} or
Erbb2 in mammary tissue. These promoters are regulated by steroid hormones [57]. One issue with GEM modeling is that the expression of these oncogenes may not reflect the levels in human breast tumors, and that the cell types that are developing into tumors in the genetically modified mice are not the same type as in human breast tumors resulting in a dissimilar model to human breast tumors [58]. In other models, tumor suppressor genes are knocked out to generate breast tumors. For example, fifty percent of BALB/c Tp53+/− heterozygous mice develop mammary tumors, making it an excellent model for Li-Fraumeni syndrome [59]. Unfortunately, as both of these modeling methods of genetic modifications are germ line inherited, the overexpression of certain oncogenes or absence of tumor suppressor genes can be developmentally lethal [54,60-63]. When these promoter driven oncogenes or knockdown/out tumor suppressors are inherited through the germ line, they are expressed (or not) in other tissues such as the lungs, brain, and salivary glands which can cause other primary tumors and also lymphomas and can compound results [64,65]. For specific control over the expression of these promoters or to conditionally knockout certain tumor suppressors to avoid developmental lethality, Cre recombinase or tetracycline regulation can be utilized [66,67]. Through the usage of conditional expression models, mammary tumors have also been generated through the combination of overexpressing oncogenes while knocking down tumor suppressor expression as in the case of MMTV-Ras; Tp53+/− mice [68] or through double tumor suppressor knockdown/out as in the case of MMTV-Cre; Brca1Co/Co; Tp53+/− mice [69]. Overall, it has been determined that these methods of modeling have distinct differences when used in comparison to human breast cancers, with emphasis that similar to xenografts in immuno-deficient mice, metastases occur more often in the lungs than in the tissues where metastases of human breast
cancers arise. Furthermore, histology of these mouse mammary tumors differs from human breast cancers, and the extent of hormone dependency varies between the two as well [70]. However, while these differences exist, these models have been invaluable in studying breast cancer [54,59,71-73].

An alternative approach to breast cancer modeling in mice, rats and other species is to use carcinogens to induce mammary tumors. Generally either alkylation agents such as N-methyl-N-nitrosourea (MNU) or polycyclic hydrocarbons such as 7,12-dimethylbenz[a]anthracene (DMBA) are used to induce mammary tumors in mice [74,75]. Tumor initiation by these carcinogens is hormone-dependent (estrogen receptor alpha and progesterone receptor positive); thus, priming the mammary gland with hormones, especially medroxyprogesterone acetate (MPA), hastens initiation and increases tumor incidence by NMU and DMBA [76-78]. Although 80% of MNU-induced tumors in rodents result in Hras mutations [79,80], around 20% of DMBA-induced mammary tumors in rodents exhibit Hras mutations [81]. The low mutation rate of Hras by DMBA is comparable to the amount of HRAS mutations in human breast cancers [82]. Carcinogen-induced mammary tumors in mice develop heterogeneous tumors, which include adenocarcinomas, squamous cell carcinomas, and myoepithelial carcinomas, and are more comparable to human breast cancers than those developed through xenografts or genetically engineered mice [83].
1.4 Breast cancer risk assessment endpoints in mouse mammary gland

Terminal end buds (TEBs) are bulbous structures in the mouse (and rat) mammary gland that are found at the growing end of mammary epithelial trees. Humans have comparable structures in breast tissue known as terminal ductal lobular unit 1 (TDLU1). As TEBs/TDLU1 have high rates of proliferation and are susceptible to DNA damage, chemical carcinogen exposure leads to malignant mammary cancer at these locations [84-87]. Because of this, the number of TEBs/TDLU1 that are found in a mammary gland are directly correlated to mammary cancer risk in rodents/humans [88,89]. Epithelial tree density and growth length have also been used as endpoints to assess breast cancer risk in rodents [90,91]

1.5 Obesity as an epidemic

More than two-thirds of Americans are overweight, half of which are obese [92]. Obesity-linked disease is a top cause of preventable death in the United States, behind tobacco use and high blood pressure [93]. An estimated 112,000 to 216,000 people die each year due to obesity-linked diseases, which is much less than the 300,000 to 400,000 that has been previously reported and determined to be inaccurate [93-95]. Nevertheless, in 2008, obesity-related health costs reached 147 billion dollars in the United States. Obesity causes a high rate of preventable death and enormous health costs, thus making it a public health crisis [92,96].
1.6 Measuring obesity

Defining obesity is a highly-debated subject. Commonly, body mass index (BMI) is used as a measurement for obesity, where BMI = mass (kg)/height (m)^2. A BMI of 18.5 to 25 is considered healthy, while a BMI under 18.5 is underweight. A BMI of 25 to 30 is overweight and over 30 is obese. People who have a BMI of over 40 are considered morbidly obese. The main debate over BMI is that people who are muscular such as athletes sometimes end up with BMIs in the overweight category, albeit they have no excess body fat. Additionally, BMI does not differentiate between where the adipose tissue is located on the body [92,97]. Waist circumference and waist to hip ratio may be a better alternative method for measuring obesity, as they are more indicative of central obesity, which, discussed in the next section, has a greater correlation to obesity-linked disease risk [98,99]. A new method of measurement known as A Body Shape Index (ABSI) was recently published by a research group at the City College in New York (CCNY) that uses waist circumference and BMI in its calculations. They reported ABSI to be an accurate risk assessment for premature mortality [100].

1.7 Central obesity and obesity linked disease

Obesity causes a variety of diseases and conditions such as metabolic syndrome, high cholesterol, insulin resistance and type II diabetes mellitus, hypertension, sleep apnea, heart disease, cancer, stroke, and overall early death [101-105]. In women, waist circumference and waist to hip ratio are a more accurate predictor of obesity-linked disease than weight or BMI because waist circumference and waist to hip ratio are a better measurement of central obesity.
Central obesity occurs when fat deposits accumulate centrally on the body (around the abdomen) in an apple-shaped pattern rather than peripherally (in the thighs, arms, and buttocks) in more of a pear-shaped pattern. Central obesity is more common in men and postmenopausal women, while obese premenopausal women accumulate fat more peripherally [98,106]. In contrast to humans, obese “premenopausal” female mice accumulate fat in abdominal depots [107], making them an appropriate model to study a link between obesity and breast cancer.

1.8 Obesity and cancer

Obesity is a risk factor for most cancers and is estimated to be responsible for 15 to 20 percent of cancer mortalities. If all adults maintained a healthy BMI of under 25, more than 90,000 cancer deaths per year could be avoided [50]. Obesity increases endometrial cancer risk by 2 to 5 fold [108]. In men, colorectal cancer risk is increased by 30 to 70 percent by obesity. In women, the evidence is weaker due to the differences in the location of fat accumulation, but a meta-analysis reported that the majority of studies still report a slight increase in risk of colorectal cancer by obesity [109]. Risk of kidney and esophageal cancers are also associated with obesity, with an increase of 1.5 to 2.5 fold and 2 to 3 fold, respectively [101].

Breast cancer risk due to obesity varies by whether or not the woman is pre- or postmenopausal. As obese premenopausal women are more likely to carry weight in the pear shape, obesity not only does not increase breast cancer risk, but actually has a protective effect. The reason for this is under study, but it may be due to obese young women commonly having amenorrhea and therefore lower levels of blood estrogens [110]. However, weight gain after age 18 of over
twenty pounds increases risk of postmenopausal breast cancer by 15 to 55 percent [111]. Postmenopausal women carry weight more centrally; thus, obesity in these women increases breast cancer risk by 30 to 60 percent [49,112-114]. Weight gain after menopause [111,115] or breast cancer diagnosis increases recurrence risk, and being obese increases risk of breast cancer recurrence in pre- and postmenopausal women with breast cancer [49].

1.9 Mechanisms linking obesity to breast cancer

The mechanisms mediating the effects of obesity on cancer risk still need to be elucidated [48,116-118]. Adipose tissue, especially centrally-stored fat, acts as an endocrine organ and immune system regulator. Hence, adipose tissue is a major source of cytokines, insulin-like growth factor 1 (IGF-1), adipokines, and hormones; therefore, central obesity can greatly affect biological processes promoting breast cancer growth. Obesity also induces changes in epigenetic regulation of gene expression. The effectors of adipose tissue are illustrated in Figure 1.1.

Inflammation

Accumulated adipose tissue recruits immune cells and causes chronic low-grade inflammation, which is associated with increased cancer risk [119,120]. Adipose tissue produces an abundance of cytokines and chemokines including interleukin 6 (IL-6), interleukin 1β (IL-1β), tumor necrosis factor alpha (TNF-α), and monocyte chemoattractant protein 1 (MCP-1).
Figure 1.1: Adipose tissue as an endocrine gland and immune system regulator.
IL-1β is highly expressed in breast cancer cell lines, breast cancer tissue, and in breast cancer microenvironment and is involved in insulin resistance, angiogenesis, apoptosis, proliferation, and migration [121-123]. Caspase-1 is responsible for processing IL-1β to its mature form; thus, mice that are deficient in caspase-1 are more insulin sensitive than wild-type mice [124].

IL-6 is produced by adipose infiltrating macrophages and adipocytes. Insulin and TNF-α stimulate production of IL-6 [125]. IL-6 activates c-Jun N-terminal kinase (JNK) and thus signal transducer and activator of transcription (STAT) and mitogen-activated protein kinase (MAPK) signaling pathways [126]; therefore, cell migration is promoted and apoptosis is inhibited in breast cancers [127]. Blood levels of IL-6 are higher in obese people [128] and are associated with poor outcomes in metastatic breast cancer [129]. Obese Il-6 and Tnfr1 knockout mice do not have increased cancer risk, insinuating that these two cytokines mediate the effects of obesity on cancer risk [130].

In adipose tissue, TNF-α is produced mainly by infiltrating macrophages [131]. Through activation of JNK and subsequently nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), dysregulation of insulin signaling and induction of insulin resistance occurs. TNF-α activation of JNK signaling also causes an increase in cell proliferation [132]. TNF-α serum levels are elevated in obese individuals [133], and these elevated levels have been linked to breast cancer [134].
MCP-1 (also known as CCL2 in humans) is a chemokine that recruits monocytes to adipose tissue. MCP-1 levels are associated with an increase in macrophage infiltration, tumor cell migration, and angiogenesis in the adipose tissue. IL-1β can stimulate production of MCP-1 [135,136]. MCP-1 overexpression in adipose tissue increases macrophage recruitment and initiates insulin desensitization [137,138].

**Insulin resistance**

Insulin resistance occurs when cells no longer respond to insulin, and thereby do not shuttle glucose into cells, resulting in high blood glucose and insulin levels. Obese people have increased levels of insulin and lower levels of IGF-binding proteins. This results in higher blood levels of free IGF-1 which then promotes tumors by inhibiting apoptosis and increasing cell proliferation through c-Myc and Cyclin D1 activation [139,140]. Treatment of MCF-7 breast cancer cells with IGF-1 increases proliferation [141]. Almost fifty percent of human breast tumors overexpress IGF-1 receptors, which enhances the proliferative and pro-survival effects of IGF-1 [142]. Both IGF-1 and insulin stimulate production of sex steroid hormones (notably estrogen) [143,144], and estrogen can increase expression of IGF-1 receptor [145].

Even more, insulin acts as an anti-inflammatory compound by suppressing circulating and hepatic levels of IL-6, IL-β, MCP-1, and TNF-α [146,147]; therefore, resistance to insulin results in inflammation [148]. Increased levels of IL-6 and TNF-α due to obesity-induced chronic inflammation are causally-linked to the development of insulin resistance [149]. Insulin
resistance is associated with an increased risk of breast cancer in pre- and postmenopausal women [150,151].

The nuclear peroxisome proliferator-activated receptor γ (PPARγ) is expressed in endothelial cells, vascular smooth muscle cells, macrophages, fibroblasts, and the majority of breast tumors. PPARγ regulates fatty acid uptake and storage, and thus is important for controlling adipogenesis and maintaining energy homeostasis [152,153]. Through the inhibition of cytokine signaling and subsequent anti-inflammatory response [154], down-regulation of insulin receptor [155], and regulation of adiponectin [156], PPARγ improves insulin sensitivity.

**Adipokines**

Leptin is an adipose-derived hormone that is produced primarily by adipose tissue, but also in others such as mammary gland and tumor, and in the stomach [157,158]. Leptin (from the obese gene Ob in mice and LEP gene in humans) is important in mammary gland development, as leptin knockout mice (Ob/Ob) have diminished epithelial growth in the mammary gland [157]. Even more, leptin binds to receptors in the hypothalamus to inhibit appetite, control energy expenditure, and promote insulin sensitivity [159-161]. Leptin receptor LEPR or Ob-R (mice) is overexpressed in mammary tumors [162], but it also is present in normal mammary glands [163]. Leptin measures how thin your body is, and because leptin levels directly correlate to the amount of adipose tissue that a person has, obese people have very high levels of leptin which can result in leptin resistance [164]. The brain reads leptin deficiency as a sign of starvation.
Ob/Ob knockout mice are extremely obese [165], but treatment with leptin returns them to a normal body weight [166-168]. Though rare, leptin deficiency via mutation in the LEP gene in humans can cause extreme obesity in early childhood [169]. Treatment with leptin in these leptin-deficient children and adults reverses obesity and insulin resistance and alters the brain’s response to food [170-174]. However, leptin treatment in obese individuals results in no differences in body weight. This is due to the high levels of leptin in obese individuals and the resulting insensitivity of the Ob-R/leptin receptor (leptin resistance) [174-176].

Leptin can induce macrophages to produce TNF-α; however, this can be inhibited by adiponectin [121]. IL-1β and TNF-α can stimulate adipocyte production of leptin [122]. Through JAK/STAT and MAPK signaling, leptin acts as a growth factor, inhibits cell cycle checkpoints, increases migration and invasion, and increases angiogenesis [161] and can also stimulate aromatase activity [177]. Leptin is associated with mammary carcinoma growth and increased breast cancer risk [157,178].

Adiponectin, an adipose derived hormone, is involved in glucose regulation (increases cellular glucose uptake and decreases gluconeogenesis) and fatty acid catabolism primarily through AMP-activated protein kinase (AMPK) signaling [179]. Adiponectin promotes insulin sensitivity [180]. Though adiponectin is secreted by adipose tissue, its circulating levels are inversely linked to obesity, perhaps due to being down-regulated by the TNF-α and IL-6 also originating from adipose tissue [179]. Low adiponectin levels are associated with increased breast cancer risk [181] and causally linked to insulin resistance [182]. However, when adiponectin levels are high,
it can increase apoptosis, decrease angiogenesis, decrease inflammation, and increase proliferation [183]. Adiponectin receptors colocalize with leptin receptor LEPR in the arcuate nucleus of the hypothalamus [184].

Adiponectin also interacts with PPAR-γ. PPAR-γ regulates adiponectin transcription through binding to conserved cis-acting regulatory DNA elements, and serum adiponectin levels strongly correlate with PPAR-γ activity [156]. Alternatively, through AMPK signaling, adiponectin can activate PPAR-γ [185].

Another PPAR-γ regulated adipokine, resistin has effects that promote adipogenesis, insulin resistance and inflammation [186]. In mice, the majority of resistin is produced by adipocytes [187], while macrophages are responsible for the resistin in humans. These genes are located on different chromosomes [188]. In mice, resistin’s primary function is in glucose metabolism regulation, whereas in humans it conducts more of a role in inflammation [189]. Resistin signals through vascular endothelial growth factor (VEGF) to increase angiogenesis [190] and inhibits AMPK signaling in muscle and liver [191]. A study in 2007 reported that resistin increased levels of TNF-α and IL-6 and increased JNK signaling, thus finding the link from resistin to inflammation [192]. Levels of resistin are increased with obesity and decreased by PPAR-γ [151] and are associated with breast cancer risk [193].

Lastly, visfatin (originally termed pre-B-cell colony-enhancing factor, PBEF, and also nicotinamide phosphoribosyltransferase, NAMPT) is an adipokine that can mimic insulin [194].
In addition to adipocytes, visfatin is produced by infiltrating macrophages [195], hepatocytes [196], skeletal muscle cells [197], and peripheral blood leukocytes [198]. While little is understood about visfatin signaling, a few studies have shown that JNK and NFκB may stimulate visfatin to activate angiogenesis by increasing levels of VEGF and matrix metalloproteinases (MMPs). Visfatin also stimulates production of the cytokines IL-1β, IL-6, and TNF-α, making it pro-inflammatory and linked to insulin resistance [199,200]. Visfatin stimulates proliferation in MCF7 breast cancer cells [201] and is overexpressed in doxorubicin resistant breast tumors [202].

*Aromatase*

Adipose tissue produces an enzyme called aromatase which converts testosterone into estrogen. This is particularly adverse for postmenopausal women with estrogen receptor alpha (ER-α) positive breast cancer, as because the ovaries are no longer producing estrogen, adipose tissue becomes the main source of estrogen in the body [203,204]. TNF-α and IL-6 stimulate aromatase activity [205]. Aromatase inhibitors are currently used as adjuvant treatment of ER-α positive breast cancers to reduce recurrence [206-208]. Resistance to the treatment with aromatase inhibitors i. e. anastrozole is common in obese women, perhaps due to high aromatase activity in adipocytes and consequently insufficient inhibition of aromatase. This results in a significant increase in breast cancer recurrence and death, suggesting that aromatase inhibitor treatment in obese women is not as effective as in women who are a healthy weight, and that weight loss is extremely important for obese women with breast cancer [209-213].
**Epigenetics**

Heritable changes in gene expression that do not involve mutations to DNA are known as epigenetics. DNA methylation and histone modifications are two of a number of forms of epigenetics that dictate gene expression. Changes in DNA methylation are caused by the activity of a group of enzymes known as DNA methyltransferases (DNMTs). DNMTs add a methyl group onto the 5-carbon of cytosine rings within CpG dinucleotides, resulting in the silencing of a target gene, either by causing de novo methylation by DNMT3a or by maintaining epigenetic patterns during cell division through DNMT3b. DNMT1 not only regulates the maintenance of epigenetic patterns, but can also induce *de novo* methylation, especially in cancer cells [214-216]. Histone deacetylases (HDACs) remove acetyl groups from histones. Acetylation of histones decreases their ability to bind to DNA. Therefore, among other functions, HDACs cause DNA to bind to histones, and thus gene transcription is blocked. This can cause silencing of tumor suppressor genes [217,218].

While there is still much to be understood about epigenetics in inflammation [219], emerging evidence indicates that inflammation induces epigenetic alterations which can lead to increased disease risk, including cancer [220,221]. Inflammation may increase DNA methylation in normal tissue and contribute to the development of autoimmune diseases [222]. This may be due to IL-1β and IL-6, which are up-regulated in the presence of excess fat and suggested to initiate epigenetic changes [220,223,224]. Even more, CMP-1 and TNF-α have been reported to be regulated by HDACs [225,226]. This hypothesis is supported through evidence that treatment
with HDAC inhibitors (HDACi) exhibits anti-inflammatory effects via suppression of cytokines such as IL-1β, IL-6, and TNF-α [227-230].

Several studies have investigated whether epigenetic changes in genes that regulate adipose cells may cause obesity [231,232]. There also is evidence that obesity induces epigenetic changes. Obesity can either elevate or lower levels of HDACs in the hypothalamus [233]. As treatment with HDACi can reverse insulin resistance [234], these obesity-induced changes in HDACs may potentiate insulin resistance development. The reversal of insulin resistance may be through the changes in cytokines that can induce insulin resistance and also though inhibition of HDAC regulated adipogenesis [235]. Moreover, a number of genes involved in tumorigenesis are regulated by obesity-related epigenetics. Studies have linked an increased likelihood that women with high waist-to-hip ratio exhibit elevated methylation of E-cadherin (CDH1) and p16 (CDKN2A) genes in the breast tumors [236], and this may contribute to increased epithelial to mesenchymal transition and metastasis, and cancer cell proliferation. Hypomethylation of TNF-α [237], LEP [238], and pyruvate dehydrogenase lipoamide kinase isozyme 4 (PDK4) [239] are also associated with obesity. Our preliminary study has shown that obese mice exhibit an increase in DNMT1 p= 0.017, DNMT3b p= 0.011 and Np95 p= 0.009 (Np95 is a histone-binding protein that possesses ubiquitin ligase activity and is a regulator of cell cycle progression [240]) expression in the uterus, and the increase can be reversed by vitamin D (VD3) that possesses anti-inflammatory [241-243] and epigenetic properties which includes the down-regulation of DNMTs [244].
1.10 Stress and cancer

Several studies in humans and animal models have been conducted to determine if stress plays a role in affecting cancer risk, but it is still unclear how to interpret all of the conflicting results due to differences between types of stress and study designs. In humans, some studies show an increased cancer risk [245-248], some show a decreased cancer risk [249], and some find no association [250-252]. These studies generally focus on bereavement, overall life events or work stress. A meta-analysis based on 27 studies from 1966 to 2002 found associations between increased amounts of stressful life events, death of a significant other, and death of a relative or friend with an increase in breast cancer risk. Results were more profound when focusing on prospective compared to retrospective studies (due to recall bias) and shows the need for more prospective studies that focus on specific types of stressors [253].

Social isolation is a potent psycho-social stressor; it initiates a robust central nervous system (CNS) and stress-hormone reaction [254]. A study in the United States that involved almost 30,000 male health professionals aged 42-77 years reported that the relative risk of mortality for the majority of socially-isolated men was increased by 19% [255], and a French study showed that socially-isolated men and women were 3.6 times more likely to die from cancer than socially-integrated people [256]. Moreover, in another study, women who were socially-isolated before cancer diagnosis had a 66% elevated risk of all-cause mortality, and over a two-fold breast cancer mortality risk than women who were socially-integrated [257]. In this study, the degree of social isolation was measured by the Berkman-Syme Social Networks Index [258]. This combines the measurements of four types of social connections: marital status (married
versus not); sociability (number and frequency of contacts with children, close relatives, and close friends); church group membership (yes versus no); and membership in other community organizations (yes versus no). This resulted in four groups: (1) socially isolated (individuals with low intimate contacts—not married, fewer than six friends or relatives, and no membership in either church or community groups), (2) moderately isolated, (3) moderately integrated, and (4) socially integrated. The reference group was composed of women with large social networks. Smoking, physical activity, BMI, age at menopause, hormone use, and many other compounding variables (but not substance abuse) were assessed by analysis of covariance. Women who had the highest degree of social-isolation were more likely to be a smoker, have lower levels of physical activity, and eat less protein [257].

Whether or not psychological interventions in women with breast cancer is effective is highly disputed. One study found that the amount of social support that a person has correlates with cancer survival [259]. Additionally, a study on relaxation and guided imagery intervention in women with breast cancer undergoing treatment found an increase in natural killer cell activity, but not with cytokine levels except IL-1β. Survival and recurrence were not followed in this study [260]. An intervention study where women with breast cancer underwent 39 hours of psychological therapy in the form of 26 sessions to reduce stress and increase quality of life which included muscle relaxation, help with problem solving, the identification of supportive family members of friends, and advice to improve health through diet and exercise, reported that breast cancer recurrence risk and breast cancer mortality were both reduced by this psychological intervention in comparison to an assessment only group [261]. However, commentaries by
Coyne et al. refuted these claims based upon the current literature that has shown little link between psychological intervention and evident study design bias and statistical analysis issues [262,263]. This obvious dispute displays the need for improved studies on psychological intervention and risk of breast cancer recurrence and survival.

Studies in mice report that isolated housing (one mouse per cage) increases the risk of colon [264], Ehrlich adenocarcinoma [265], hepatocellular carcinoma [266,267], and mammary tumorigenesis [268-270]. A study by the McClintock group on socially-isolated Sprague-Dawley rats showed an 84-fold increase of spontaneous mammary tumor burden versus group-housed controls at 15 months of age [271]. This group also found an increase in T-antigen derived mammary tumors in mice due to social isolation stress [270]. Surprisingly, a recent study showed that up-regulation of p53 may mediate the adverse effects of social isolation stress on mammary tumorigenesis; thus, reduced levels of p53 have emerged as a protective factor against social isolation stress-induced mammary cancer in mice [272]. Our study described in Chapter 2 suggests that these findings may be explained via altered activity of p53-mediated apoptosis and autophagy.

Some stressors cause overeating and, thus, weight gain and central obesity in humans, whilst some have the opposite effect [273]. Sometimes this weight gain is inconsistent with the number of calories consumed [274]. Diagnosis and treatment of breast cancer is stressful, so it comes to no surprise that after a year from diagnosis, most women gain weight, some up to 50 pounds [275]. In rodents, a mild stressor has no effect on food intake; however, moderate and severe
stressors such as noise, restraint and immobilization reduce food consumption, and thus body weight. These stressors produce increased levels of norepinephrine, but neuropeptide Y levels do not change [276-278]. Conversely, some chronic stressors like an exposure to cold water, foot shock, or social isolation induce body weight gain in mice and directly enhance the growth of adipose tissues through NPY, promoting central obesity. In these mice, however, glucocorticoid and catecholamine levels either do not change or are even down-regulated [277,279-281].

1.11 The hypothalamus-pituitary-adrenal axis and sympo-adrenomedullary system

The hypothalamus-pituitary-adrenal axis (HPA-axis) is an important pathway in the body’s response to stress and is the primary activator in conjunction with the sympo-adrenomedullary system (SAS) of the fight or flight response. The fight or flight response initiates an increase in blood sugar through gluconeogenesis, an increase in blood pressure, and temporary immune suppression. The increase in blood sugar allocates glucose to be used in muscles rather than in the gut, preparing the body for a fast response to the perceived threat. The increase in blood pressure similarly brings blood to the brain, heart, and muscles. Stress signals to the hypothalamus to release corticotrophin-releasing hormone (CRH) which then either activates cortisol (or corticosterone in rodents), a glucocorticoid that is synthesized from cholesterol, or the catecholamine epinephrine. For cortisol stimulation, CRH goes to the anterior pituitary gland which then produces adrenocorticotropic hormone (ACTH). ACTH acts upon the adrenal glands to produce cortisol. Cortisol acts as a negative feedback regulation loop on the hypothalamus and pituitary gland, but chronic stress can disrupt this feedback. Studies have
found that early life stressors such as parental loss or maltreatment can cause increased levels of waking cortisol levels many years after the exposure [282-284] by permanently making changes in HPA-axis signaling [285,286]. In mice, social isolation stress significantly elevates corticosterone levels in the blood [287]. Cortisol inhibits apoptosis in the mammary gland [288] and stimulates prosurvival pathways in MCF10A mammary epithelial cells [289]. Higher serum cortisol levels have been found to induce aromatase activity [290].

As a part of the SAS initiation of the fight or flight response, CRH also stimulates the release of the catecholamines epinephrine and norepinephrine from the adrenal medulla and sympathetic nerves, respectively [291]. Norepinephrine and epinephrine activate adrenoreceptors (AR) α and β. Activation of β-AR initiates angiogenesis, which is the main pathway in which these catecholamines exert their effects on tumorigenesis. An increase in metastases through stimulation of β-AR has also been reported and is most likely through production of metalloproteinases MMP-2 and MMP-9 [292].

1.12 Neuropeptide Y

Produced by neurons in the hypothalamus, neuropeptide Y (NPY) is the most abundant peptide in the brain but is also released in peripheral sympathetic nerves during stress which gives NPY the ability to have direct effects on tumors [293-295]. NPY has 4 receptors in humans, Y1R, Y2R, Y4R (also called PPYR1), and Y5R [296]. Additionally Y6R is expressed in mice [297]. Affinity to specific receptors is regulated by the proteolytic cleavage of NPY by dipeptidyl peptidase IV (DPPIV) and aminopeptidase P (APP), where a shorter form of NPY binds to Y2R
and Y5R instead of Y1R [298]. A chronic stress induced hormone [299], NPY’s primary functions in the brain are in regulation of appetite, alcohol consumption, circadian rhythms, memory retention, epilepsy, angiogenesis, and cardiovascular and lung function [300-302]. However, through its peripheral actions, NPY promotes tumor growth and angiogenesis in neural-crest derived tumors [303] and augments central obesity, metabolic syndrome, and angiogenesis through its Y2R receptors [279]. In Ewing’s sarcoma, NPY induces cell death through its Y1R/Y5R receptors [304]. Y1R is the predominant NPY receptor in primary human breast carcinomas [305,306] and breast cancer cell lines, [296]. Estrogen induced proliferation of MCF7 cells can be blocked by NPY treatment through its Y1R receptors [305], but through Y5R can stimulate proliferation, angiogenesis, and migration in other breast cancer cell lines [307-309]. Y2R is the primarily expressed receptor in normal breast tissue [306].

Leptin inhibits NPY in the hypothalamus [160]. Adiponectin receptors and NPY colocalize in the arcuate nucleus of the hypothalamus [310], insinuating that adiponectin and NPY may interact, as other adipokines are known to regulate NPY expression, as well [160,311]. NPY is also expressed in macrophages in abdominal adipose tissue [312], but it is not known whether adiponectin and NPY interact in this tissue. Moreover, NPY plays an integral role in abdominal adipose inflammation mediation. Through stimulation of macrophages and neutrophils, NPY induces production of IL-6, TNF-α, IL-1β, and other pro-inflammatory cytokines [313]. NPY also inhibits natural killer cell activity, which are responsible for the surveillance and decimation of virus-infected and tumor cells [314].
1.13 Stress and epigenetics

Growing evidence suggests that epigenetic regulation alters gene expression in the brain in relation to memory [315], circadian rhythms [316], neurological and psychological diseases such as Fragile X syndrome, schizophrenia, and bipolar depression [317,318]. Accordingly, the HDAC inhibitor valproic acid is used to treat bipolar depression [319]. Epigenetic changes also regulate the stress response as HDAC6 can modulate glucocorticoid receptor (GR) signaling in the brain [320]. On the other hand, stressors can change epigenetic patterning. Early life stresses alter epigenetic-controlled expression of GR [321], and forced-swim stress induces epigenetic modulation of acetylcholinesterase through HDAC4 [322]. Moreover, immobilization stress and also fear conditioning through foot shock therapy in rats alters the gene profile of brain-derived neurotrophic factor (BDNF) [323]. Chronic stress through social defeat decreases levels of HDAC5, which is important in regulating behavioral response to emotional stimuli [324]. Ten days of chronic social defeat stress (introduction of an aggressive male mouse into the cage of the resident male mouse) in mice produced a significant increase in expression of Dnmt3a mRNA in the brain [325]. Another study in chronically-stressed mice through social defeat decreased levels of DNMT3b, which in turn increased levels of CRF; thus, potentiating the stress response. Taken together, these studies suggest a quintessential role not only for epigenetic regulation in the stress response, but also for chronic stress in epigenetic alterations.

1.14 The cell cycle and p53-mediated apoptosis and autophagy

Cell cycle and cell death control are essential in tumorigenesis. Tumor cells acquire the ability to activate the cell cycle while evading apoptosis [326]. During the cell cycle, there are several
checkpoints that must be bypassed to continue cell proliferation. A group of proteins known as cyclins, which expression levels vary throughout the cell cycle, form a complex with and activate cyclin dependent kinases (CDKs) which is necessary to pass through checkpoints. Of particular interest, cyclin D binds to CDK4/6 to continue through the G1/S checkpoint [327,328]. Cyclin D expression is induced through many signaling pathways including MAPK [329,330], NFκB [331,332], and JAK/STAT [333,334], by growth factors [335], estrogen [336], hypoxia [337], cytokines [333,334], and β-catenin [338]. During the G1/S checkpoint, retinoblastoma tumor suppressor protein (Rb) binds to E2F transcription factors which regulate S phase gene expression until Rb is phosphorylated by the cyclin D-CDK4/6 complexes which then allows cell cycle progression into S phase through the induction of cyclin E (which forms a complex with CDK2) and further phosphorylation and subsequent inactivation of Rb. p16 INK4A (and other INK4 CDK inhibitors like p15, and p18) inhibits CDK4/6, making p16 INK4A a tumor suppressor [326,339,340]. As cyclin D levels are directly correlated to external stimulation of the cell cycle, increases in cyclin D are a commonly used measurement of increased cellular proliferation.

DNA damage and replication mistakes are sensed by ataxia-telangiectasia (ATM), ataxia-telangiectasia and Rad3-related (ATR), CHK1, and CHK2. They in turn activate the tumor suppressor p53, the “guardian of the genome,” which then through its transcription factor function induces other tumor suppressors like p21Cip1 and p27Kip1 that inhibit a number of CDKs, thus causing cell cycle arrest and activating DNA repair mechanisms [341,342]. If repairs are successful, cell cycle will begin to progress; however, if not, apoptosis is induced [343]. p53 can also be activated in response to hyperactivation of oncogenes, hypoxia, and cellular stress.
Cellular levels of p53 are kept low due to mouse double minute 2 (MDM2) which is activated through the phosphoinositide 3-kinase kinase (PI3K) pathway. MDM2 binds to p53, which inhibits the transcriptional activation functions of p53 [344,345]. p53 is then transported from the nucleus where it performs the majority of its functions to the cytosol where MDM2 acts as ubiquitin ligase, marking p53 for degradation by the proteasome [346,347]. ATM and p14ARF (p19ARF in mice) can inactivate MDM2, but p53 can also induce MDM2 expression [348]. Upon activation, p53 can initiate production of pro-apoptotic members of the B-cell lymphoma gene 2 (BCL2) family such as p53 up-regulated modulator of apoptosis (PUMA), NOXA, BAX, BID, BIM, or BIK which then either block anti-apoptotic agents or open channels in the mitochondria to release cytochrome c and thus initiate the caspase cascade and apoptosis [349-351]. Cytosolic p53 can aid with the permeabilization of the mitochondrial membrane through non-transcription factor mechanisms such as activating BAX or binding to and inhibiting BCL2 or BCL-XL, as well. Anti-apoptotic BCL2 and BCL-XL are stimulated through PI3K signal and inhibit apoptosis via hindering the release of cytochrome c from the mitochondria [326,343,352,353].

Autophagy is a homeostatic process that allows a cell to digest its own damaged proteins and organelles [354]. Beclin 1, which is activated through JNK signaling, is a key regulator of autophagy and is required for the initiation and nucleation of the autophagosome in autophagy through activation of p53 [355]. Upon autophagy initiation, autophagosomes engulf the damaged proteins or organelles and fuse with lysosomes to form autophagolysosomes and complete degradation [356]. This process relies on autophagy related proteins (ATG). During this process, one such ATG, microtubule-associated protein-1 light chain 3 (LC3I) converts to LC3II, the
membrane-bound version of LC3I [357]. The ratio of LC3II to LC3I can be used as a measurement of autophagic initiation. p62 is an adaptor protein that recruits ubiquitinated proteins or organelles to LC3II to be taken into the autophagosome for degradation, and following an increase in autophagic flux, p62 is degraded [358]. A decrease in p62 protein levels is thus a marker of the completion of autophagy. While normal autophagic maintenance functions as a tumor suppressing mechanism, cellular stress-induced autophagy actually acts as a prosurvival pathway for tumor cells. p53 maintains autophagic homeostasis and can both induce and prevent autophagy. p53-mediated autophagy prevention is induced through accumulation of p53 in the cytosol [359]. Through up-regulation by nutrient stress, cytokines, rapamycin, or hypoxia, nuclear p53 can stimulate the production of autophagic-inducers damage-regulated autophagy modulator (DRAM) and Sestrin1 to promote cell survival [357,359-361]. Previous studies have found increased autophagy in adipose tissue of obese individuals, and this is proposed to be an attempt by the cells to inhibit obesity-induced inflammatory changes [362].

1.15 DNMT and HDAC inhibition for cancer treatment

Aberrant DNA methylation status is common in breast tumors [363], especially in relation to chemotherapy resistance. The overexpression of DNMT3b is associated with triple negative breast cancers, and treatment with DNMT inhibitors (DNMTi) increases sensitivity to cytotoxic chemotherapy [364] The DNMTi decitabine (an analog of 5-azacytidine) has been used in clinical trials to treat chronic myelogenous leukemia that are resistant to imantanib, but cytotoxic side effects limit its usage [365].
HDACs play a large role in the regulation of many cancer-related pathways including p53, p21, STAT3, Rb, and NFκB [366-369], and aberrant HDAC regulation is commonly found in tumor cells [370]. Like DNMTi, HDAC inhibitors (HDACi) are also in clinical use to treat cancer. Suberoylanilide hydroxamic acid (SAHA, also called vorinostat) was the first HDACi approved for usage in cancer treatment and is currently in clinical use to treat B-cell lymphoma [371,372]. Cell cycle arrest and apoptosis can be achieved by treatment with SAHA and other HDACi such as panobinostat [367,373,374]. However, treatment of cancer cells with such HDACi induces autophagy too and thus cancer cell survival, especially when apoptosis and caspase activity are inhibited [375]. When panobinostat is used in combination with an autophagy inhibitor chloroquine, tumor burden decreases and animal survival increases from 37 days postimplantation to 60 days in a xenograft model of MDA-MB-231 breast cancer cells [376]. These results are due to cellular stress that is induced by both panobinostat and chloroquine. Although cellular stress can activate both apoptosis and autophagy, chloroquine blocks the autophagy-induced cell survival, allowing for apoptosis to be dominate; consequently, decreasing tumor burden and increasing survival in these mice [375-378].

DNMTi in conjunction with HDACi have better outcomes than either alone and are used in clinical trials to sensitize tumors (breast, ovarian, cervical, lung, and more) to cytotoxic chemotherapy [379,380]. For example, a combined treatment with hydralazine (DNMTi) and valproic acid (HDACi) increases the sensitivity of HeLa cancer cells to chemotherapeutic agents gemcitabine, cisplatin and adriamycin. These results were more profound with both drugs versus either alone [381]. Another study treated hormone therapy-resistant breast cancer patients with
SAHA and tamoxifen and found a positive reversal of resistance [382]. The combination of the DNMTi decitabine and HDACi phenylbutyrate has been used to prevent tobacco carcinogen-induced lung tumors in mice [383].
1.16 Hypothesis and Aims

Social isolation is a potent psychosocial stressor and is associated with obesity and increased breast cancer risk and mortality. In this thesis, I investigated the combined effects of social isolation stress and obesity on insulin resistance, mammary tumorigenesis, and the mechanisms involved.

The research presented in this thesis is based on the following hypothesis: *Social isolation in combination with an obesity-inducing diet (OID) increases mammary cancer.*

In order to test this hypothesis, the following specific aims were established:

**Aim 1.** Investigate whether social isolation stress and an OID, alone or in combination, increase NPY levels, and induce central obesity and insulin resistance, and increase breast cancer risk in female C57BL/6 mice (*Chapter 2*).

**Aim 2.** Determine if the increase in mammary cancer tumorigenesis in socially-isolated, OID-fed mice is due to epigenetic changes (*Chapter 3*).
Chapter 2

Social isolation in combination with an obesity-inducing diet increases mammary cancer in female c57bl/6 mice through altered p53 signaling

2.1 Abstract

Social isolation is a potent psychosocial stressor and is associated with obesity and increased breast cancer risk and mortality. In a pilot study, we found that in female C57BL/6 mice, two weeks of social isolation stress (one mouse per cage) was a sufficient enough stressor to not only increase neuropeptide Y (NPY) levels in serum (p= 0.0475), but also significantly increase breast cancer risk via an increase in the number of terminal end buds (p= 0.0399) when compared to group-housed (5 mice per cage) mice. Therefore, we conducted an extended study to investigate if the effects of social isolation on mammary cancer are potentiated in C57BL/6 mice fed obesity-inducing diet (OID) and the mechanisms involved. Social isolation, with or without OID, increased food intake and body weight, and caused impaired insulin and glucose tolerance. Mammary tumorigenesis, initiated by 7,12-dimethylbenz[a]antracene, was significantly higher in the socially isolated OID fed mice than in the other three groups (p< 0.001). Serum leptin levels were increased by OID, but not by stress, whilst Ppara mRNA expression in the mammary gland was reduced (p= 0.036) and serum NPY levels (p= 0.013) were increased only in socially isolated OID fed mice. Cell proliferation in mammary glands, measured by Ki67, was significantly increased in socially isolated mice (p= 0.002), but apoptosis, measured by Tunel assay, was not altered. Social isolation, but not OID, significantly increased Tp53 (p< 0.001),
Mdm2 (p = 0.001) and Ccdn1 (p < 0.001) mRNA expression in the mammary gland as well as Jnk1 (p = 0.003) and Dram1 (p = 0.016) mRNA that are linked to p53-induced autophagy. Protein levels of Beclin1 were increased by obesity (p = 0.003) and p62 levels were down-regulated by social isolation stress (p = 0.003), whilst LC3II was increased only in socially isolated control diet fed mice (p = 0.052). These results confirm that mammary cells in stressed mice exhibited elevated levels of autophagy. In summary, we found that obesity potentiates the effects of social isolation on mammary carcinogenesis, and social isolation increased p53 expression and induced autophagy (perhaps in conjunction with OID) but not apoptosis.

2.2 Pilot Study

2.2.1 Materials and Methods

Animals

Female C57BL/6 mice were utilized in this study. The mice were housed in a temperature- and humidity-controlled room and were under a 12 hour light-dark cycle. All animal procedures were approved by the Georgetown University Animal Care and Use Committee and followed the National Institute of Health guidelines for the proper and humane use of animals in biomedical research.

Stress exposure

Female C57BL/6 mice were socially-isolated (1 mouse per cage) or stayed in group-housing (5 mice per cage) at 6 weeks of age for 2 weeks (n= 9-11 mice per group). These mice were fed only control diet.
**Tissue collection**

At 8 weeks of age, the mice were anesthetized using methoxyflurane inhalant. Blood was collected via cardiac puncture, placed in serum separator tubes, and then centrifuged at 1,000 x g for 10 minutes at room temperature. The serum was stored at -80°C until use. The mice were then sacrificed by carbon dioxide asphyxiation. Mammary glands were removed and placed in a fixative solution for whole mount preparation.

**Whole mount preparation**

After being stretched onto a slide, the mammary glands were placed in a fixative solution which contained 25% glacial acetic acid (EM Science, Gibbstown, NJ) and 75% methanol (Fisher Scientific Co.) and kept in this solution for no less than one week. Mammary glands were then washed in 70% ethanol (Fisher Scientific Co.) for 2 hours, rinsed in distilled water, and stained in carmine aluminum solution overnight. The carmine aluminum solution was made by mixing 1 gram of carmine (Sigma Chemical Co.) and 2.5 grams aluminum potassium sulfate (Sigma Chemical Co.) in 500 ml distilled water and boiling for 20 minutes. The following day, mammary glands were washed in 70%, 95% and 100% ethanol solutions (Fisher Scientific Co.) for 2 hour each, and then placed in xylene (Fisher Scientific Co.) overnight. Finally, the mammary glands were covered with Permount (Fisher Scientific Co.) and cover-slipped. All procedures were conducted at room temperature.
**Terminal end buds and mammary gland growth length**

The whole mounts of the mammary glands were examined under an Olympus bright field microscope, and the number of terminal end buds (TEBs) were counted per gland (n= 9-11 per group). To assess growth of the epithelial tree, the distance from the center of the lymph node to the TEBs was measured using a ruler (n= 9-10 per group).

**NPY Enzyme immunoassay (EIA)**

C18 Sep-column and buffers kit (Peninsula Laboratories, S-5000) were used to perform the extraction on the mouse serum samples in preparation for the NPY (Peninsula Laboratories S-1145) EIA by freeze-drying the eluent in a methanol/dry ice bath, and then evaporating it with a centrifugal concentrator. Both the extraction and EIA for NPY were completed according to kit instructions (n= 3-4 per group).

**Statistical Analysis**

The student’s t-test was performed on all data to determine differences. All statistical analysis was carried out using SPSS SigmaStat software, and differences were considered significant if P was less than 0.05. All probabilities are two-tailed. Data are expressed as mean ± SEM (standard error of mean).
2.2.2 Results and Discussion

Confirmation of stress induction by social isolation:

To confirm that social isolation of female C57BL/6 mice for 2 weeks is sufficient to induce a stress response, serum levels of NPY were measured by EIA. NPY was increased in the socially-stressed mice versus the group-housed control (p= 0.0475; Figure 2.1).

Assessing mammary cancer risk attributable to social isolation stress

As the number of TEBs are directly associated with mammary cancer risk in mice [88,89], TEBs were counted in the mammary glands of mice that were socially-isolated for 2 weeks and compared to their group-housed counterparts. The number of TEBs was significantly higher in the stressed mice versus the control mice (p= 0.0399; Figure 2.2), indicating that social isolation stress increases mammary cancer risk in female mice. The distance from the center of the lymph node to the TEBs was also measured, and no difference was found between the group-housed when compared to the socially-isolated mice (p= 0.118; Figure 2.3).

Discussion

These findings align with the current body of literature that social isolation stress not only elicits a potent stress response through an increase in NPY signalling [254], but also significantly increases breast cancer risk in mice [268,271]. Therefore, we conducted an extended study to investigate if the effects of social isolation on mammary cancer are potentiated in C57BL/6 mice fed obesity-inducing diet (OID) and the mechanisms involved, which is detailed in the later part of this chapter.
Figure 2.1: Serum NPY levels by EIA. After two weeks of social isolation, serum neuropeptide Y (NPY) levels were measured by EIA. NPY was increased in the socially-stressed mice versus the group-housed control p= 0.0475, indicating that 2 weeks of social isolation stress is sufficient to induce NPY expression. n= 3-4 per group. Data is expressed as mean ± SEM. The student’s t-test was performed to analyze differences, and differences are considered significant when p< 0.05. Significance is denoted by *. 
Figure 2.2: Number of terminal end buds (TEBs) in mammary glands. Mammary cancer risk due to social isolation stress was assessed by counting the number of TEBs present in the mammary glands. Quantication of TEBs (A), and photos of representative mammary glands (3x) from the group-housed (B) and socially-isolated (C) mice are shown. The number of TEBs was significantly higher in the stressed mice versus the control mice p= 0.0399, indicating that social isolation stress increases mammary cancer risk in female mice. n= 9-11 per group. Data is expressed as mean ± SEM. The student’s t-test was performed to analyze differences, and differences are considered significant when p <0.05. Significance is denoted by *.
**Figure 2.3: Distance from lymph node to TEBs in mammary glands.** Mammary cancer risk due to social isolation stress was assessed via distance from lymph node to TEBs in mammary glands. Measurement of distance was performed using a ruler. No significant difference was observed. n= 9-10 per group. Data is expressed as mean ± SEM. The student’s t-test was performed to analyze differences, and differences are considered significant when p< 0.05.
2.3 Extended Study

2.3.1 Materials and Methods

*Animals*

Female C57BL/6 mice were utilized in this study. The mice were housed in a temperature- and humidity-controlled room and were under a 12 hour light-dark cycle. All animal procedures were approved by the Georgetown University Animal Care and Use Committee and followed the National Institute of Health guidelines for the proper and humane use of animals in biomedical research.

*Dietary and stress exposure*

At weaning (postnatal day 21), the mice were either group-housed (5 mice/cage) or introduced to social isolation stress by housing them singly. In addition, mice were fed either an AIN93G control based diet (Harlan Teklad, TD.09029; Table 2.1) or an obesity-inducing high fat diet (OID, Harlan Teklad, TD.09030; Table 2.2). 17.2% of the calories in the control diet were from fat (2% lard), resulting in a caloric content of 3.8 kcal/g. The OID contained 58.8% calories from fat (30% lard), with a caloric content of 5.4 kcal/g. This resulted in four groups: group-housed mice consuming control diet (G-C), group-housed mice consuming OID (G-OID), socially isolated mice consuming control diet (I-C), and socially isolated mice consuming OID (I-OID).
Table 2.1: Composition of control diet.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/Kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200.0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3.0</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>357.486</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>132.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>50.0</td>
</tr>
<tr>
<td>Lard</td>
<td>20.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral Mix, AIN-93G-MX (94046)</td>
<td>36.0</td>
</tr>
<tr>
<td>Vitamin Mix, AIN-93-VX (94047)</td>
<td>10.0</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
</tr>
<tr>
<td>TBHQ, antioxidant</td>
<td>0.014</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>% by weight</th>
<th>% kcal from</th>
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</thead>
<tbody>
<tr>
<td>Protein</td>
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<td>18.8</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>60.1</td>
<td>63.9</td>
</tr>
<tr>
<td>Fat</td>
<td>7.2</td>
<td>17.2</td>
</tr>
</tbody>
</table>

Kcal/g 3.8

1) Values are calculated from ingredient analysis or manufacturer data.
Table 2.2: Composition of high fat/high sugar obesity-inducing diet (OID).

<table>
<thead>
<tr>
<th>Formula</th>
<th>g/Kg</th>
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<tbody>
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<td>Casein</td>
<td>288.0</td>
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<tr>
<td>L-Cystine</td>
<td>2.0</td>
</tr>
<tr>
<td>Sucrose</td>
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</tr>
<tr>
<td>Maltodextrin</td>
<td>150.55</td>
</tr>
<tr>
<td>Lard</td>
<td>300.0</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral Mix, AIN-93G-MX (54046)</td>
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</tr>
<tr>
<td>Vitamin Mix, AIN-93-VX (54047)</td>
<td>14.2</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>3.85</td>
</tr>
</tbody>
</table>

Selected Nutrient Information:

<table>
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<tr>
<th>Nutrient</th>
<th>% by weight</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
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<td>18.6</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>31.0</td>
<td>22.8</td>
</tr>
<tr>
<td>Fat</td>
<td>35.3</td>
<td>58.6</td>
</tr>
</tbody>
</table>

Kcal/g 5.4

*Values are calculated from ingredient analysis or manufacturer data*
Figure 2.4: Study design.

Social Isolation Stress and High Fat Diet DMBA MPA Insulin/Glucose Tolerance Testing Sacrifice, Tissue/Blood Collection

21 days/weaning Wk Wk Wk Wk Wk Wk 12/13 Wk

6 7 8 9 10

Monitor mammary tumorigenesis
**Mammary tumor induction**

Mice were treated with a single subcutaneous dose of 15 mg of medroxyprogesterone acetate (MPA, Pfizer) on postnatal week 6 and then exposed to 1 mg of 7,12-dimethylbenz[a]anthracene (DMBA, Sigma) on weeks 7, 8, 9, and 10 by oral gavage to induce mammary tumors. Study design is illustrated in Figure 2.4.

**Weight gain and food consumption**

Body weight was measured weekly from weaning until sacrifice. Before the induction of mammary tumors by MPA-DMBA, food was weighed in a subgroup of mice (n= 5 mice/group) every 2-3 days for the first 3 weeks of the dietary and stress exposures. Caloric intake was calculated using the caloric content of the diets.

**Insulin and glucose tolerance testing**

When mice were 12-13 weeks of age, we assessed parameters linked to insulin resistance in 4-10 mice per group. After a 6 hour fast, un-anaesthetized mice were injected intra-peritoneally with 0.75 U/kg of body weight of recombinant human insulin (Sigma) or 2g/kg of 20% glucose in molecular grade water. Blood samples via tail vein puncture were taken at baseline (0 min) and 30, 60, 90 and 120 minutes after the insulin/glucose challenge. Blood glucose levels were measured using a FreeStyle portable glucose meter (TheraSense). All insulin and glucose tolerance testing was performed by me with the assistance of other members of the Hilakivi-Clarke lab or Animal Shared Resources.
**Mammary tumorigenesis**

Starting one week after the last DMBA administration, the mice were palpated and mammary tumors were measured weekly with calipers. When the tumor burden exceeded 10% of the weight of the mice, they were sacrificed before the end of the monitoring period. Otherwise, mice were monitored for 13 weeks after receiving the last DMBA dose and sacrificed at 23 weeks of age. Tumor incidence, latency, multiplicity, size and growth were recorded. Numbers of mice per group were the following: G-C: 21 G-OID: 19 I-C: 17 I-OID: 15.

**Tissue collection**

At sacrifice, blood was obtained via cardiac puncture. Serum was separated, frozen, and kept at -20°C until assayed. Mammary glands and tumors were collected at sacrifice. Abdominal adipose tissue, pancreas, liver, muscle, ovaries, fallopian tubes and uterus, and brain including the hypothalamus were also collected for future studies. Tissues were either fixed in 10% buffered formalin, embedded into paraffin blocks, and sectioned (5µm), or flash frozen in liquid nitrogen and stored at -80°C until assayed.

**Enzyme Immunoassay (EIA) and Procarta Immunoassay Multiplex**

Adipocetin (Millipore, EZMADP-60K) (n= 4-6/group) and Leptin (Millipore EZML-82K) (n= 7/group) EIA were performed according to manufacturer’s protocol. C18 Sep-column and buffers kit (Peninsula Laboratories, S-5000) were used to perform the extraction on the mouse serum samples in preparation for the NPY (Peninsula Laboratories, S-1145) EIA by freeze-drying the eluent in a methanol/dry ice bath, and then evaporating it with a centrifugal
concentrator. Both the extraction and EIA for NPY were completed according to kit instructions (n= 7/group). Serum levels of the cytokines (n=9-10/group) GM-CSF, IL-1β, IL-6, LIF, MCP-1, MIP-1α, RANKL, and TNF-α were measured by Procarta Immunoassay Multiplex kit (Affymetrix, PC3009M) using metallic beads according to kit instructions.

**Histopathology**

Tumor sections were stained with hematoxylin and eosin to determine histopathology, and classification was assessed by a trained pathologist.

**Immunohistochemistry**

*Immunohistochemistry (IHC) for ERα, progesterone receptor (PgR), and CD68:* Mammary tumor tissue sections (n=4-6/group for ERα; n=9-17/group for PgR; n= 6-7 for CD68) were deparaffinized with xylene and rehydrated through a series of descending graded ethanols. Antigen retrieval was achieved by microwaving in target retrieval solution citrate buffer, pH 6.0 (Dako, S2369) for 15 minutes with 20 minutes of cooling. Blocking was performed using 3% H$_2$O$_2$ in phosphate buffered saline (PBS) for 20 minutes. Tissue sections were incubated overnight at 4°C in primary antibodies ERα (Santa Cruz, H-184) at a dilution of 1:600, PgR (Dako, A0098) at a dilution of 1:400, and CD68 (ABD Serotec, MCA1957GA) at a dilution of 1:100 in 3% bovine serum album (BSA) in tris-buffered saline with tween 20 (TBST). A corresponding mouse primary antibody to IgG (Dako, X0931) was used for negative controls. Mouse uterus sections were stained concurrently as a positive control. The LSAB+ System-HRP, DAB+ (Dako, K0679) kit was used to develop the sections as instructed by the manufacturer,
and they were counterstained with Harris Hematoxylin (Fisher Scientific). All washes were performed using TBST. The sections then went through ascending graded ethanol and xylene and mounted using Permount (Fisher Scientific). Quantification for these IHC was performed by scoring intensity and percentage of positive cells modified from Allred et al.[384].

*Tunel assay in mammary glands:* Mammary gland tissue sections (n= 4-7/group) were deparaffinized with xylene and rehydrated through a series of descending graded ethanols. Tissues were then pretreated with 20 µg/ml proteinase K in PBS for 15 minutes, and then blocked in 3% H2O2 in PBS for 5 minutes. The ApopTag® Plus Peroxidase In Situ Apoptosis Kit (Millipore, S7101) was then followed according to manufacturer’s protocol. Positive controls were included in the kit (Millipore, 90422). Negative controls were not treated with TdT enzyme (reaction buffer only). All washes were performed using TBST. They were counterstained with 0.5% methyl green in 0.1 M sodium acetate, pH 4.0. Finally, these sections were washed in 3 changes of n-butanol for 30 seconds each, dehydrated in xylene, and mounted with Permount. Scoring in the mammary glands was based the percentage of cells that stained positive in the glands, with an average of six frames (a frame is the field of view at 40x which can include multiple glands) per slide assessed.

*Immunohistochemistry for Ki67 in mammary glands:* Antigen retrieval was achieved by using Tris/EDTA pH 9 for 20 minutes at 100°C followed by 20 minutes of cooling. The mammary gland sections (n= 4-7/group) were incubated in Ki67 primary antibody (Novus Biologicals, NB600-1252) at a dilution of 1:40 for 1 hour. They were then incubated in HRP-rabbit secondary
(Dako, K4003) for 30 minutes, followed by DAB and counterstaining with hematoxylin. Instead of using an antibody, the protein buffer alone was used as a negative control. Scoring was based on percent positive staining of the overall tissue. Staining was performed by the Histopathology and Tissue Shared Resources, but we assessed the scoring.

Photographs (40x) and score assessment for ERα, PgR, p53, tunel, and Ki67 were all performed using brightfield microscopy on an Olympus BX61 with Image-Pro Plus (Media Cybernetics) software.

**cDNA synthesis and qRT-PCR**

Complementary DNA synthesis and quantitative real-time PCR analysis: 200 ng of total RNA per sample was used as a template for random primed cDNA synthesis with a recombinant Moloney murine leukaemia virus reverse transcriptase via RNase Inhibitor High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, 4374967), according to manufacturer's instructions. An RT enzyme-minus control reaction was also included. The cDNA samples were then used as templates for quantitative real-time PCR analysis with specific primers for the target gene using EvaGreen 2X qPCR MasterMix-ROX (Applied Biological Materials Inc.) and an ABI Prism 7900 Sequence Detection System. The primer sequences are shown in Table 2.3. RNA for these assays was from 4-7 mice per group. Each sample was run in triplicate. Absolute gene expression levels were determined using SDS2.3 software (Applied Biosystems) and the standard curve method. Concentration of each sample was normalized the mRNA levels of the reference gene *Hprt1*.
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccnd1</td>
<td>CCTCCATAAAGTCTGTGTCCC</td>
<td>AGGGTACTTTGGTCTGTTTTCC</td>
</tr>
<tr>
<td>Dram1</td>
<td>CATCGGAATGGGCATCGTAG</td>
<td>GGGACAGGATTTGTAGGAGATG</td>
</tr>
<tr>
<td>Hprt1</td>
<td>CCTCATGGACTGATTATGGACAG</td>
<td>TCAGCAAAGAAACTTATAGCCCC</td>
</tr>
<tr>
<td>Il-1β</td>
<td>ATGGGCAACCACCTACCTATTT</td>
<td>GTTCTAGAGAGTGCTGCTAATG</td>
</tr>
<tr>
<td>Il-6</td>
<td>CTCCATCCAGTTGCTTTCT</td>
<td>CTCCGACTTGTAAGGTGATAG</td>
</tr>
<tr>
<td>Jnk1</td>
<td>TCCAGCACCACATACATCAAC</td>
<td>CTCTCATCTAACTGCTTGTCCG</td>
</tr>
<tr>
<td>Mdm2</td>
<td>GCGTGGAATTTGAAGTGGAGTC</td>
<td>CTGTATCGCTTTCTCCTGTCTG</td>
</tr>
<tr>
<td>Npy1R</td>
<td>GAATGACAATGAAAAAGTCTGAAGC</td>
<td>GGCTGTGCTTTTTTCCCCAC</td>
</tr>
<tr>
<td>Pparγ</td>
<td>AGAAGTGCCCCCTGTGG</td>
<td>CAACAGCTTCTCCTCTCAG</td>
</tr>
<tr>
<td>Tp53</td>
<td>ATTGGGACCACCTGGCTGTAG</td>
<td>CGAGGCTGATATCCGACTGTGA</td>
</tr>
<tr>
<td>Tnf-α</td>
<td>CTACCTTGCTGCCTCCTCTTT</td>
<td>GAGCAGAGGTTCCAGTGATGTA</td>
</tr>
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Table 2.3: Forward and reverse primer sequences used during qRT-PCR.
**Western blot analysis**

In a separate experiment, changes in autophagy-related proteins Beclin 1, LC3II and p62 were investigated in the mammary glands of mice that were not exposed to DMBA. In this study, 40 mice were divided into the same four groups as above (n= 10 per group) upon weaning: group-housed mice consuming control diet (G-C), group-housed mice consuming OID (G-OID), socially isolated mice consuming control diet (I-C), and socially isolated mice consuming OID (I-OID). Four weeks later, all mice were sacrificed and their mammary glands were harvested, snap frozen and stored in -80 C until used for Western blot assays.

Snap frozen tissues (n= 4-6 for each group) were ground and lysed in RIPA lysis buffer with protease inhibitor pellet (Roche Diagnostics, Indianapolis, IN) and 10mM glycocerophosphate (Sigma-Aldrich, St. Louis, MO), 1mM sodium orthvandate (Sigma-Aldrich), 5mM pyrophosphate (Sigma-Aldrich), and 1mM PMSF (Sigma-Aldrich). The following primary antibodies were used in a 1:1000 dilution: anti-LC3II and Beclin 1 antibodies were from Cell Signaling Technology (Danvers, MA), anti-β-actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-p62 antibody was obtained from BD Biosciences (San Jose, CA). Western blots were developed using chemiluminescent substrate (Denville Scientific, Metuchen, NJ) and quantified with Quantity-One software (Bio-Rad, Hercules, CA). For LC3II, the lower band of LC3II intensity was normalized to the upper band of LC3I. For Beclin 1 and p62, band intensity was normalized to β-actin.
Statistical Analysis

2-way ANOVA was used to determine significance between groups with diet (control or OID) and stress (group or isolated housing) as variables. An interaction between the two variables which is denoted as p(interaction) throughout this dissertation, indicates that the effect of stress is different with or without OID (or OID acts differently in the presence of stress). Pairwise Multiple Comparison Procedures (Holm-Sidak method) was performed posthoc when significant differences were found and further elucidation between which groups the significance was occurring was necessary. Differences between groups are illustrated by marking bars that differ from each other with different letters (a/b/c) which is a commonly used method and also used by us in previous publications to show differences between multiple groups [385,386]. This method is utilized in order to keep the graphs from becoming overcrowded with crossing lines and consequently uneasy to read. When letters are different between bars, for example one is “a” and the other is “b” (or “c” in some cases), then the groups are different from one another. However, if both bars have an “a” above them, then there is no significant difference between these groups. Repeated Measures ANOVA was performed on the percent change in body weight data from week 1 to 6. The Kaplan-Meier survival curve and subsequently the log-rank test were used to compare the differences in tumor incidence. All statistical analysis was carried out using SPSS SigmaStat software, and differences were considered significant if P was less than 0.05. All probabilities are two-tailed. Data are expressed as mean ± SEM (standard error of mean).
2.3.2 Results

*Caloric intake, and body weight gain:*

As OID is more energy dense than the control diet, mice on the control diet ate more grams of food than those on the OID (p< 0.001). The mice that were housed in social isolation also ate more grams of food than group-housed mice (p< 0.001; Figure 2.5A), and consequently, socially-stressed mice consumed more kcal/day than group-housed mice (p< 0.001). Further, regardless of consuming less food, the mice on the OID consumed more kcal/day than control diet fed mice (p< 0.001; Figure 2.5B). Mice that were socially-isolated and fed OID gained the most weight (determined during weeks 1-6 from starting different diets; group x weeks 1-6: p< 0.001; Figure 2.5C). At the end of the study, both social isolation (p< 0.001) and OID (p= 0.045) led to a significantly increased body weight in mice (Figure 2.5D). These findings support other studies that have shown that social isolation induces body weight gain through an increase in appetite [280].
Figure 2.5: Food consumption, caloric intake, and body weight gain. Food consumption (A), caloric intake (B), and body weight gain (C) were measured in female C57BL/6 mice that had been fed either a control or obesity-inducing diet (OID) and either group-housed (5 mice/cage) or socially-isolated (1 mouse/cage). The final percent body weight gain (D) is also shown. Statistical analysis was performed using 2-way ANOVA for A, B, and D. Repeated Measures ANOVA of weeks 1-6 was utilized in C. (A) Food consumption (g) in a sub-group of mice (n=5/group) was measured by weighing the amount of food in the cages every 2-3 days over the first three weeks of dietary and stress exposures. Mice that were on OID ate less than the ones on control diet, and socially-isolated mice ate more than those that were group-housed. OID: p<
(B) Caloric intake (kcal) was calculated based upon the amount of food consumed by these mice and the caloric content of the food. The isolated, OID-fed mice had the highest caloric intake. OID: p< 0.001; stress: p< 0.001. (C) Mice were weighed weekly from weaning until sacrifice, and percent body weight gain over time was calculated. Mice that were socially-isolated and fed OID gained the most weight as determined during weeks 1-6 from starting different diets; group x weeks 1-6: p< 0.001 (n= 14-21 mice/group). (D) Mice that were socially-isolated and fed the OID had the highest percent weight gain at the end of the study. OID: p = 0.045; stress: p< 0.001. Error bars are shown as standard error of mean (SEM). Different letters denote significant differences. For example, in panel (A), I-C is labeled as “a,” and I-OID is labeled as “b,” indicating that there is a significant difference between them. However, G-C and G-OID both have the letter “c,” indicating that these groups are not different. Differences are considered significant when p< 0.05. G-C = group-housed control mice, G-OID = group housed obese mice, I-C = isolated and control diet fed mice, and I-OID = isolated and obese mice.
**Insulin tolerance**
When assessed at different time-points, there were significant differences among the groups at 60 minutes and 120 minutes. At both time points, obese mice had higher glucose levels than mice fed the control diet (p= 0.01 and p= 0.031, respectively). In addition, at 60 minutes, socially-isolated mice exhibited higher glucose levels (p= 0.044). Post hoc analysis indicated that compared with group-housed control mice, the increase was significant only in the socially-isolated obese mice (60 minutes: p= 0.009 and 120 minutes: p= 0.036; Figure 2.6A). Similarly, area under the curve (AUC) in the insulin tolerance test was significantly increased in the obese mice (p= 0.021), but post-hoc individual comparisons among the groups indicated that only socially-isolated obese mice had higher AUC than the group-housed control mice (p= 0.051); Figure 2.6B), demonstrating that stress potentiates the effects of obesity in impairing insulin sensitivity.

**Glucose tolerance**
In the glucose tolerance testing, socially-isolated mice fed the control diet exhibited elevated baseline glucose levels (p(interaction)= 0.013). Obese mice had lower baseline glucose levels than mice fed the control diet (p= 0.044). At 60 min time-point, the obese mice exhibited higher glucose levels than the control diet fed mice (p= 0.015), but the highest levels were seen in the socially-isolated mice that received control diet (p(interaction)= 0.021). This group also exhibited highest glucose levels at 90 min (p(interaction)= 0.016), and 120 min (p(interaction)= 0.038; Figure 2.6C). Reflecting these differences, AUC was significantly higher in the group-housed obese mice (p= 0.018) and socially-isolated control diet fed mice (p= 0.015) than in the
control mice (Figure 2.6D), but not in socially-isolated obese mice. These findings suggest that obesity and social isolation both impair glucose sensitivity, but in combination they do not.
Figure 2.6: **Insulin and glucose tolerance testing.** Insulin tolerance (A) and glucose tolerance (C) testing was performed on a sub-group of mice (n= 4-10/group) at 12-13 weeks of age by fasting these mice for 6 hours, and then instigating the insulin or glucose challenge by intraperitoneal injection of 0.75 U/kg of body weight of recombinant human insulin or 2g/kg of 20% glucose in molecular grade water and monitoring blood glucose levels at baseline (0 mins, before injection) and 30, 60, 90, and 120 mins after injection. (A) OID: p= 0.01 at 60 min, and p= 0.031 at 120 min. (C) OID: p= 0.044 at baseline, p= 0.015 at 60 min; interaction: p= 0.013 at baseline, p= 0.021 at 60 min, p= 0.016 at 90 min, and p= 0.038 at 120 min. Area under the curve analysis (AUC) for the insulin tolerance test (B) OID: p= 0.021 and glucose tolerance test (D)
are presented as well (D) p(interaction) = 0.013. These findings suggest that stress potentiates the effects of obesity in impairing insulin sensitivity, and that obesity and social isolation both impair glucose sensitivity, but in combination they do not (thus the interaction). Different letters denote significant differences. For example in panel (B), I-C is labeled as “a,b” and I-OID is labeled as “a,” indicating that there is no significant difference between them. However, G-C and I-OID have different letters “a” and “b,” indicating that these groups are significantly different from each other. Differences are considered significant when p< 0.05. Error bars are shown as standard error of mean (SEM). Statistical analysis was performed using 2-way ANOVA and posthoc analysis when necessary. G-C = group-housed control mice, G-OID = group housed obese mice, I-C = isolated and control diet fed mice, and I-OID = isolated and obese mice.
NPY and adipokine levels in serum

Serum levels of NPY, leptin and adiponectin were measured via EIA. NPY levels were significantly increased in mice fed OID and kept in social isolation (p(interaction)= 0.013), but not in group-housed OID fed mice or socially-stressed mice fed the control diet (Figure 2.7A). In contrast, leptin levels were significantly increased by OID (p< 0.001), but not by stress (Figure 2.7B). Adiponectin levels were lowered both by OID (p= 0.011) and social isolation stress (p= 0.0422; Figure 2.7C). These findings show that NPY levels are increased by stress but only in obese mice and leptin levels are elevated only by obesity, whereas adiponectin levels are reduced both by social isolation stress and obesity.

Expression of NPY and adiponectin linked genes in the mammary gland

To determine if certain signaling targets of NPY and adiponectin were affected by social isolation stress, mRNA expression of Ppar-γ, NPY receptor Y1R, Il1β, Il6 and Tnfa were measured in the mammary gland. Both social isolation stress and obesity decreased mRNA levels of Ppar-γ (p= 0.036 and p= 0.010, respectively; Figure 2.7D), consistent with an impairment in insulin sensitivity in these mice and decrease in serum adiponectin level. No significant differences were observed in obese or stressed mice for Y1R (Figure 2.8A), Il1β (Figure 2.8B), Il6 (Figure 2.8C), or Tnfa (Figure 2.8D). Y2R and Y5R were measured, but the primers did not work.
Figure 2.7: Serum NPY and adipokines by EIA and Pparγ in mammary glands. At the end of tumor monitoring period, serum extraction and then EIA was performed to determine levels of NPY (n= 7/group); interaction: p= 0.013, indicating that NPY is only increased when there is social isolation in combination with OID (A), leptin (n= 4-6/group); OID: p< 0.001, indicating that only the OID increases leptin (B) and adiponectin; OID: p= 0.011, and stress: p= 0.042 (n= 7/group), indicating that both social isolation stress and OID down-regulate adiponectin (C). mRNA expression in the mammary glands of Pparγ (n= 5-7/group); OID: p= 0.010, and stress: p= 0.036. (D) were measured by qRT-PCR relative to Hrtp1 by using the standard curve method, and we found that Pparγ is decreased by both the social isolation stress and diet. Different letters
denote significant differences. For example in panel (A), I-C is labeled as “b” and I-OID is labeled as “a,” indicating that there is a significant difference between them. However, G-OID and I-OID have the same letters “a” and “a,” indicating that these groups are not significantly different from each other. Differences are considered significant when p< 0.05. Error bars are shown as standard error of mean (SEM). Statistical analysis was performed using 2-way ANOVA and posthoc analysis when necessary. G-C = group-housed control mice, G-OID = group housed obese mice, I-C = isolated and control diet fed mice, and I-OID = isolated and obese mice.
**Figure 2.8:** NPY and adiponectin linked genes in mammary gland. At the end of tumor monitoring period, mRNA expression levels of *NpyIR* (n= 3-7/group) (A), *IL-1β* (n= 3-7/group) (B), *IL-6* (n= 5-7/group) (C), and *Tnf-α* (n= 3-5/group) (D) were measured by qRT-PCR relative to *Hrpt1* mRNA expression by using the standard curve method from the mammary glands of the mice. No differences were observed. Error bars are shown as standard error of mean (SEM). Statistical analysis was performed using 2-way ANOVA, and posthoc analysis was not performed as no initial differences were observed by 2-way ANOVA. G-C = group-housed
control, G-OID = group-housed obese, I-C = isolated and control diet fed, and I-OID = isolated and obese.
**Mammary tumorigenesis**

The socially-isolated, OID-fed mice exhibited a significant increase in carcinogen-induced mammary tumor incidence when compared to the three other groups (p< 0.001; Figure 2.9A). Social isolation in the control diet fed mice caused increased tumor incidence at a similar rate as did OID in the group-housed mice, but due to a lower number of mice in the first than second group, the difference reached statistical significance only between the group-housed obese and control diet fed mice (p= 0.048). Additionally, tumor latency was significantly shortened by social isolation stress (p= 0.047) but not by OID (Figure 2.9B). No differences were seen in tumor multiplicity (Figure 2.9C). Despite a trend showing that social isolation stress and OID increased tumor burden, it was not significantly different among the groups due to a large within group variation (Figure 2.9D). Taken together, these data demonstrate that social isolation stress and obesity both increase carcinogen-induced mammary cancer risk in female mice, and show that there is an additive effect between the two.

**Histopathology, ERα and PgR characterization of mammary tumors**

The mammary tumors were examined histopathologically by a pathologist, and all of them were found to be malignant. Adenocarcinomas, adeno-squamous carcinomas, and adenomyoepitheliomas were identified at the same proportion in the four groups. Further, histopathological grades of the tumors also were similar across the groups (Table 2.4). All tumors were ERα and PgR positive, and no differences among the groups were seen in the expression of the two receptors (Figure 2.10).
Figure 2.9: Mammary tumorigenesis. Mammary tumors were induced by using the MPA-DMBA method. Mice were palpated and measured for tumors by calipers weekly after the final week of DMBA treatment until sacrifice. Tumor incidence, or the percent of mice that get tumors; OID+stress vs all other groups: p< 0.001 by log-rank test, indicating that the I-OID have a significantly higher tumor incidence than the other groups (A), tumor latency, or the time until the first measurable tumor appears is increased by social isolation; stress: p<0.048 (B), tumor multiplicity, the number of tumors per mouse (C), and tumor burden, the total area of the tumors per mouse, (D) were analyzed (n= 15-21/group), but no differences were observed. Error bars are shown as standard error of mean (SEM). For A, statistical analysis was performed by using the
Kaplan-Meier survival curve and log-rank test. For B-D, statistical analysis was performed using 2-way ANOVA, and only in (B) were posthoc tests performed. In (B), different letters denote significant differences. For example G-OID is labeled as “a” and I-OID is labeled as “b,” indicating that there is a significant difference between them. However, G-OID and G-C have the same letters “a” and “a,” indicating that these groups are not significantly different from each other. Differences are considered significant when p< 0.05. G-C = group-housed control mice, G-OID = group housed obese mice, I-C = isolated and control diet fed mice, and I-OID = isolated and obese mice.
Table 2.4: Tumor histopathology. Tumor histopathology was performed on H&E stained slides of collected tumors (n= 11-21/group) by a trained pathologist. No significant differences were observed between groups in the type of tumor or tumor grade. Differences are considered significant when p< 0.05. Statistical analysis was performed using 2-way ANOVA. G-C = group-housed control, G-OID = group-housed obese, I-C = isolated and control diet fed, and I-OID = isolated and obese.
**Figure 2.10: ERα and PgR staining in mammary tumors.** Immunohistochemistry and scoring was performed in our lab on mammary glands and tumors, and photographs were taken by brightfield on an Olympus BX61 at 40x. ERα (n= 4-6/group) (A) and PgR (n= 9-17/group) (B) expression was quantified by scoring intensity and percentage of positive cells. Error bars are shown as standard error of mean (SEM). Statistical analysis was performed using 2-way ANOVA. No differences were found for either receptor between the groups; therefore, posthoc analysis was not necessary. G-C = group-housed control, G-OID = group housed obese, I-C = isolated and control diet fed, and I-OID = isolated and obese.
**Inflammation in the mammary glands and adipose tissue**

As inflammation is an integral component to the link between obesity and cancer risk [120], and the stress response pathways regulate key inflammatory agents [313,314], serum levels of cytokines GM-CSF, IL-1\(\alpha\), IL-1\(\beta\), IL-6, LIF, MCP-1, MIP-1\(\alpha\), RANKL, and TNF-\(\alpha\) were measured by Procarta Immunoassay Multiplex kit. As there were too many errors in the readouts, the data was unusable. CD68, a measurement for macrophage infiltration, was assessed by IHC in mammary gland and abdominal adipose tissue. High background staining, perhaps due to nonspecific staining of the antibody, occurred in both tissues, making it impossible to determine differences between groups. Further development of these assays need to be completed to correctly assess this parameter. This could be accomplished in the IHC by using a different CD68 antibody or an antibody for MAC387, which also stains for macrophages.

**Assessment of proliferation and apoptosis in the mammary glands**

To determine if differences in mammary tumor incidence between socially-isolated and group-housed mice arose from differences in cell proliferation or apoptosis, Ki67 (proliferation) and Tunel staining (apoptosis) were assessed in the normal mammary glands. Social isolation stress (p= 0.002), but not diet, significantly increased cell proliferation in the mammary gland (Figure 2.11A). No changes in apoptosis were observed by stress or diet via tunel staining of the mammary gland (Figure 2.11B).
Figure 2.11: **Ki67 and Tunel staining in mammary gland.** At the end of tumor monitoring period, cell proliferation using staining for Ki67 (performed by the Histopathology and Tissue Shared Resource); stress: p= 0.002, indicating that cell proliferation is increased by stress (A), and apoptosis by TUNEL assay, which there were no differences (B) were assessed by immunohistochemistry in the mammary glands, and both were quantified (n= 4-7/group) by scoring percent positive staining of the overall tissue in the lab. Error bars are shown as standard error of mean (SEM). Differences were determined through 2-way ANOVA and designated as significant when p< 0.05. In panel (A), the groups with “a” are significantly different than the
groups with “b,” but those with the same letter such as “a” and “a” are not different. G-C = group-housed control mice, G-OID = group-housed obese mice, I-C = isolated and control diet fed mice, and I-OID = isolated and obese mice.
Because of the significant changes by social isolation stress on proliferation, mRNA expression levels of cyclin D1 (*Ccnd1*) by qRT-PCR were investigated. Consistent with the increase in cell proliferation by social isolation stress, cyclin D1 was significantly up-regulated by social isolation stress (p< 0.001), but not diet (Figure 2.12A). p53 and expression of its signaling targets. Since mammary tumorigenesis is not increased by social isolation stress in p53 knockout mice [272], suggesting that p53 is required for social isolation to affect mammary cancer risk, we studied p53 expression in the mammary gland. Consistent with the earlier study, social isolation stress (p< 0.001), but not diet, increased levels of p53 (Figure 2.12B). MDM2, which closely interacts with p53 and is generally suppressed by up-regulation of p53, was found to be up-regulated in socially-stressed mice (p< 0.001; Figure 2.12C). The increase in Mdm2 mRNA expression indicates that the negative feedback between it and p53 may have been disrupted by social isolation stress.

As apoptosis was not affected by social isolation stress, we investigated whether p53 might have activated autophagy pathways. Indeed, social isolation stress, but not diet, increased mRNA expression of both *Dram1* (p= 0.016; Figure 2.13A), which is a direct target of p53, and *Jnk1* (p= 0.003; Figure 2.13B); these two genes are proposed to mediate the effects of p53 on autophagy [360,361].
Figure 2.12: Assessment of cell cycle and cell death in the mammary gland. Levels of Ccnd1 (n= 5-7/group); stress: p< 0.001 (A), Trp53 (n = 5-7/group); stress: p< 0.001 (B), and Mdm2 (n= 4-7/group); stress: p< 0.001 (C) were measured by qRT-PCR relative to Hrpt1 mRNA expression by using the standard curve method from the mammary glands of the mice. Cyclin D1, MDM2, and p53 are all up-regulated by social isolation stress. Error bars are shown as standard error of mean (SEM). Differences were determined through 2-way ANOVA and designated as significant when p< 0.05. Different letters denote significant differences. In all of the panels, the groups with “a” are significantly different than the groups with “b,” but those with the same letter such as “a” and “a” are not different. G-C = group-housed control mice, G-OID =

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group-housed obese mice, I-C = isolated and control diet fed mice, and I-OID = isolated and obese mice.
Figure 2.13: Evaluation of autophagy in the mammary gland. *Dram1* (n= 4-5/group); stress: p= 0.016 (A) and *Jnk1* (n= 5-7/group) stress: p< 0.003 (B) were measured by qRT-PCR relative to *Hrpt1* mRNA expression by using the standard curve method from the mammary glands of the mice. Both DRAM1 and JNK1 are up-regulated by social isolation stress, but not by diet which indicated the initiation of autophagy in those mammary cells. 2-way ANOVA was performed, and significance is achieved when p< 0.05. Error bars are shown as standard error of mean (SEM). Different letters denote significant differences. In both of the panels, the groups with “a” are significantly different than the groups with “b,” but those with the same letter such as “a” and
“a” are not different. G-C = group-housed control mice, G-OID = group-housed obese mice, I-C = isolated and control diet fed mice, and I-OID = isolated and obese mice.
Social isolation stress induces autophagy

As mammary gland samples ran out due to a large portion of them being used in western blots for various targets that had antibodies that needed a large amount of optimization but that ultimately did not work (i.e., DNMT1, DNMT3a, DNMT3b, and leptin and adiponectin receptors), and to a high incidence of mammary tumors causing the initial quantity of collected mammary glands to be low in this study, a separate set of mice that were not treated with a carcinogen, but were given control diet or OID, and group-housed or socially-isolated were generated. Protein levels of genes that are associated with autophagy were measured in the mammary glands of these new mice. Beclin 1 levels were significantly elevated in obese group-housed (p= 0.003; Figure 2.14A), indicating that obesity, not stress was responsible for the increase. Previous studies have found increased autophagy in adipose tissue of obese individuals [362], and this is proposed to be an attempt by the cells to inhibit obesity-induced inflammatory changes, though we did not find any changes in inflammation. In contrast, social isolation stress increased LC3II levels, but the increase was seen only in mice fed a control (interaction between stress and diet: p= 0.052; Figure 2.14B). Social isolation stress also decreased the levels of p62 (p= 0.003; Figure 2.14C); these changes were seen both in lean and obese socially isolated mice. Together these findings can be interpreted to indicate that mammary cells in socially isolated mice undergo autophagy. OID may also induce autophagy as it increased levels of Beclin 1.
Figure 2.14: Assessment of autophagy by immunoblotting. The levels of autophagy markers Beclin1; OID: p= 0.003 (A), LC3II (LC3II (bottom band) ratio to LC3I (top band)); interaction: p< 0.052 (B), and p62; stress: p= 0.003 (C) were determined by immunoblotting in the mammary glands of 4-6 mice per group that did not receive carcinogens, but were either group-housed and fed control diet (G-C), group-housed and fed OID (G-OID), isolated and fed control diet (I-C) or isolated and fed OID (I-OID) for 4 weeks. OID increased Beclin 1 levels, and stress decreased p62 increased LC3II, but only when OID was not present. The blot is shown in (D). Error bars are shown as standard error of mean (SEM). Different letters denote significant differences when p< 0.05. 2-way ANOVA was utilized to determine significance with posthoc analysis when
necessary, as in panel (B). In (B), G-C and G-OID are not different as they both have “b,” whereas I-C and I-OID are different as they have different letters “a” and “b.”
2.3.3 Discussion

A potential role for stress in breast cancer etiology has been studied extensively in human populations as well as in animal models. Although some anecdotal and experimental evidence supports a link [245], most human studies have failed to establish a connection [250] and some suggest a reduction in risk [249]. Findings from animal studies are equally contradictory, with one type of stress increasing and another reducing the risk [387]. Here we confirmed previous results obtained in women [257] and animal studies [268-271,388] that social isolation stress increased mammary cancer risk. Although it is known that obesity is more common among socially-isolated individuals than those who are well-connected to others [389,390], none of these studies have explored a possible interaction between social isolation stress and obesity in affecting breast cancer risk. We found that mice housed in social isolation and fed obesity-inducing diet had higher body weight and mammary cancer incidence than group-housed obese mice or mice housed in social isolation and fed the control diet. Thus, obesity promoted the effect of social isolation in increasing mammary cancer risk.

In addition to higher mammary cancer risk, socially-isolated obese mice exhibited insulin resistance, and the effect was more profound in these mice than in group-housed obese mice. Glucose tolerance testing, in turn, indicated that obesity and social isolation stress both separately, but not together, impaired tolerance. Previous studies have not directly investigated the effect of social isolation on insulin or glucose tolerance, but have measured systemic metabolic changes by assessing energy intake, body weight development as well as differences in circulating glucose, insulin and leptin levels [391]. Similar to our finding, socially-isolated
mice fed a standard laboratory diet consumed more energy and had higher body weights than group-housed mice but their circulating leptin levels were not altered [391]. Further, metabolic gene expression in the mammary adipocytes of socially-isolated mice mimicked expression seen in cancer cells; i.e., they exhibited up-regulation of hexokinase 2 (HK2), ATP citrate lyase (ACLY) and acetyl-CoA carboxylase alpha (ACACA). These changes were not seen in the adipose cells in other locations, including in abdominal or peripheral adipose depots [391].

There are several pathways that, when altered, can lead to insulin resistance, including an increase in NPY levels [279] and a reduction in adiponectin levels [182] or PPARγ expression [392]. NPY levels have been reported to be elevated by cold stress [279], and we found that socially-isolated obese mice also exhibited an increase in NPY levels, although neither obesity nor social isolation alone increased NPY. This is likely due to leptin resistance in the socially-isolated obese mice, as leptin inhibits NPY in the hypothalamus [160]. Since leptin levels were normal in the isolated, control diet fed mice, leptin was able to adequately inhibit chronic stress induced NPY; thus, NPY levels were not increased in these mice. However, since the socially-isolated obese mice had high levels of leptin, and OID is known to induce leptin resistance in mice [164], it can be deduced that the NPY expression was no longer regulated by leptin and therefore levels were increased. These findings suggest that NPY promoted insulin resistance in socially-isolated obese mice. Obesity is known to reduce adiponectin levels [393] but increase PPARγ expression in the liver [394,395]. In this study, both obesity and stress reduced circulating adiponectin levels and mammary Pparγ expression, suggesting that these changes may also have contributed to insulin resistance in the socially-isolated obese mice. The
possibility that increased NPY levels and reduced adiponectin and Ppar\(\gamma\) levels affected susceptibility to mammary tumorigenesis remains to be studied, as only low adiponectin levels are consistently linked to elevated breast cancer risk [181]. Together, findings obtained in the earlier study [391] and our current study suggest that social isolation stress induces local metabolic changes in the mammary gland.

Reduced expression of p53 has emerged as a protective factor against social isolation stress-induced mammary cancer in mice [272]. This seems paradoxical, as p53 is the “guardian of cellular genome” and activated by various cellular stressors to orchestrate responses that prevent or repair genomic damage, or eliminate damaged cells by apoptosis. Consistent with the loss of p53 in protecting from social isolation induced mammary cancer in p53 knockout mice, we found that social isolation stress up-regulated Tp53 expression in the mammary gland, but obesity did not. In addition, expression of Mdm2 was elevated, although it is generally down-regulated by p53, as it induces degradation of p53.

p53 maintains autophagic homeostasis and can both induce and prevent autophagy. Induction occurs when p53 is expressed in the nucleus [359]. Autophagy, in turn, may result in apoptosis or cell survival. We found no evidence of increased apoptosis in socially-isolated mice that exhibited increased p53 expression. In cancer cells, nutrient stress can up-regulate p53 which then induces autophagy to promote cell survival [357] via DRAM1 which is a direct target of p53 [360]. Another p53 associated gene that induces autophagy is JNK [361]. JNK activates Beclin 1 that in turn activates p53 [355,396]. Beclin 1 also plays a key role in orchestrating
autophagy [356]. In our study, the expression of Dram1 and Jnk1 mRNA were up-regulated by social isolation stress, and Beclin 1 was up-regulated by stress. Even more, protein levels of LC3II (a marker of initiation of autophagy) were increased, and p62 levels were decreased by stress, indicative that autophagy flux has been completed. As Dram1 and Jnk1 mRNA were measured in the mammary glands of the original mice (treated with MPA-DMBA), while the Beclin 1, LC3II, and p62 protein levels were assessed in second set of mice (no MPA-DMBA, and “only” 4 weeks of isolation/diet exposure), p53, Dram1 and Jnk1 will be measured in the mammary glands from the latter group of mice by qRT-PCR and immunoblot, as well. Even more, the western blots need to be repeated on one gel, as the one presented used two gels which may compound the findings. LC3II will also be normalized to actin at this time. Together these findings suggest that up-regulation of p53 by social isolation stress may be linked to increased breast cancer risk via increased autophagy that may have induced cancer cell survival, instead of inducing apoptosis. This increase in autophagy may be augmented in the socially-isolated, OID-fed mice through up-regulation of Beclin 1 by the OID.

When considering other biological pathways affected by stress and possibly mediating the effects of social isolation on increased mammary cancer risk, it is well established that stress leads to increased activation of the sympathetic nervous system, including increased release of catecholamines and the activation of hypothalamic-pituitary-adrenocortical (HPA) axis which in turn increases cortisol secretion. However, the effects of social isolation are unlikely to be mediated through an increase in these stress hormone levels, as these changes are transient and normalize during chronic social isolation [391]. In a pilot study, corticosterone levels did not
change in mice that were socially-isolated for 2 weeks; however, this may be in fact due to differences in the time of day that the mice were euthanized. Moreover, while NPY was increased in mice that were socially isolated for 2 weeks and on a control diet; prolonged exposure in the socially-isolated, control diet fed mice of the extended study resulted in no change in NPY levels. This may be a result of leptin inhibition of NPY in these animals versus the isolated, OID fed mice where high levels of leptin may be desensitizing the leptin receptors to leptin; therefore, NPY levels are still elevated. Changes in sex steroid hormone levels also have been reported by stress [397]. We studied the expression of the estrogen and progesterone receptors in the mammary glands, but did not find differences among socially-isolated and group-housed mice. Other possible mediators of the effects of social stress, especially in combination with consumption of a high fat diet, include impairments in the immune system. Although we did not systematically investigate them, the mRNA expression levels of \textit{Il1\beta}, \textit{Il6} and \textit{Tnfa} in the mammary gland were assessed. For \textit{Il1\beta} and \textit{Il6}, non-significantly elevated levels were seen in the mammary glands of obese, socially-isolated mice.

In conclusion, our data suggest that high fat intake-induced obesity potentiates the effects of social isolation stress on mammary carcinogenesis, and together, perhaps by up-regulating p53 and inducing expression of genes that stimulate autophagy, increase mammary tumorigenesis in mice. Future directions for this study will be thoroughly discussed in Chapter 4.
Chapter 3

HDAC and DNMT inhibitors reverse weight gain and insulin resistance in socially stressed mice, but have no effect on increased mammary cancer risk

3.1 Abstract

To determine whether epigenetic changes are causing the observed increase in mammary cancer risk in socially-isolated, obesity-inducing diet (OID)-fed female mice that were described in Chapter 2, qRT-PCR was performed on their and the other 3 groups’ mammary glands to measure mRNA levels of DNA methyltransferase 1 (Dnmt1), Dnmt3a, and Dnmt3b. While no differences were found in Dnmt3a or Dnmt3b by stress or diet, Dnmt1 levels were significantly elevated by stress (p< 0.001). In a separate study, obese group-housed mice that previously were found to exhibit increased levels of DNMT1 in the uterus were treated with vehicle or DNMT inhibitor hydralazine (DNMTi) and histone deacetylase inhibitor valproic acid (HDACi) (called here EPI treatment for inhibitors of "EPI"genetic regulators) in drinking water, and changes in body weights and insulin resistance were assessed. The EPI treatment reversed body weight gain and insulin resistance in the OID fed mice. In a follow-up study, socially-isolated, OID-fed mice were treated with hydralazine and SAHA (another HDACi that does not have the anti-anxiety properties possessed by valproic acid) or a vehicle control in drinking water, and changes in body weight, insulin resistance, and carcinogen-induced mammary tumorigenesis were assessed. Treatment of EPI drugs to socially-isolated, obese mice reduced body weight gain (t-test, week
9: p= 0.003, week 10: p= 0.009, week 11: p= 0.025) and reversed insulin resistance as the area under the curve is significantly different between the two groups (t-test, p= 0.009). However, tumor incidence, latency, and multiplicity were not affected. Taken together, these data suggest that insulin resistance in obese mice may result from epigenetically-induced changes and therefore DNMTi/HDACi drugs may be useful in preventing weight gain and improving insulin sensitivity, but the increase in mammary tumorigenesis in socially-isolated obese mice is not caused by an increase in DNMT and HDAC activity.

3.2 Dnmt expression in mammary glands from Chapter 2 mice

3.2.1 Materials and Methods

cDNA synthesis and qRT-PCR

cDNA synthesis and qRT-PCR was conducted as described in Chapter 2.3.1. The primer sequences are displayed in Table 3.1. RNA for these assays was from 3-7 mice per group. Concentration of each sample was normalized the mRNA levels of the reference genes 18s.

Statistical Analysis

2-way ANOVA was used to determine significance between groups with diet (control or OID) and stress (group or isolated housing) as variables. Pairwise Multiple Comparison Procedures (Holm-Sidak method) was performed posthoc when necessary. All statistical analysis was carried out using SPSS SigmaStat software, and differences were considered significant if P was less than 0.05. All probabilities are two-tailed. Data are expressed as mean ± SEM (standard error of mean).
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dnmt1</td>
<td>TCTCGTCTCCATCTTCGT</td>
<td>ACACGAGGTCCCCCTATTC</td>
</tr>
<tr>
<td>Dnmt3a</td>
<td>GGACTTTATGAGGGTGACTGGC</td>
<td>GATGTCCCTCCTGTCACCTAACG</td>
</tr>
<tr>
<td>Dnmt3b</td>
<td>TGACAGGAGATGGAGACAGAG</td>
<td>ATTGCTATGTCGGGTTCG</td>
</tr>
<tr>
<td>Hdac1</td>
<td>GAGATGACCAAGTACCACAGTG</td>
<td>AAACACGCCATCAAAACACCG</td>
</tr>
<tr>
<td>18s</td>
<td>TCGGAACTGAGGCCATGATT</td>
<td>CCTCCGACTTTTCGTTTTGATT</td>
</tr>
</tbody>
</table>

Table 3.1: Forward and reverse primer sequences used during qRT-PCR.
3.2.2 Results

Analysis of Dnmt mRNA expression in mouse mammary gland

To elucidate whether epigenetic changes are causing the observed increase in mammary cancer risk in the socially-isolated, OID fed female mice that were described in Chapter 2, qRT-PCR was performed on the mammary glands of the four diet/stress exposure groups to measure mRNA levels of Dnmt1, Dnmt3a, and Dnmt3b. While Dnmt3a and Dnmt3b were not significantly elevated by stress or diet, Dnmt1 levels were significantly increased by stress (p< 0.001; Figure 3.1). These findings suggest that stress may up-regulate DNMT1 activity, and DNMT1 may incur changes on the epigenome that induce an increase in mammary tumorigenesis.
Figure 3.1: Mammary gland mRNA expression of Dnmts. Dnmt1 (n= 3-7/group); (stress: p<0.001) (A), Dnmt3a (n= 3-6/group) (B), and Dnmt3b (n= 4-6/group) (C) were measured by qRT-PCR relative to 18s mRNA expression by using the standard curve method from the mammary glands of the mice. DNMT1 is increased by social isolation stress, but no changes were found in DNMT3a or DNMT3b. Error bars are shown as standard error of mean (SEM). Different letters denote significant differences when p< 0.05 by 2-way ANOVA. In panel (A), groups that a labeled with the same letter like “a” and “a” are not different, whereas if they are “a” and “b” then they are significantly different. G-C = group-housed control mice, G-OID =
group-housed obese mice, I-C = isolated and control diet fed mice, and I-OID = isolated and obese mice.
3.3 Effects of treatment with DNMTi and HDACi on body weight gain and insulin resistance in group-housed OID-fed mice

3.3.1 Materials and Methods

*Animals and Diet*

The methods utilized for the animal care is as described in Chapter 2.3.1. The female C57BL/6 mice were fed the OID that is presented in Table 2.2 and group-housed for the entirety of this study. No carcinogen was utilized and tumorigenesis was not assessed here.

*DNMTi and HDACi in Drinking Water*

The mice were administered a combination of the HDACi valproic acid (1.2 mg/kg/day) and DNMTi hydralazine (5 mg/kg/day) in drinking water or plain water as control (n=5-6/group). The body weights of these mice were followed for 6 weeks and insulin resistance was assessed.

*Body Weight and Insulin Tolerance Testing*

The weights of the mice were measured weekly for the 6 weeks of the study. Insulin tolerance testing was performed at the end of the study as described in Chapter 2.3.1

*Statistical Analysis*

The student’s t-test was performed on all data to determine differences. All statistical analysis was carried out using SPSS SigmaStat software, and differences were considered significant if P
was less than 0.05. All probabilities are two-tailed. Data are expressed as mean ± SEM (standard error of mean).

3.3.2 Results

Effects of DNMTi and HDACi on body weight and insulin resistance in obese mice

To determine if a combination of DNMTi and HDACi in drinking water can affect obesity, body weight was monitored for the duration of the study. The final percent weight gain was significantly lower in mice that were treated with DNMTi and HDACi than in obese mice receiving vehicle (p =0.014; Figure 3.2A). Even more, insulin resistance was assessed and found to be reversed in these treated mice (p< 0.001 at 90 and 120 minutes; Figure 3.2B), and the area under the curve is different between the treated and the control groups (p< 0.001; Figure 3.2C). Together, these data suggest that body weight control and insulin resistance are epigenetically regulated.
Figure 3.2: Effect of HDACi/DNMTi on final percent body weight change and insulin tolerance in obese mice. Final percent weight change (p= 0.014) (A), insulin tolerance testing p< 0.001 at 90 min and 120 min (B), and area under the curve analysis p< 0.001 (C) were conducted on a sub-group of obese mice that either received vehicle (control) or valproic acid and hydralazine (HDACi and DNMTi, respectively) (treated) in drinking water (n= 6/group). HDACi/DNMTi reversed body weight gain and restored insulin sensitivity. Error bars are shown as standard error of mean (SEM). The student’s t-test was performed to analyze differences. Significance is denoted by * when p< 0.05.
3.4 Effects of DNMTi and HDACi on socially-isolated, obese mice

3.4.1 Materials and Methods

*Animals, Diet, Stress Exposure, and Tumor induction*

The methods utilized for the animal care are as described in Chapter 2.3.1. At weaning, the female C57BL/6 mice were fed the OID that is presented in Table 2.2 and were socially-isolated also as described in that section both for the entirety of the study. No mice were fed control diet nor were they group-housed. Tumors were induced by the MPA-DMBA method detailed in Chapter 2.3.1. The Study Design is depicted in Figure 3.3.

*DNMTi and HDACi in Drinking Water*

Instead of using valproic acid, I used another HDACi for this study; i.e., suberoylanilide hydroxamic acid (SAHA), also called Vorinostat. This is because valproic acid has antidepressant and antianxiety properties and could thus affect the results by reducing stress induced by social isolation rather than acting as HDACi. The HDACi SAHA was solubilized at a 1:5 ratio with 2-Hydroxypropyl)-β-cyclodextrin (HOPS) into water by boiling and stirring on a hotplate and then cooling at room temperature until no longer extremely hot followed by cooling on ice until the solution was room temperature as published in other studies [398,399]. A combination of the HDACi SAHA (100 mg/kg/day) in HOPS and the DNMTi hydralazine (5 mg/kg/day) or just the HOPS in water at a similar concentration as the treatment solution as a control were administered in drinking water to the mice starting one week after the last DMBA treatment until the end of the study.
Figure 3.3: Study design.
**Body weight and insulin and glucose tolerance testing**

Mice were weighed weekly from weaning until the end of the study. At 12 weeks of age, insulin tolerance testing was performed on a subset of the mice n= 7-11/group as previously described in Chapter 2.3.1.

**Mammary tumorigenesis**

Starting one week after the last DMBA administration, the mice were palpated and mammary tumors were measured weekly with calipers. When the tumor burden exceeded 10% of the weight of the mice, they were sacrificed before the end of the monitoring period. Otherwise, mice were monitored for 17 weeks after receiving the last DMBA dose and sacrificed at 27 weeks of age. Tumor incidence, latency, multiplicity, size and growth were recorded. n= 19-23 mice/group.

**Tissue collection**

At sacrifice, blood was obtained via cardiac puncture. Serum was separated, frozen, and kept at -20°C until assayed. Mammary glands and tumors were collected at sacrifice. Abdominal adipose tissue, pancreas, liver, muscle, ovaries, fallopian tubes and uterus, and brain including the hypothalamus were also collected for future studies. Tissues were either fixed in 10% buffered formalin, embedded into paraffin blocks, and sectioned (5µm), or flash frozen in liquid nitrogen and stored at -80°C until assayed.


cDNA synthesis and qRT-PCR

cDNA synthesis and qRT-PCR was conducted as described in Chapter 2.3.1. The primer sequences are displayed in Table 3.1. RNA for these assays was from 3-8 mice per group. Concentration of each sample was normalized the mRNA levels of the reference genes \( Hprt1 \).

3.4.2 Results

Reversal of body weight gain and insulin resistance by HDACi and DNMTi in socially-isolated obese mice

To further confirm that epigenetic regulation plays a role in the development of obesity and insulin resistance, body weight gain and insulin tolerance testing were assessed in these mice. Combination treatment of DNMTi and HDACi administered to socially-isolated, obese mice reduced body weight gain (week 9: \( p = 0.003 \), week 10: \( p = 0.009 \), week 11: \( p = 0.025 \); week 13: \( p = 0.043 \) (Figure 3.4A). Moreover, insulin resistance was reversed in the mice that were given the EPI treatment water (60 mins \( p = 0.003 \) and 90 mins \( p = 0.007 \); Figure 3.4B). The area under the curve was also significantly different between the two groups (\( p = 0.009 \); Figure 3.4C). These data are consistent with the results in Chapter 3.3.2 in showing that treatment with HDACi and DNMTi reverses body weight gain and insulin resistance in obese mice, regardless whether they are group-housed or housed in social isolation.
Figure 3.4: **Body weight and insulin resistance:** Treating socially-isolated, OID-fed mice with HDACi and DNMTi significantly reduced weight gain compared with the untreated mice n= 19-23 mice/group at week 9: p= 0.003, week 10: p= 0.009, and week 11: p= 0.025 and week 13: p= 0.043 (A). Additionally, insulin resistance n= 7-11/group was reversed by the HDACi/DNMTi treatment. Significant differences were found at 60 mins p= 0.003 and 90 mins p= 0.007 (B). The area under the curve was significantly different between the two groups as well, p= 0.009 (C). Error bars are shown as standard error of mean (SEM). The student’s t-test was performed to analyze differences. Significance is denoted by * when p< 0.05.
Effects of HDACi and DNMTi treatment on mammary tumorigenesis in socially isolated, obese mice

Tumor incidence (Figure 3.5A), latency (Figure 3.5B) and multiplicity (Figure 3.5C) were analyzed in socially-isolated, obese mice (n= 19-23/group) and found to be unchanged by the combined treatment of HDACi and DNMTi.

Targets of HDACi and DNMTi in the mammary gland

Alterations of mRNA levels in the mammary gland of DNMTi and HDACi targets were measured by qRT-PCR. No changes were observed in Dnmt1 (Figure 3.6A), Dnmt3a (Figure 3.6B), Dnmt3b (Figure 3.6C), or Hdac1 (Figure 3.6D). n= 7-8/group

Expression of NPY and adiponectin linked genes in the mammary gland

To determine if some NPY related genes and were epigenetically regulated, mRNA expression of Ppar-γ (Figure 3.7A), NPY receptor Y1R (Figure 3.7B), Il1β (Figure 3.8A), Il6 (Figure 3.8B) and Tnfa (Figure 3.8C) were measured in the mammary gland. Treatment with HDACi and DNMTi did not alter mRNA levels of any of these genes. n= 7-8/group
Figure 3.5: Mammary tumorigenesis in socially-isolated, obese mice treated with EPI drugs. Mammary tumors were induced by using the MPA-DMBA method. HDACi SAHA and DNMTi hydralazine were added to drinking water one week after the last DMBA administration and kept on it or vehicle control until the end of the study. Tumor incidence (percent of mice that get tumors) (A), tumor latency (time until the appearance of the first measurable tumor) (B), and tumor multiplicity (number of tumors per animal) (C) were analyzed, but no differences were found between treatment groups (n= 19-23/group). Error bars are shown as standard error of mean (SEM). For A, statistical analysis was performed by using the Kaplan-Meier survival curve.
and log-rank test. For B-C, the student’s t-test was performed to analyze differences, and differences are significant when $p < 0.05$. 
Figure 3.6: No changes in targets of HDACi and DNMTi in the mammary gland of socially-isolated, obese mice. mRNA levels of n= 7-8/group $Dnmt1$ (A), $Dnmt3a$ (B), $Dnmt3b$ (C), and $Hdac1$ (D) were measured by qRT-PCR relative to $Hprt1$ mRNA expression by using the standard curve method from the mammary glands of the mice treated with vehicle or HDACi/DNMTi. No significant differences were observed, although there was a trend for lower $Dnmt1$ and $Dnmt3b$ expression, both of which were previously found to be up-regulated in the mammary glands of socially-isolated, obese mice (see Figure 3.1). Error bars are shown as
standard error of mean (SEM). The student’s t-test was performed to analyze differences, and
differences are significant when $p < 0.05$. 
Figure 3.7: Effect of HDACi/DNMTi on *Pparγ* and *Npy1r* mRNA expression in mammary gland of socially-isolated, obese mice. At the end of tumor monitoring period, mRNA expression of Ppar-γ (A) and Npy1R (B), n= 7-8/group for both, were measured in the mammary glands by qRT-PCR relative to *Hrptl* by using the standard curve method. No differences were observed. Error bars are shown as standard error of mean (SEM). The student’s t-test was performed to analyze differences, and differences are significant when p< 0.05.
Figure 3.8: No changes in inflammatory cytokines in the mammary glands of socially-isolated, obese mice. At the end of tumor monitoring period, mRNA expression of Il1β (A), Il6 (B) and Tnfα (C), n= 7-8/group for both, were measured by qRT-PCR relative to Hrpt1 mRNA expression by using the standard curve method from the mammary glands of socially-isolated obese mice. No differences were observed. Error bars are shown as standard error of mean (SEM). The student’s t-test was performed to analyze differences, and differences are significant when p< 0.05.
Effects of HDACi/DNMTi treatment on cell cycle and cell death in the mammary gland

In order to identify if the changes in cell proliferation in the mammary glands of socially-isolated obese mice were epigenetically regulated, I determined the expression of Ccnd1 (Figure 3.9A) in the mammary glands of mice treated with vehicle or HDACi/DNMTi treatment. No changes were observed. Further, expression of p53 was not altered (Figure 3.9B), and neither were Mdm2 (Figure 3.9C) and Jnk1 (Figure 3.9D) mRNA levels. The lack of reversal of cyclin D1, p53 or its target genes by treatment with EPI drugs may indicate that these changes were not epigenetically driven.
Figure 3.9 Effects of HDACi/DNMTi treatment on cell cycle and cell death in the mammary glands of socially-isolated, obese mice. Levels of Ccnd1 (A), Trp53 (B), Mdm2 (C), and Jnk1 (D), n=7-8/group for all, were measured by qRT-PCR relative to Hrpt1 mRNA expression by using the standard curve method from the mammary glands of the socially-isolated, obese mice that received control water or water containing HDACi and DNMTi. Error bars are shown as standard error of mean (SEM). No differences were found. The student’s t-test was performed to analyze differences, and differences are significant when p< 0.05.
3.4.3 Discussion

Over the past few decades, epigenetics has emerged as a highly important field in cancer [400]. DNMTs and HDACs work in concert with each other to cause gene silencing which can lead to the suppression of tumor suppressor genes (TSG). E-cadherin and p16 were some of the first of the TSGs to be discovered as commonly hypermethylated in cancer [401] and silencing of these TSGs may contribute to increased cancer cell proliferation and epithelial to mesenchymal transition and metastasis. Aberrant DNA methylation is common in all types of cancers, including in breast tumors [401,402]. DNA hypermethylation in breast tumors is particularly associated with chemotherapy resistance [363] and resistance to antiestrogen therapy [382]. A new synthetic HDAC inhibitor, MHY218, induces apoptosis or autophagy-related cell death in tamoxifen-resistant MCF-7 breast cancer cells [403].

Lifestyle factors play a large role in determining epigenetic changes. For example, obesity-induced inflammation is associated with epigenetic changes that lead to an increase in cancer risk [220,221]. This may be partially through the excessive amounts of cytokines that are found in abdominal adipose tissue depots and circulation, and have been observed to mediate epigenetic changes [220,223,225,226,404]. Stress can also initiate epigenetic changes, as seen in early life stressor-induced alteration of epigenetic regulation of GR [321], and the modification of the BDNF gene profile through immobilization stress and foot shock therapy fear conditioning [323]. Because of these findings, we hypothesized that the increase in mammary cancer that we observed in socially-isolated, obese mice in Chapter 2 may have been due to epigenetic changes.
To investigate this hypothesis, we first measured mRNA levels of *Dnmt1, Dnmt3a, and Dnmt3b* in the mammary gland. *Dnmt1* was significantly elevated by stress. *Dnmt3a* and *Dnmt3b* showed a trend towards a stress response, but did not reach significance due to a high level of variation between samples. These data are consistent with the hypermethylation of TSGs that is commonly found in most breast tumors [363], and up-regulation of DNMTs in many breast cancer cells [364]. As DNMT1, known for its role in epigenetic pattern maintenance, but also importantly, *de novo* methylation in cancer cells [214], was up-regulated by stress in the mice that were socially-isolated and/or fed an OID in Chapter 2, it can be extrapolated that social isolation stress is inducing new methylation patterns in the mammary cells. Thus, the silencing of some tumor suppressor genes may be occurring, though we have not identified any at this time.

In a pilot study, female group-housed mice that were fed OID were administered DNMTi and HDACi in drinking water or plain water as a control. OID-induced body weight gain was reversed and insulin sensitivity was restored in the mice that were treated with the EPI drugs. These findings are similar to what others have found [234]. The reversal of insulin resistance may be through the changes in cytokines that can induce insulin resistance and also though inhibition of HDAC regulated adipogenesis [235] or through changes in PPARγ and related adipokines adiponectin and leptin, as they are intricately involved in insulin sensitivity [155,182]. Our results suggest that epigenetic changes played a notable part, although the identity of the epigenetically-regulated genes remains to be revealed. We measured mRNA levels of insulin sensitivity genes in the mammary glands, such as PPARγ and inflammatory
cytokines, but no differences were found with EPI treatment. However, further investigation of these insulin sensitivity mediators in other tissues, such as abdominal adipose cells, liver and muscle is warranted.

Together, our findings led us to conduct a study on the effects of DNMTi and HDACi treatment in socially-isolated, obese mice on with MPA-DMBA induced mammary tumors. We monitored mammary tumorigenesis in the socially-isolated, obese mice to answer whether or not the increase in mammary cancer risk that we saw in socially-isolated, OID fed mice was due to epigenetic changes. While a previous study reported prevention of tobacco carcinogen-induced in mouse lung tumors through a combination of HDACi and DNMTi treatment [383], we did not observe any changes in mammary tumor incidence, latency, or multiplicity. mRNA levels of Dnmt1, Dnmt3a, Dnmt3b and Hdac1 in the mammary gland were also not altered, though this may be somewhat due to high variation between samples, at least for Dnmt1 and Dnmt3b.

From these results and the discovery that p53 mediated autophagy is increased in the socially-isolated, OID fed mice detailed in Chapter 2, it can be inferred that the increase in mammary tumorigenesis in these mice is due to the autophagic induced cell survival and perhaps other pathways that we have not investigated yet. These changes did not seem to be epigenetically regulated. One such pathway may be angiogenesis, as NPY has a well-established role in angiogenesis and adipogenesis [303,405] and NPY was greatly increased in those mice. Npy1r mRNA levels were also measured in the mammary glands of the HDACi/DNMTi treated mice, but no changes were found. To determine whether cell cycle and cell death pathways were
altered with the HDACi/DNMTi treatment, mammary gland mRNA levels of *Tp53*, *Mdm2*, *Ccnd1*, and *Jnk1* were assessed. As no changes were observed in any of these genes, though high variation between samples was present, regulation of these genes through epigenetic modulation is not indicated in these mice.

In conclusion, our data suggests that while DNMT1 may be up-regulated in the mammary glands of socially-isolated, obese mice, the observed increase in their mammary tumorigenesis more likely is caused by p53-mediated increase in autophagic cell survival and perhaps other pathways such as NPY induced angiogenesis. Further, through the observations that HDACi/DNMTi administration repeatedly reverses body weight gain and restores insulin sensitivity, this study adds to the current hypothesis that HDACi/DNMTi treatment would be useful in treatment for type II Diabetes. Future directions for this study will be thoroughly discussed in Chapter 4.
Chapter 4  Summary and Perspectives

This chapter summarizes the findings in chapters 2 and 3, and provides perspectives of their implications in breast cancer prevention.

4.1 Perspectives on Chapter 2

4.1.1 Summary of Chapter 2

In chapter 2, I show that social isolation stress in combination with an obesity inducing diet (OID) increased MPA-DMBA induced mammary tumorigenesis in female C57BL/6 mice. Further, these mice gained more weight than the other groups of mice and developed insulin resistance. Since in a pilot study I did not find any changes in corticosterone levels in the socially isolated mice, in accordance with studies done by others showing that increased activation of sympathetic nervous system by social isolation stress is transient and normalizes during chronic social isolation [365], I searched for other biological mediators for isolation. Serum levels of NPY were increased, while serum adiponectin and PPARγ in the mammary gland were decreased, which is consistent with insulin resistance. Cytokine levels in the mammary gland were not altered, but this may be due to high variation between samples. Cell proliferation through Ki67 assessment and Cyclin D1 levels in the mammary gland were increased, but apoptosis via tunel staining was unaffected. p53 and MDM2 were both elevated in the mammary gland, as well. As apoptosis was unchanged, yet p53 and MDM2 were altered, p53 mediated autophagy pathways were examined. Both DRAM1 and JNK1 levels were increased in
mammary gland, which is indicative of autophagy. Further, Beclin 1 protein levels were increased, while p62 was decreased, indicating that autophagy took place in the mammary cells. These findings suggest that social isolation stress induces autophagy in the mammary gland, and it might be linked to elevated expression of p53. Figure 4.1 summarizes these findings.
Figure 4.1: Summary of findings in Chapter 2 of the effects of social isolation stress and obesity on autophagy.
4.1.2 Implications for breast cancer prevention

Though many studies, including this one, show a link between social isolation stress and cancer [257,268-271,388], in general, the role of stress in cancer risk is highly disputed due to contradictory data that can be explained in part due to differences in stressors and poor study designs [249,250,387]. As the effectiveness of psychological interventions in women with breast cancer is also highly disputed (for some of the same reasons), [259,261-263], there is a need for improved studies on psychological intervention and risk of breast cancer recurrence and survival, especially among socially isolated patients. Studies like the one that has been described in Chapter 2 demonstrating an association between social isolation stress, obesity and increased mammary cancer risk with proposed mechanism of action are imperative for the field to gain insight of the relationship between breast cancer and stress. With this knowledge, better understanding for the general populace can be obtained, and, hopefully, more awareness can lead to effective means to prevent breast cancer.

4.1.3 Future directions

While the data that were presented in Chapter 2 are fairly encompassing, many experiments can still be conducted to further elucidate the mechanisms behind the observed increase in breast cancer risk through social isolation stress and obesity. Further, the findings may have implications for attempts to prevent breast cancer recurrence. Most importantly, although the results show that the social isolation stress-induced increase in mammary tumorigenesis leads to
up-regulation of p53 and autophagy, and increased cell proliferation but not apoptosis in the mammary gland, these data are correlative and cannot be interpreted to indicate a causal link between p53 and autophagy. We have currently started to investigate a causative association by using p53+/- heterozygous knockout mice which are resistant to social isolation-induced increase in mammary carcinogenesis [254] and Atg7-/- mice that are defective for autophagy, as Atg7 is essential for ATG conjugation, LC3 modification systems, and autophagosome formation. If up-regulation of p53 is required for social isolation stress to induce autophagy, we do not anticipate any changes in Dram1, Beclin1, LC3II and p62 in p53+/- mice housed in social isolation and fed an OID. Further, if autophagy is essential for inducing an increase in mammary tumorigenesis, we expect that socially isolated, obese Atg7-/- mice do not exhibit an increase in mammary tumorigenesis.

While inflammation assessment was attempted, adequate evaluation was not met. The cytokine multiplex immunoassay should be repeated, as the data from the first attempt was unusable. Moreover, CD68 IHC staining in mammary gland and adipose tissue needs to be further developed to decrease background, allowing for accurate measurement of macrophage infiltration. mRNA levels of the cytokines can be measured in the new mammary gland samples from the mice that underwent stress and diet exposures without mammary tumor induction. As NPY levels were increased in the socially-isolated, OID-fed mice, and NPY directly stimulates angiogenesis, VEGFR mRNA in mammary glands and von Willebrand Factor and VEGFR IHC staining in the mammary gland should be evaluated.
As we found that socially isolated, obese mice exhibited insulin resistance, IGF-1 may be increased in these mice, as seen in other studies of insulin resistant mice [234]. Therefore, IGF-1, its receptor IGF1R, and IGF1BP levels should be assessed in serum, mammary gland, and liver tissues. If IGF-1 signaling is altered in these mice, then this may also explain the increase in cell proliferation and cyclin D1 that we observed.

Lastly, a clinical intervention study of group counseling, meditation or relaxation therapy could be conducted for women who are socially-isolated and at high risk of developing breast cancer, and perhaps also for newly diagnosed breast cancer patients.

4.2 Perspectives on Chapter 3

4.2.1 Summary of Chapter 3

HDAC inhibitors valproic acid and SAHA, in combination with DNMT inhibitor hydralazine were administered in drinking water to group-housed, OID fed mice. Insulin resistance and body weight gain were reversed with drug treatment. Further, HDACi/DNMTi were given to socially-isolated, OID fed mice that also received MPA-DMBA to induce mammary tumors. Once again, body weight gain was inhibited and insulin sensitivity was restored in the EPI drug treated mice. However, the EPI drug treatment did not reverse an increase in mammary tumorigenesis. Similarly, levels of DNMTs and HDAC, cytokines, PPARγ, and genes that regulate cell cycle and cell death genes were unchanged upon drug treatment. Because insulin sensitivity was restored and body weight gain was reversed, we know that treatment with HDACi/DNMTi is
working in some tissues. Taken together, these data disprove our hypothesis that the observed mammary cancer increase in socially-isolated, OID fed mice was due to epigenetic changes.

**4.2.2 Implications for breast cancer prevention**

HDAC and DNMT inhibitors are regularly used in cancer treatment, including in breast cancer, especially in tumors that are resistant to chemotherapy or antiestrogens [379,380,382]. While this study did not support the use of HDACi/DNMTi in our model for cancer prevention, it powerfully supported the usage of these drugs as treatment for Diabetes type II.

**4.2.3 Future Directions**

Some experiments should still be performed to investigate the role of epigenome in mediating the effects of obesity on mammary tumorigenesis and social isolation on insulin resistance in this project. A global methylation analysis on the mammary glands and adipose tissue could be performed to understand the effects of the HDACi/DNMTi on insulin resistance. Additionally, the global methylation analysis could be performed on the mammary glands from the mice in Chapter 2 to identify potential tumor suppressor genes that are perhaps being silenced through the up-regulation of DNMT1 by social isolation stress.

Serum levels of NPY, adiponectin, and leptin should be assessed in the HDACi/DNMTi treated mice compared to the control mice to determine changes in their levels as this may help explain the insulin sensitivity restoration. Better inflammation analysis through assessment of CD68 or cytokines in mammary gland, adipose tissue, and serum is also needed. To confirm that the EPI
drugs were present in the mammary glands, mass spectroscopy could be performed on them. Serum levels of the drugs could also be measured to verify that the mice received the adequate dose. As a study reported that the HDACi panobinistat in combination with the autophagy inhibitor chloroquine decreased tumor burden and increased survival in MD-231 xenograft mice [376], treatment of an autophagy inhibitor to these socially-isolated, OID-fed mice alone or in addition to the DNMTi/HDACi drugs could compliment the results of our ongoing study of socially-isolated, OID-fed mice in Atg7−/− mice.

It is unclear whether a change in body weight or the alteration of the epigenome is responsible for the observed reversal of insulin resistance in the HDACi/DNMTi treated mice. While it is most likely due to the effects of HDAC alteration because HDAC2 has been shown to regulate insulin signaling by binding to insulin receptors in the liver, and inhibition of HDAC2 by trichostatin A partially restores insulin signaling [406], a number of experiments should be performed to confirm that weight loss is not causing the restoration of insulin sensitivity that we observed. As the change in body weight in these mice may be through appetite changes, food consumption should be measured. In a previous study, we found that HDACi/DNMTi had no effect on body weight in lean mice that were on a control diet. Therefore, treating mice that are insulin resistant but not obese with HDACi/DNMTi to observe if insulin resistance is still reversed despite no change in body weight. To do this, mice with a conditional knockdown of insulin receptor in the liver could be utilized as these mice exhibit insulin resistance [407]. A conditional knockdown is necessary as complete loss of insulin receptor results in early life death from diabetes-induced ketoacidosis [408,409]. Lastly, as this study and others [234,406] have
implicated the usage of HDACi/DNMTi treatment for Diabetes type II, a clinical trial of HDACi/DNMTi in an obese population could be designed.
REFERENCES


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