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PSYCHOSOCIAL STRESS EXPOSURE IMPAIRS MAMMARY GLAND DEVELOPMENT AND PRESERVES MAMMARY STEM CELL POPULATIONS

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Dedication

To my mom for teaching me to be curious.
To Clarissa, Susie, and Petra for teaching me to work hard and follow my dreams.
To the House: Lola, Katie, Lolo, Marissa, Sari, Anna, and Moe for teaching me to be myself.

And to Dahlia for teaching me to choose adventure, to take care of myself, and to cherish the time we have.

Epigraph

Success means not giving up halfway but resolutely pursuing the path you have chosen. To this end, it is also important [to] realize that the place where you work is a place for forging your character and growing as a human being.

Table of Contents

List of Figures viii

List of Supplementary Figures x

Acknowledgments xi

Abstract xiii

Chapter 1: Introduction 1

1.1 Breast cancer 1

1.2 Nulliparous mammary gland development 6

1.3 Mammary stem cells 11

1.4: Reproductive hormones and mammary gland development 17

1.5: Psychosocial stress and breast cancer risk 23

Chapter 2: Psychosocial stress exposure disrupts mammary gland development 31

2.1: Abstract 31

2.2: Introduction 32

2.3 Methods 35

2.3.1: Sprague-Dawley rats 35

2.3.2: Experimental design 36

2.3.3: Mammary whole mount preparation and analysis 36

2.3.4: H&E staining of ductal structures (TEB, TD, AB, and Lobules), fat, and stroma 37

2.3.5: Immunohistochemistry 38

2.3.6: Ovarian steroid exposure 38

2.3.7: Corticosterone stress reactivity 39

2.3.8: Behavioral response to exploration stressor 40

2.3.9: Statistical analysis ... 40
2.5: Results ... 40
2.4.1: Mammary ductal extension and not TEB Number is altered by social isolation ... 40
2.4.3: Social isolation alters central mammary gland composition: ducts, stroma, and fat . 45
2.4.4: Ovarian steroid exposure is altered by social isolation ... 48
2.4.5: Increased vigilance and glucocorticoid stress reactivity in isolated rats ... 50
2.5: Discussion ... 53
Chapter 3: Glucocorticoid Receptor Activation Inhibits Proliferation and Promotes Survival of Mammary Stem and Progenitor Cells ... 61
3.1: Introduction ... 61
3.2: Materials & Methods ... 64
3.2.1: Sprague-Dawley rats ... 64
3.2.2: Experimental design ... 65
3.2.3: Mammary cell isolation ... 65
3.2.4: MaSC classification & quantification. ... 66
3.2.5: Three-dimensional cell culture of enriched mammary progenitor populations ... 66
3.2.6: Three-dimensional cell culture of mammary cells ... 67
3.2.7: Mammary adipose tissue conditioned media ... 67
3.2.8 MCF-10A mammosphere formation and cell death. ... 68
3.2.9 Quantitative RT-PCR ... 69
3.2.10 Targeted analysis of lysophosphatidylcholine (LPC) and lysophosphatidic acid (LPA) 69
3.3: Results ... 70
3.3.1: Psychosocial stress exposure increases the population of $CD24^{+}CD49f^{+}$ Cells ... 70
3.3.2: Corticosterone reactivity but not progesterone and estrogen exposure correlates to $CD24^{+}CD49f^{+}$ population size ... 71

3.3.3: Corticosterone impairs differentiation of mammary progenitor cells 74

3.3.4: Mammary adipose tissue secretome from isolated rats increases survival of mammary epithelial cells in suspension 75

.... 76

3.3.5: Corticosterone treatment increases secretion of pro-survival factors from mammary adipose tissue 78

3.4 Discussion 80

Chapter 4: Conclusions and Future Directions 83

4.1: Pseudotemporal analysis of mammary epithelial cells and its implications 84

4.2: Expanding research to include nulliparous mammary gland development. 87

4.3: Individual variation in ovarian cycle hormones and breast cancer risk 89

4.4: Psychosocial interventions: rodents and beyond 90

References 93

List of Figures

Figure 1.1: Diagram of a human breast. 2

Figure 1.2: Rat mammary gland development. 7

Figure 1.3: Diagram of mammary stem cell hierarchy 13

Figure 1.4 Temporal concentrations of estrogen and progesterone and resulting vaginal cytology 21

Figure 2.1: Effect of social environment on mammary gland development of terminal end buds, ductal extension, and distal ductal area (measured in mammary gland whole mounts). 42

Figure 2.2: Differentiation of ductal structures in four different sectors of the distal rat mammary gland (S1 nearest the nipple to S4 most distal). 46

Figure 2.3 Characterization of mammary gland composition. 48

Figure 2.4: Ovarian steroid exposure (measured by vaginal cytology and ovarian weight). 50

Figure 2.5: Vigilance testing, glucocorticoid stress reactivity, and adrenal weights. 52

Figure 2.6: Characterization estrogen, progesterone, and glucocorticoid receptors. 54

Figure 3.1: Cytometric analysis of rat mammary epithelial cell subtypes according to cell surface marker expression. 71

Figure 3.2: Correlation of CD24+CD49f+ progenitor cell population to progesterone, estrogen, and glucocorticoid exposure. 73

Figure 3.3 : Short-term glucocorticoid receptor increases spheroid formation. 74

Figure 3.4 : Glucocorticoid receptor activation decreases spheroid formation, and increases forming efficiency in subsequent generations. 76

Figure 3.5. Mammary adipose tissue conditioned media from isolated rats promotes mammospheres formation and decreases cell death in MCF10A cells. 79

Figure 4.1: Experimental overview of pseudotemporal ordering of mammary epithelial cell differentiation 87

List of Supplementary Figures

Supplementary Figure 2.S1: Mammary gland whole mounts. 43

Supplementary Figure 2.S2: Color deconvolution for quantification of mammary gland structures. ductal structures, stroma, and adipose tissue. 47

Supplementary Figure 2.S3: Association between stroma and ductal structures in grouped and isolated rats. 49

Figure 3.S1: Cytometric analysis of rat mammary epithelial cell subtypes at 13-17 weeks of age. 72

Supplemental Figure 3.S2: Acetyl CoA Carboxylase gene expression and LPC secretion from grouped and isolated rats mammary adipose tissue. 78

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Abstract

Exposure to psychosocial stressors and ensuing stress physiology have been associated with invasive mammary tumors in rodent models of human breast cancer. Many physiologic and environmental exposures that influence breast cancer risk occur during mammary gland development. However, the effect of psychosocial stress exposure on mammary gland development remains unknown. In a nulliparous Sprague-Dawley rat model of breast cancer, we demonstrate that social isolation increased glucocorticoid reactivity to everyday stressors and reduced alveolobular differentiation during late puberty and young adulthood. Moreover, glucocorticoid stress reactivity and not reproductive steroid exposure was positively associated with the population of mammary progenitor and stem cells, which are the purported cell-of-origin in breast cancer. *In vitro* analysis demonstrated the glucocorticoid exposure decreased differentiation of mammary progenitor cells. The work in this thesis demonstrates that exposure to psychosocial stress and resulting elevated glucocorticoid reactivity disrupts both mammary gland growth and differentiation of mammary epithelium, which could contribute to a later increase in mammary cancer risk.

Chapter 1: Introduction

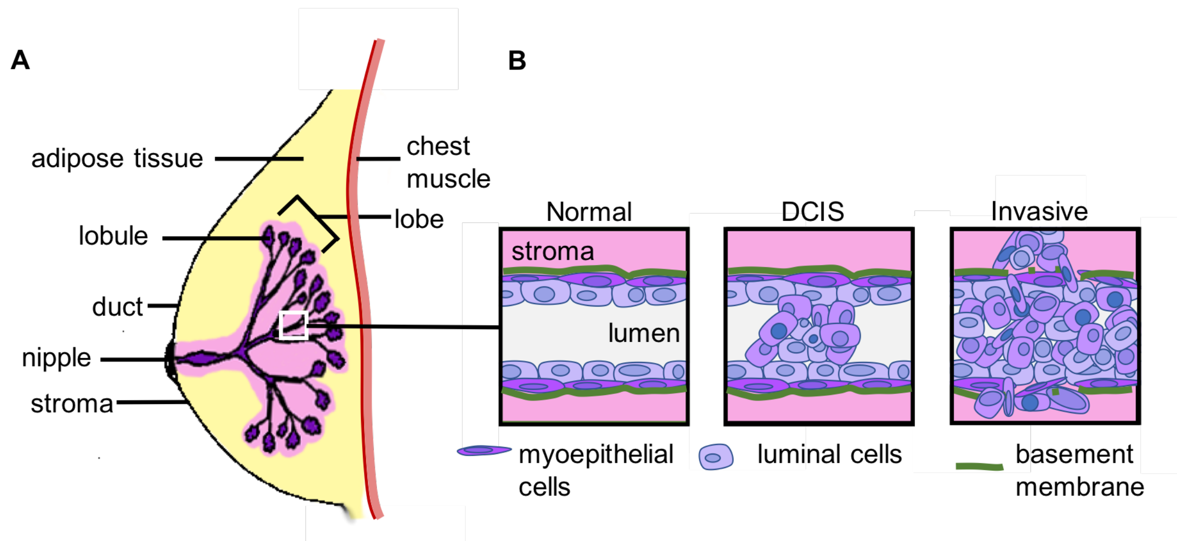
1.1 Breast cancer

Breast cancer is the most common non-skin malignancy in women (Siegel, Miller, and Jemal 2017). In 2017, an estimated 252,710 women will be diagnosed with invasive breast cancer, constituting 15% of all new cancer cases and over 40,000 women will die of the disease (Siegel, Miller, and Jemal 2017). Breast cancer is a heterogeneous disease that derives from the breast epithelial cell and has a variety of prevention strategies and treatments.

A woman's breast is made of 15-20 lobes containing smaller sections called lobules (Figure 1.1) (Pandya and Moore 2011). Adipose tissue and non-adipose stroma fill the space between lobules and ducts (thin tubes that connect the lobes and nipples; Figure 1A). The mammary ducts are made of two layers of epithelial cells with a single basally located myoepithelial cell layer and a single luminal layer that line the empty lumen at the center of the duct (Figure 1B; (Pandya and Moore 2011; Javed and Lteif 2013). The evolutionary purpose of these ducts is to carry milk from a milk-producing lobule to the nipple during breast feeding (Javed and Lteif 2013).

Breast neoplasm occurs when the cells of the breast grow out of control and form a tumor (Bombonati and Sgroi 2011). These tumors may be cancerous (malignant) or not cancerous (benign). Breast cancer arises from the breast epithelium which includes the myoepithelial cells and luminal cells that constitute the ducts and lobules of the breast (Bombonati and Sgroi 2011). The progression of breast cancer begins with proliferative changes that result from pro-growth signals or decreased apoptosis (Robbins, Kumar, and Cotran 2010). This results in a disruption of the two layers of ductal cells present in the normal breast (Figure 1.1B). The otherwise empty lumen begins to fill with epithelial cells and are classified (in order of increasing number and mitotic index) as: proliferative disease, atypical hyperplasia, low/moderate grade ductal carcinoma *in situ* (DCIS), and high grade DCIS (Figure 1.1B) (Bombonati and Sgroi 2011). Invasive breast cancer

Figure 1.1: Diagram of a human breast.
The breast is comprised of three main components epithelium (ducts and lobules), adipose tissue, and other stroma (e.g. connective tissue, fibroblasts, immune cells, etc). The ducts begin at the nipple and extend into the adipose tissue. **B.** The normal duct is made up of two layers of cells, myoepithelial and luminal cells that separate an empty lumen from stroma. As DCIs develops, epithelial cells begin to fill the lumen of the duct. Invasive breast cancer occurs when the cells break through the basement membrane.



is the transition of carcinoma *in situ* to invasive carcinoma in which the proliferating cells break through the basement membrane of the duct and into the surrounding tissue (Allison 2012). This transformation is likely due to the accumulation mutations and/or epigenetic events in the breast epithelium and microenvironment (Cowell et al. 2013). Invasive breast cancer can also spread to other organs in a process called metastasis that is the cause of death among women who die of breast cancer (Colzani et al. 2011).

The diverse pathological outcomes associated with and histological appearance of carcinomas are manifestations of genetic and epigenetic changes in these cells that occur over the lifespan. One model of carcinogenesis postulates that a normal cell must acquire proliferative and invasive capabilities to become malignant (Bombonati and Sgroi 2011). Moreover, it has been hypothesized that the cell of origin of breast cancer is a mammary stem or progenitor cell that has unique properties that distinguish them from differentiated cells; the less differentiated the cell of

origin, the higher-grade disease (Ercan et al. 2014; Visvader 2011). Although the majority of tumor cells would consist of non-stem-like progeny, under this hypothesis only the malignant stem or progenitor cells would contribute to tumor progression or recurrence (Iseghohi 2016). It would follow that effective treatment would only need to target this population. The stem/progenitor model will be elaborated later in the chapter.

The treatment of breast cancer varies based on the stage, grade, and subtype of the pathology at diagnosis. The majority of invasive carcinomas are ductal carcinomas (70-80%) and will be the focus of this section. There are rarer types of invasive carcinomas including: invasive lobular, medullary, mucinous, tubular, invasive papillary, and metaplastic carcinoma (Robbins, Kumar, and Cotran 2010).

Upon diagnosis, the tumor is characterized by the TNM scale which defines the size and extent of the primary tumor (T), whether the cancer has reached nearby lymph nodes (N), and whether the cancer has metastasized, or spread to local lymph nodes (M) (Singletary and Connolly 2006). This combination of factors is categorized as the “stage” of the cancer, with Stage 0 being DCIS, Stage I-III as invasive cancer with no metastases, and Stage IV as metastatic breast cancer that has by definition spread to distant organs or lymph nodes outside of the breast (Singletary and Connolly 2006). Excision of DCIS is largely curative and breast cancer deaths are due to the subsequent development of invasive carcinoma from DCIS that was not resected. Stage I-III cancer treatment usually includes resection of the tumor including effected lymph nodes, radiation therapy, chemotherapy, and other drug therapies before or after surgery (Robbins, Kumar, and Cotran 2010). Stage IV breast cancer has the poorest prognosis and treatment includes surgery and systemic drug therapies (Robbins, Kumar, and Cotran 2010). Once distant metastases are present, a cure is unlikely, although long-term remissions can be achieved (Singletary and Connolly 2006; Miller et al. 2016).

The grade of breast cancer is defined as how similar or different the cancer cells appear from the growth pattern of the normal, healthy breast (Elston and Ellis 1991). Low grade breast cancer (Grade 1) cells grow in a slow, well-organized pattern. High grade breast cancer (Grade 3) look very different from normal cells and grow in a disorganized, irregular pattern with many cells dividing to make new cancer cells (Elston and Ellis 1991). The presentation of the cells from Grade 1 to 3 is characterized as differentiated in appearance to undifferentiated in appearance. Women with Grade 1 breast cancer have a 70% 24-year survival rate, while 46% of women with Grade 3 cancers die within 10 years of diagnosis (Robbins, Kumar, and Cotran 2010).

Breast cancers are also categorized based on genetic and protein expression profile which make up five major subtypes: luminal A, luminal B, normal, basal-like, and human epidermal growth factor receptor 2 (HER2) positive. The subtype correlates with prognosis and response to therapy and therefore is clinically important (Yersal and Barutca 2014). Breast cancer subtypes are defined by the histologic appearance as well as the presence of estrogen receptor (ER) and progesterone receptor (PR) and overexpression of HER2, which are targets for some breast cancer therapies. Approximately 80% of carcinomas that are ER and PR positive respond to hormonal manipulation as compared to 40% of cancers that express only one of those receptors (Robbins, Kumar, and Cotran 2010). HER2 overexpression can also be targeted through therapies that interrupt the HER2 pathway (Tai, Mahato, and Cheng 2010). Breast cancers that are “triple-negative” for these markers are highly aggressive and have no targeted treatment option (Yersal and Barutca 2014; Robbins, Kumar, and Cotran 2010). Furthermore, triple-negative breast cancer (TNBC) generally presents as a high-grade cancer with a high proliferation rate. The aggressive phenotype in combination with the lack of targeted therapies results in poor prognosis for TNBC patients. Genetic similarities between mammary stem and progenitor cell populations and malignant subtypes will be discussed in Section 1.3.

While non-Hispanic white women have the highest rates of breast cancer, women of African and Hispanic ancestry have higher rates of poorly differentiated, ER- tumors. There is also an increased incidence of TNBC in low-income and young women (Li et al. 2003; Vona-Davis and Rose 2009; Carey et al. 2006). As a result, there is a significant disparity in mortality between racial/ethnic minorities and low-income women who are already medically underserved. Health disparities in breast cancer will be expanded upon in Section 1.5.

There are several factors beyond race/ethnicity that substantially increase risk of developing breast cancer and include reproductive (e.g. age at menarche, first live birth, and breast feeding) and non-reproductive factors (e.g. environmental toxins, radiation exposure, and diet). Many of these risk factors underscore that the environmental impact on later breast cancer risk begins during mammary gland development (Pike et al. 1983; Colditz and Frazier 1995; Rosner and Colditz 1996; Peeters et al. 1994). From a breast cancer perspective, bountiful data has demonstrated several periods of the life span in which environmental exposures disproportionately increase breast cancer vulnerability: *in utero*, during puberty, pregnancy, postpartum involution, and menopausal-related involution (Colditz and Frazier 1995; Pike et al. 1983; Milanese et al. 2006; Martinson et al. 2013; Pepper Schedin 2006). The hallmark of these "windows of vulnerability" is tissue remodeling driven by developmentally regulated programs that engage the mammary epithelium, stroma, and adipose tissue (Martinson et al. 2013).

Risk factors vary between the subtypes of breast cancer. Some lifecycle events that reduce ER+ breast cancer risk, such as age at first birth, increase risk for ER- breast cancer. However, younger age at menarche increases breast cancer risk across all subtypes (Ambrosone et al. 2015; Peeters et al. 1994; Turkoz et al. 2013). Ostensibly this association exists due to the increased time in which the developing mammary gland is exposed reproductive hormones or deleterious environmental exposures (Pike et al. 1983). Yet, the mechanism by which exposure during early-life impacts breast cancer risk has not been thoroughly investigated.

1.2 Nulliparous mammary gland development

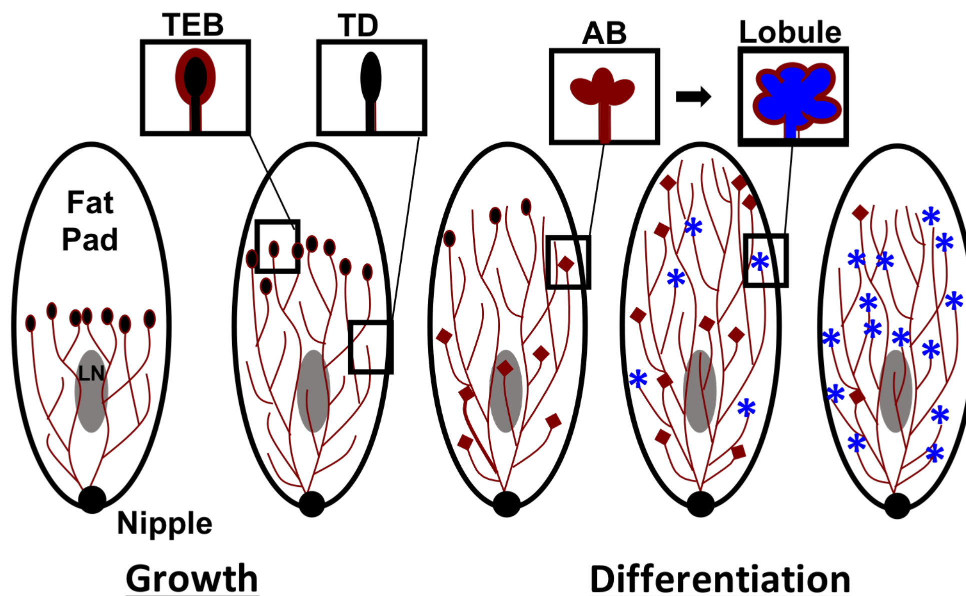
Mammary gland development begins *in utero* with the propagation of a rudimentary ductal tree. However, the majority of mammary gland development occurs during puberty. Between birth and the onset of ovarian cycles, the mammary ductal tree undergoes hormone independent growth that is relative to overall body development (Javed and Lteif 2013; Astwood, Geschickter, and Rausch 1937). With the onset of ovarian cycles in female mammals, proliferation and expansion of the mammary ductal tree occurs under the influence of hormones and growth factors (Macias and Hinck 2012; Cathrin Brisken 2002; Cathrin Brisken and O'Malley 2010; Marshall and Tanner 1969; Grumbach 2002; Javed and Lteif 2013; Beatrice and Barry 2000). This process occurs in two stages: growth and differentiation (Figure 1.2).

During the growth phase, the mammary ductal tree infiltrates the mammary fat pad led by highly proliferative, club-shaped terminal end buds (TEBs) (Paine and Lewis 2017). TEBs are identified and categorized based on the size of the club which is comprised of multiple layers of highly proliferative body cells that are protected from the fat pad by a layer of cap cells. Cycles of proliferation and apoptosis in the TEB are necessary for ductal elongation (Barres et al. 1992; Fata, Chaudhary, and Khokha 2001). The proliferation of the body cells drives the TEB forward and subsequent apoptosis clears the lumen of the newly formed duct (Mailleux, Overholtzer, and Brugge 2008). The apoptosis also provides space for subsequent proliferation and further propulsion of the duct into the fat pad. The extension of the mammary ductal tree continues until the mammary ductal tree reaches the end of the fat pad (I. H. Russo and Russo 1978). When the TEBs reach the end of the mammary fat pad, the proliferative index of the structures decrease, the body cells apoptose, and the TEBs cavitate to form terminal ducts (Gjorevski and Nelson 2011; I. H. Russo and Russo 1978). Over the course of ductal elongation, branching and elaboration of the ductal tree occurs. With each ovarian cycle, the primary ducts branch and

segment into subsidiary ducts (Arendt and Kuperwasser 2015; Cathrin Brisken and O'Malley 2010a).

The microenvironment surrounding the epithelial-lined mammary ducts includes mature adipocytes, pre-adipocytes, fibroblasts, blood vessels, inflammatory cells, and the extracellular matrix, all of which provide instructive and permissive signals to the developing mammary gland (Celis 2005; R C Hovey, McFadden, and Akers 1999). For example, it has been observed that transgenic mice that lack white adipose tissue are unable to develop normal mammary ductal structures and have reduced ductal branching, indicating a role for the adipocyte in pubertal ductal development (Couldrey et al. 2002). The mammary gland is thus increasingly appreciated as a

Figure 1.2: Rat mammary gland development.
Mammary gland development is broadly distinguished into two processes: growth and differentiaton. During the growth phase, the mammary gland develops from the main lactiferous duct, beginning at the nipple, and extends through the mammary fat pad. The bulbous end of these ducts is called terminal end buds (TEBs). TEBs are most numerous at approximately three weeks of age in the rat. During the differentiation phase, the ductal tree branches and alveolar buds (ABs) begin to form. As differentiation continues, ABs elaborate to include more acini and are recategorized as lobules. Over the course of this process, the mammary ductal tree comprises progressively more space in the mammary gland.



mammary epithelial ductal tree surrounded by an interactive adipocyte-laden microenvironment.

Adipose depots, endocrine organs themselves, differ depending on their anatomical location including in the mammary gland (Roca-Rivada et al. 2011; Porter et al. 2009; O'Rourke et al. 2009; Volden et al. 2013). However, few studies have directly addressed the influence of local mammary adipocyte-derived signals in mammary development and stem cell biology, which is surprising considering the proximity of adipocytes to mammary epithelium and the necessity for mammary adipocyte signals in normal mammary ductal development (Landskroner-Eiger et al. 2010; Humphreys et al. 1997).

Most literature defining puberty focuses on vaginal opening, the initiation of ovulation, and the capacity for pregnancy and therefore labels 4-7 weeks of the rat lifespan as puberty, typically referring to the subsequent stages of the lifespan as "adulthood" (Dziedzic et al. 2014; Russell D Romeo, Lee, and McEwen 2004; Laroche et al. 2009; Sengupta 2013). However, this time is only the beginning of reproductive function and does not represent the end of mammary gland development. In subsequent weeks, mammary ductal extension continues through the remaining portion of the fat pad (J Russo, Wilgus, and Russo 1979; I. H. Russo and Russo 1978). During this time, the mammary ductal tree branches and differentiates into alveolae and lobules (I. H. Russo and Russo 1978).

The extent of elaboration of the mammary ductal tree varies across mammals. In humans, the branched ducts further subdivide at the tips to form acini, a cluster of smaller ducts. This collection of ducts and surrounding stroma is termed a terminal duct lobular unit (TDLU) and is equivalent to the TEB in rodents (Javed and Lteif 2013). In contrast to the adult breast, during early development ductal structures are truly embedded in and in close proximity to mammary adipose tissue (Howard and Gusterson 2000). As the grouping of the ducts continue to branch and the number of ducts increases, the cluster of acini becomes larger and the TLDUs are recategorized as lobules. The lobules can vary in terms of size and number of acini and are further categorized based on this characteristic. Lobules type 1 is the smallest, least differentiated of

these structures and consists of a small cluster of terminal ducts and acini; lobule types 2-4 consists of increasing number of acini. In the nulliparous woman, the breast reaches maturation by 18 to 20 years of age and contains mainly lobules type 1, but also type 2 and 3 (Javed and Lteif 2013; Honeth et al. 2015). Final differentiation, characterized by the most elaborated and largest lobules, are only attained in women who have undergone further hormonal-induced changes during pregnancy and lactation (J Russo et al. 1990; J Russo, Wilgus, and Russo 1979). The course of change in morphology is designated as differentiation.

Similar to humans, the nulliparous rat mammary gland contains a rudimentary ductal tree as postnatal ovarian development begins. As puberty progresses, the ductal tree becomes more elaborate and contains a wide range of morphologies including terminal ducts, alveolar buds, and lobules (Young 2013). Indeed, alveolar buds are morphologically similar to lobules type I found in the human breast (P Schedin, Mitrenga, and Kaeck 2000; J Russo et al. 1990; Javed and Lteif 2013). Over the course of subsequent ovarian cycles, the alveolar buds continue to develop into more elaborate lobules.

Pubertal development in the mouse is similar to that of rats with several important exceptions. The nulliparous mouse has few, if any, alveolar buds and requires pregnancy and lactation for final alveolobular development (Cathrin Brisken and Ataca 2015). Therefore, the majority of pubertal mammary gland development is complete after TEBs cavitate into terminal ducts. While mice have been long used to model human mammary gland development, particularly in relation to pathogenesis, the developing mammary ductal tree of the rat is more similar to that of humans and includes more elaborate ductal structures. As observed in humans, the rat mammary gland continues to develop for a greater proportion of the lifespan in the nulliparous animal, long after the onset of ovarian cycles.

As described above, the majority of the mammary gland develops postnatally and is developmentally plastic through the estrous cycle. Terminal differentiation is conditional and only

occurs after multiple pregnancies. Studies in women demonstrate that puberty and young adulthood, is a key time during which the developing mammary is particularly sensitive to environmental and metabolic insults (Pike et al. 1983; Rosner and Colditz 1996; Wolff et al. 1996; Biro et al. 2006; Soliman, Sanctis, and Elalaily 2014; Koprowski et al. 1999; Young 2013). This period of sensitivity continues during young adulthood mammary gland development and into a woman's early 20s unless pregnancy occurs (Javed and Lteif 2013).

The majority of rodent models that are used to study the effect of the environment on mammary gland development have focused on early puberty when TEBs are present. While, a second body of literature focuses later in the life-span and includes the protective effects of pregnancy and post-partum involution which reduces mammary cancer risk in rodents and women (Bernstein 2002; MacMahon et al. 1970).

Prior to the advent of genetically engineered rodent models, carcinogen-induced neoplasia was used to understand the etiology of mammary cancer. This mode of inducing cancer has shaped research on the relationship between mammary gland development and breast cancer risk to focus on early puberty when TEBs are present. Due to their high proliferative index, TEBs are more susceptible to DNA-damage from carcinogens than other ductal structures. Exposure to carcinogens induces malignant transformation of the ductal epithelium likely through impaired DNA-repair and/or epigenetic alterations (I. H. Russo and Russo 1978; Cavalieri and Rogan 1978). Moreover, women exposed to carcinogens or radiation during the time at which they have terminal ductal lobular units, the human equivalent to TEBs, have significantly higher risk of developing breast cancer later in life (Colditz and Frazier 1995; Goodman et al. 1997). Thus research focused on the environmental causes of mammary cancer have effectively used TEB number as a read-out of breast cancer risk (Frenkel, Wei, and Wei 1995; Abba et al. 2016). For example, endocrine disrupting chemicals that effect ovarian hormones and/or modulate

ovarian steroid receptors can increase TEB number and are therefore hypothesized to increase breast cancer risk (Fenton 2006).

In recent years with the advent of genetically engineered rodent models, the TEB has remained the focus of environmental exposure models of breast cancer. This is partially due to the rich history of carcinogen-based research, but it is also due to historical limitations in genetic engineering technology. Until recent years, there has been a larger genetic 'toolbox' for mice, including embryonic-stem-cell based targeting (Ellenbroek and Youn 2016; Zan et al. 2003). Therefore, genetically engineered mice have been preferentially used in breast cancer research. Because mice do not undergo substantial nulliparous alveolobular development unlike their rat and human counterparts, there persists a limited understanding of how environmental factors affect nulliparous alveolobular development and how these changes could mediate breast cancer risk.

There has also been a rich literature on the protective effects of pregnancy and lactation on breast cancer risk. Final differentiation occurs with full-term pregnancy and breast feeding. The biological and morphological changes in the breast resulting from final differentiation in full-term pregnancy and breast feeding generally reduces later breast cancer risk. It is hypothesized that lactation suppresses ovulation and triggers terminal differentiation of milk-producing luminal epithelial cells, removing them from the potential pool of cancer precursors (J. Russo, Balogh, and Russo 2008). Moreover, a mammary gland that remains relatively undifferentiated is at greater risk for developing most types of breast cancer (Bernstein 2002; MacMahon et al. 1970; D. K. Sinha, Pazik, and Dao 1988; Hilakivi-Clarke et al. 2006).

1.3 Mammary stem cells

In mammals, the luminal epithelial and the basal/myoepithelial cells in the ductal tree are populated by mammary stem cells and lineage-restricted progenitor cells; together these cells

form the stem cell hierarchy (Figure 1.3). Mammary stem cells are long-term renewing cells (LT-RC) that possess properties including self-renewal and multi-directional differentiation into lineage restricted progenitor cells. It has been hypothesized that different subtypes of breast cancer may derive from distinct mammary stem cell or progenitor cell populations and is an emerging area of research (Ginestier and Wicha 2007; Ercan et al. 2014; Visvader 2011; Rangel et al. 2016). Because these populations are dynamic through the lifetime, and are a long-lived target for errors, these cells provide an attractive but poorly understood link between developmental insults and cancer risk in adulthood (Rangel et al. 2016; Joshi et al. 2010; Asselin-Labat et al. 2010). At the time of writing, the populations of mammary stem cells and progenitor cells are still being defined.

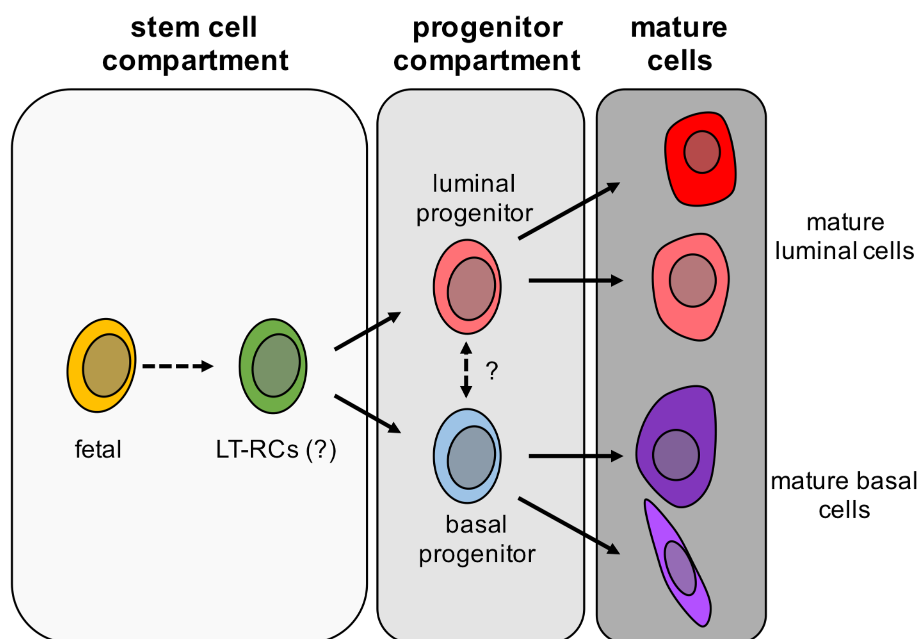
The molecularly defined “stem cell state” in adult tissues has been largely defined by the blood system. In this system, a single cell is capable of generating mature blood cells in addition to daughter cells with the same lineage differentiation capability (Clarke and Frampton 2016). Therefore, these cells can regenerate and sustain the entire blood system. The existence of similar cells in the adult mammary gland remains controversial. Yet, lineage restricted progenitor cells have been well characterized (Chen, Liu, and Song 2017).

Mammary stem cells are the parent cells of all mammary epithelial progenitor and differentiated cells necessary to populate the mammary ductal tree throughout its growth and differentiation (Visvader and Stingl 2014). Because these are long-living cells, mammary stem/progenitor cells provide a link between mammary gland development, environmental exposure early in life that modifies the stem and progenitor population, and later breast cancer risk.

The ability of adult mammary ductal cells to regenerate an entire mammary ductal tree has been demonstrated as early as the 1950s through transplanting ductal fragments into pre-pubertal mammary fat pads cleared of endogenous ductal epithelium (Deome et al. 1959). In 1998, it was discovered that a single dissociated cell could repopulate both luminal and basal

lineages of the mammary gland (Kordon and Smith 1998). Cell fractionation strategies have determined that primarily basal cells expressing high levels of CD49f and CD29 with reduced expression of luminal markers (CD24, EpCAM, etc) have the ability to repopulate the mammary gland (Makarem et al. 2013). Therefore, this population is often referred to as the mammary stem cell population. Indeed, enriching for these markers has allowed for the isolation of mammary repopulating units (MRUs) at a purity of ~1-2%. Luminal populations expressing CD49f (CD24 hi CD49f Lo) also have MRU activity, albeit to a much lesser extent (0.001%) (Shackleton et al. 2006). Human cells with high MRU activity have also been identified using immunodeficient mice as recipients. Like their mouse counterparts, these cells share markers of basal cells (CD49f Hi and EpCAM Lo) and are found at a frequency of ~0.01-0.1% (Makarem et al. 2013).

Figure 1.3: Diagram of mammary stem cell hierarchy



Lineage tracing studies have demonstrated that luminal populations contain only lineage-restricted progenitors capable of producing luminal progeny (Van Keymeulen et al. 2011; Lafkas et al. 2013; Rodilla et al. 2015; Tao et al. 2014). Conversely, the data for basal cells is conflicted with some studies supporting the view of bipotential basal cells that could be considered true

mammary stem cells (Fu et al. 2017; Wuidart et al. 2016; Wang et al. 2014) and others supporting lineage restricted cells (Van Keymeulen et al. 2011; Lafkas et al. 2013; Rodilla et al. 2015; Wuidart et al. 2016; Prater et al. 2014). It is hypothesized that basal cells that contain MRUs may de-differentiate and therefore are not true stem cells (Trejo et al. 2017). Indeed, microenvironmental factors that influence MRU activity have remained largely uninvestigated (Gyorki et al. 2009; Makarem et al. 2013). Therefore, it is unclear if the purported adult mammary stem cell is simply a result of de-differentiation induced by the microenvironment.

In vitro methods to detect the clonogenic potential of mammary epithelial cells were first described for rat and human cells and eventually for mouse cells (Emerman et al. 1996; Zaunreiter et al. 2005; J. Stingl et al. 2001; John Stingl, Eaves, and Emerman 2000). These methods rely on plating cells at a low density under conditions that prevent the growth of mammary cells to adhering to plastic. This assay, called a mammosphere forming assay, has significant limitations. For example, long-term quiescent stem cells (LT-SC), thought to rest in G0 phase, will not experience the precise combination of factors that would favor activation *in vivo* (Lombardo et al. 2015). The mammosphere assay instead enables the growth of cells either poised for mitotic division or already dividing, which is more common among lineage-restricted progenitors (Pastrana, Silva-Vargas, and Doetsch 2011; Chen, Liu, and Song 2017). Additionally, because of the very strong tendency of mammary epithelial cells to adhere to one another, the resultant clusters obtained (mammospheres), may represent a variable mixture of aggregation and proliferation. Unless such cultures are initiated with a single isolated cell or the cells are immobilize in a semi-solid medium such as Matrigel, the number of structures obtained cannot be used to quantify clonogenic cells (John Stingl et al. 2006).

For the purposes of simplicity, the CD24+CD49fhi basal MRUs will be referred to as mammary stem cells, while the CD24+CD49f lo luminal population that has clonogenic potential will be referred to as luminal progenitor cells.

There is a bounty of evidence supporting the theory that these luminal and basal populations, whether unipotent progenitors or true mammary stem cells, are the target of transformation leading to invasive breast cancer. While still controversial, the “stem cell theory” postulates that stem or progenitor cells that acquire pro-oncogenic mutations over the lifespan are the cells-of-origin in oncogenensis. It has been hypothesized that long-term survival and expansion of these cells increase susceptibility to neoplastic transformation (Ginestier and Wicha 2007). In addition, sequential accumulation of deleterious genetic and/or epigenetic alterations in mammary stem and progenitor cells that persist over the lifespan may render them as the source of breast cancer initiation or relapse.

Molecular regulators of mammary stem cell differentiation include the Wnt/beta-catenin and Notch signaling pathways. Elevated Wnt signaling induces expansion of and increases the MRUs within the mammary stem cell population and therefore has been indicated as a factor that promotes mammary stem cell self-renewal (Shackleton et al. 2006; Y. A. Zeng and Nusse 2010; Wang et al. 2014). Conversely, Notch signaling induces luminal progenitor cell fate of mammary stem cell progeny and inhibition of Notch promotes self-renewal of the mammary stem cell population with high MRU (Bouras et al. 2008). Other molecular pathways and transcriptional regulators that have been indicated in mammary stem cell self-renewal and lineage commitment include: Bim, c-myc, Hedgehog, Slug, and Sox9. In addition, all of these factors have also been indicated in mammary cancer development (Chen, Liu, and Song 2017).

Gene expression profiling of different mammary epithelial cell populations has uncovered similarities to specific subtypes of breast cancer. Relatively mature luminal cell gene signatures shared most similarities to Luminal A and B breast cancer profiles. Mammary stem cells display a genetic similarity to the ‘claudin-low’ subtype; the luminal progenitor cells that express Lgr5 and Tspan8 also have a transcriptome similar to claudin-low tumors (Fu et al. 2017; Rangel et al. 2016; Chen, Liu, and Song 2017). Contrary to previous hypotheses, the genetic signature of other

luminal progenitor cells was most similar to TNBC (Lim et al. 2009). Indeed, these cells are candidate cells-of-origin for BRCA1-associated basal-like breast cancer (Lim et al. 2009).

The ability of luminal progenitors to switch to a basal-like mammary cancers has been demonstrated using BRCA1/p53-deficient transgenic mouse model (Molyneux et al. 2010; Lim et al. 2009; Proia et al. 2011; Bai et al. 2013). Lineage switching in breast cancer progression is not exclusive to luminal progenitors nor to the BRCA1 background. For example, expression of oncogenic $PIK3CA^{H1047R}$ in lineage-restricted luminal or basal cells triggers dedifferentiation of the cells and leads to the development of multi-lineage mammary tumors with intratumoral heterogeneity (Van Keymeulen et al. 2011; Koren et al. 2015; Chen, Liu, and Song 2017).

Decades of research has made a clear association between morphological differentiation and reduced cancer risk. Yet, how this association affects the stem and progenitor pool remains unknown. Nulliparous women and women with the BRCA1 mutation, who are at high risk for developing breast cancer, have less differentiated mammary morphology and have a proportionally higher amount of mammary stem cells to differentiated cells, using the ALDH1A1 stem cell marker (Honeth et al. 2015). Data from the preneoplastic MMTV-wnt-1 mice also suggests that an increase in mammary stem cells increases cancer risk (Shackleton et al. 2006). Interestingly, stem cell number in this and other mouse models is reported as the percentage of stem cells to CD24+ epithelial cells, and therefore, it may be *either* absolute stem cell number *or* the proportion of stem cells to differentiated cells that confers risk.

The regulation of mammary stem and progenitor populations during mammary gland development is under the control of factors both present in the systemic circulation and derived from the mammary microenvironment. As discussed earlier, the presence of mammary adipose tissue is required for mammary ductal differentiation as loss of this fat depot hinders mammary ductal differentiation (Couldrey et al. 2002). However, the role of mammary adipose tissue in mammary stem and progenitor cell biology remains unclear (Cunha et al. 1997). Mammary

adipocytes are a dynamic endocrine cell type that secrete a host of factors that can be broadly broken down into: 1) steroid hormones; 2) lipid species; and 3) adipocyte-specific proteins termed adipokines (Rosen and Spiegelman 2014). Recent work has indicated that adipocyte secreted factors (e.g. leptin, adiponectin) modify clonogenic proliferation *in vitro* and therefore could play a role in mammary stem cell biology (Cathrin Brisken and O'Malley 2010a; Cathrin Brisken and Ataca 2015; Inman et al. 2015). Moreover, adipocyte-derived lipids have been indicated in proliferation and self-renewal in stem cells of other tissues (Cathrin Brisken and Ataca 2015; Pébay, Bonder, and Pitson 2007; Dottori et al. 2008).

Ovarian hormones estrogen and progesterone also play a key role in mammary stem cell biology (Joshi et al. 2010; Asselin-Labat et al. 2010). For example, the mammary stem cell population transiently increases 14-fold over each ovarian cycle, when progesterone is secreted by the corpus luteum or when an animal is exposed to exogenous progesterone (Joshi et al. 2010). Notably, mammary stem cell subpopulations are negative for ER, PR, and ErbB2, similar to cells in TNBC (Shackleton et al. 2006; Lim et al. 2009). Therefore, the involvement of reproductive steroids is mediated through accessory cells (Visvader and Stingl 2014).

1.4: Reproductive hormones and mammary gland development

In the 1950s, seminal experiments in rodents defined the minimal hormonal requirement of mammary gland development (Nandi 1958; Lyons 1958). Endocrine ablation and hormone replacement experiments have established that additive and sequential treatment with 17-β estradiol, progesterone, prolactin, glucocorticoids, and growth hormone, is necessary for mammary gland development. As these hormones act on multiple target tissues including the mammary microenvironment, they directly and indirectly affect the mammary gland. In fact, functional receptors for estrogen, progesterone, and glucocorticoids are present in the mammary glands of fetus and neonate rodents and humans (Russell C. Hovey, Trott, and Vonderhaar 2002).

Extensive work in transgenic mice have further elucidated the mechanisms of hormone-dependent mammary gland development. ERα and ERβ are expressed in both the mammary epithelium and stroma (Daniel, Silberstein, and Strickland 1987). Through transplantation studies in transgenic mice, researchers determined that ERα expression in the mammary epithelium, but not the mammary fat pad, is necessary for mammary morphogenesis in puberty and pregnancy (Feng et al. 2007). However, ERα activation signals proliferation of the mammary ductal tree by paracrine mechanisms (Mueller et al. 2002). The role of ERβ has not been well defined. Side branching is delayed in *Erβ-/-* mice, but this may be secondary to altered ovarian cycles (Antal et al. 2008).

Progesterone signaling in the developing mammary gland occurs through both direct and paracrine mechanisms and is involved in ductal branching and alveolobular development (Cathrin Brisken and O'Malley 2010b). PR is co-expressed with ERα in a subset of luminal epithelial cells (Feng et al. 2007). There are two isoforms of PR, yet careful studies in mice demonstrate that PR-β is the pertinent isoform in the mammary gland (Cathrin Brisken and Ataca 2015). Knockout and transplantation models have further demonstrated that it is PR signaling specific to the mammary epithelium that is necessary for side-branching and alveologenesis (Cathrin Brisken and Ataca 2015).

Many of the models defining the role of estrogen and progesterone in mammary gland development have utilized ovarectomized and knockout mice that have a complete absence of these hormones and their receptors. Yet, there are large differences in the exposure of estrogen and progesterone within the individual across phases of the ovarian cycle (e.g. luteal versus follicular phase). The effects of these reproductive steroids on mammary gland development may be additive over the course of several ovarian cycles.

The level of estrogen and progesterone is dynamic throughout the ovarian cycle (Figure 1.4) and is under the control of the hypothalamic-pituitary-gonadal (HPG) axis (Cora, Kooistra,

and Travlos 2015). While there is individual variation, the ovarian cycle occurs over the course of 4-5 days in rats and 28 days in humans. At the beginning of the ovarian cycle, the anterior pituitary secretes luteinizing hormone (LH) and follicle-stimulating hormone (FSH) to promote development of the ovarian follicle. FSH causes the proliferation of the granulosa cells in the ovary, which secrete estrogen (Rao et al. 1978). LH increases the secretion of androstenedione from the thecal cells in the ovary (Barbieri 2014). Androstenedione is converted to estrogen which contributes to the significant increase in estrogen secreted by the ovary (Barbieri 2014; McLean et al. 2012).

The significant increase in estrogen causes proliferation of the vaginal epithelium that makes up the uterine lining and also signals to the hypothalamus and anterior pituitary to inhibit the secretion of LH and FSH (Barbieri 2014). As the follicle continues to develop and the granulosa cells continue to produce estrogen, they also begin to secrete progesterone and inhibin. Inhibin further decreases the secretion of FSH from the brain. After estrogen secretion reaches a critical level, its action on the pituitary gland changes from inhibiting to promoting FSH and LH secretion (Barbieri 2014). Because inhibin is also being secreted from the developing follicle, the net effect is a slight increase in FSH and a large increase in LH. This is called the luteal surge, which causes the egg to be expelled from the ovarian follicle in a process called ovulation. Twelve hours prior to the LH surge, there is a sharp transient increase in progesterone (Hoff, Quigley, and Yen 1983). It has been proposed that the pre-ovulatory progesterone surge originates from either the adrenal gland or the ovary (De Geyter et al. 2002). However, its regulation and origin remains controversial. The beginning of the ovarian cycle until ovulation is referred to as the follicular phase of the ovarian cycle and as the proestrus and estrus phases of the estrous cycle (Cora, Kooistra, and Travlos 2015; Barbieri 2014).

After ovulation, the luteal phase (also known as metestrus and diestrus) begins. LH and FSH concentrations in the blood are high and cause the remaining follicular body to become a

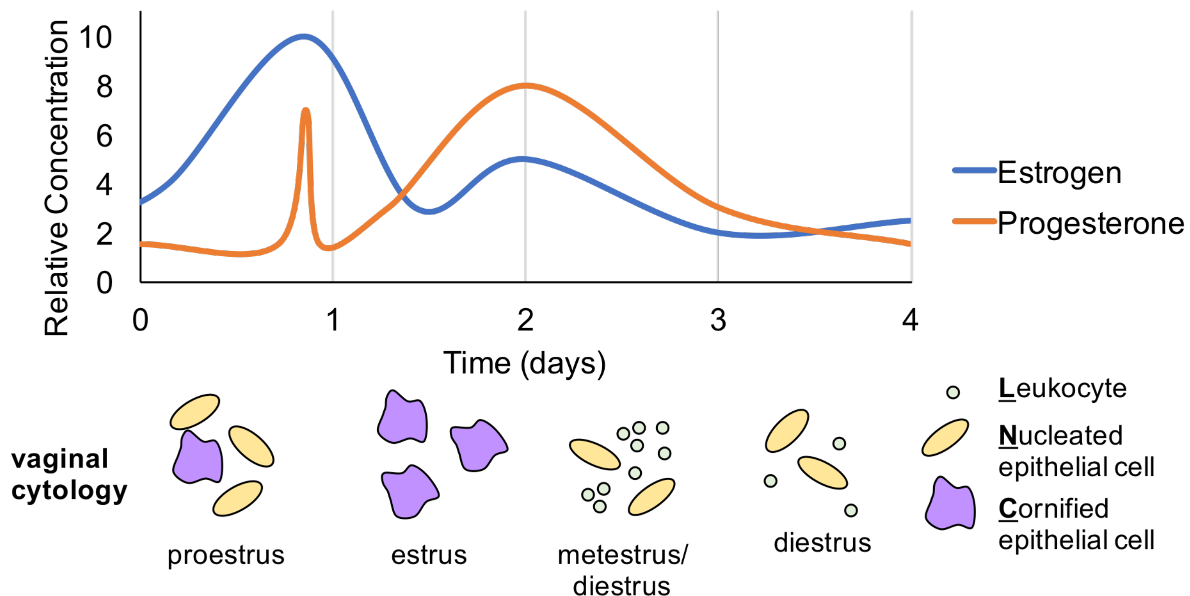
corpus luteum (Barbieri 2014). During this time, the corpus luteum slows its production of estrogen, begins to increase its secretion of progesterone and inhibin (Strauss, Barbieri, and Yen 2014). This combination of progesterone with estrogen prepares the uterine lining for egg implantation. The secretion of progesterone, estrogen, and inhibin by the corpus luteum also suppresses LH and FSH production and secretion by anterior pituitary. Without LH and FSH and in the absence of pregnancy, the corpus luteum begins to atrophy. This causes estrogen and progesterone levels to drop, which signals the end of the luteal phase. When estrogen and progesterone levels drop, LH and FSH begin to rise again and a new ovarian cycle is set in motion.

The vaginal epithelium is responsive to the fluctuation of estrogen and progesterone throughout the estrous cycle (Figure 1.4). Sampling the cells of the vagina (vaginal cytology) can be used as a dynamic bioassay to determine where a female is in the estrous/ovarian cycle and relative exposure to these reproductive steroids. During proestrus and estrus, when estrogen is relatively high, the vaginal epithelium proliferates and therefore the vaginal cytology includes largely vaginal epithelial cells. Nucleated epithelial cells are actively proliferating and are most abundant during proestrus. Cornified cells are epithelial cells that are undergoing degeneration and apoptosis after the post-ovulatory decrease in estrogen and therefore are most abundant during the estrus phase. Leukocytes filter through the wall of the vagina when progesterone receptor activation inhibits estrogen-induced proliferation. Therefore, leukocytes are most abundant when estrogen is relatively low and progesterone is being actively secreted by the corpus luteum (Cora, Kooistra, and Travlos 2015).

The morphology of the ductal tree is influenced by the fluctuation of ovarian steroids after the onset of ovarian cycles and as the ovary matures (P Schedin, Mitrenga, and Kaeck 2000). Vaginal opening is an outward indicator of ovarian development. Immediately after vaginal opening, rats have irregular, anovulatory cycles that do not produce a mature, progesterone secreting, corpus luteum. Therefore, the hormonal milieu is one of estrogen with relatively low

levels of progesterone, and this period of the lifespan corresponds to mammary ductal extension (Cathrin Brisken and Ataca 2015).

Figure 1.4 Temporal concentrations of estrogen and progesterone and resulting vaginal cytology



With the onset of regular cycles, the mammary ductal tree shifts from growth to differentiation. During the follicular phase when progesterone levels are low, the mammary ductal tree is relatively quiescent. Its proliferative index is low and is comprised of alveolobular units with few acini (P Schedin, Mitrenga, and Kaeck 2000; Joshi et al. 2010). As the hormonal milieu shifts in the luteal phase and the ductal epithelium is exposed to both progesterone and estrogen, the proliferation of the epithelium increases. The number of acini per alveolobular unit also increases (P Schedin, Mitrenga, and Kaeck 2000). Alveolobular differentiation occurs over the course of repeated sequential ovarian cycles.

There is variance in the reproductive life history (e.g. onset of ovarian cycles, ovarian cycle length, length of each phase) between individuals. Researchers attribute the association of

younger age at menarche and breast cancer risk to the increased exposure of the mammary gland to progesterone and estrogen due to an increased number of estrous cycles (Pike et al. 1983). Indeed, postmenopausal hormone replacement therapy (HRT; exogenous estrogens) increases the risk of breast cancer 1.2 - 1.7 fold (Easton et al. 1997; Tavani and La Vecchia 1999; Rangel et al. 2016). However, association between younger age at menarche and more menstrual cycles may not explain the association between age of menarche and breast cancer risk. For example, older age at menopause increases breast cancer risk by less than 1% once adjusted for age at menarche and other demographic factors (Collaborative Group on Hormonal Factors in Breast Cancer 2012). Moreover, while the increased risk from HRT ameliorates within two years of cessation, reducing the age of menarche from age 11 to age 10, increases annual risk of breast cancer 30 years later by 10% (Hsieh et al. 1990). Therefore, there are likely changes in the mammary gland that mediate the risk conferred from earlier reproductive maturity.

The mechanisms by which natural variation in ovarian cycle onset and maturity can affect mammary gland development is widely understudied. As mentioned earlier in the section, knockout and hormonal supplementation models have largely been utilized to determine the effect of estrogen and progesterone on mammary gland development. While informative, these models are blunt tools that may not integrate the temporal factors that influence mammary gland development. For example, women who are younger at menarche, and therefore at greater risk for developing breast cancer, tend to have accelerated ovarian maturity and shorter cycles (Wallace et al. 1978). Because accelerated ovarian maturity results in a shorter anovulatory phase, it could potentially decrease mammary ductal extension that occurs during this time in the life course.

Social environment, particularly exposure to stressors, contributes to individual variation in reproductive life history. Exposure of stress hormones, such as glucocorticoids, can affect the HPG axis in direct and indirect mechanisms. For example, mammals that undergo psychosocial

stress exposure have accelerated ovarian development in early puberty including earlier onset of cycles and regular cycles earlier in the life span (Surbey 1990; Sung et al. 2016; Fenster et al. 1999; Gretchen L. Hermes and McClintock 2008). While the mechanisms that drive this association remain unclear, glucocorticoids do have direct effects on other reproductive processes. Acute stressors can potentiate ovulation through the induction of a LH surge (Mahesh and Brann 1998; Tarín, Hamatani, and Cano 2010). Indeed, infusion of adrenocorticotropic hormone (ACTH), an upstream regulator of glucocorticoid secretion, stimulates LH release in women undergoing estrogen replacement (Puder et al. 2000). Glucocorticoids can also directly signal in the reproductive tract. For example, glucocorticoid receptor (GR) activation of pre-ovulatory granulosa cells increases progesterone production (Huang and Shirley Li 2001). Moreover, GR activation of the vaginal epithelium inhibits ER-mediated proliferation (Whirledge and Cidlowski 2017). It may be a combination of these mechanisms that result in differences in reproductive processes between stressed and unstressed individuals.

1.5: Psychosocial stress and breast cancer risk

Psychosocial stressors cause reproducible changes in neuroendocrinology that increase susceptibility to certain pathologies. The physiologic response to stress results in activation of the sympathetic nervous system which increases the blood concentration of noradrenaline, adrenaline, and adrenal glucocorticoids (Stratakis and Chruousos 1995). Effective coping implies that the stress response is activated rapidly and efficiently terminated; this process is called allostasis (Ramsay and Woods 2014). If the stress response is inadequate, excessive, or prolonged, the result is a disruption of homeostasis that ranges from the behavioral to molecular level. The accumulation of homeostatic dysregulation is called the “allostatic load” (Djuric et al. 2008).

The hypothalamic-pituitary-adrenal (HPA) axis is responsible for integrating environmental changes and coordinating the stress response. On a physiological level the HPA axis regulates homeostatic systems including the metabolic, cardiovascular, immune and reproductive system (Stratakis and Chruousos 1995). Upon stimulation, the hypothalamus releases corticotrophin releasing hormone (CRH), resulting in secretion of ACTH from the anterior pituitary. The adrenal gland then releases corticosteroids (primarily cortisol in humans and corticosterone in rodents). During the acute stress response, corticosteroid signaling results in an increased availability of energy through gluconeogenesis and a suppression of the metabolic processes of the immune system resulting in a greater availability of glucose (Del Rey, Chrousos, and Besedovsky 2008). The increased availability of glucose has an evolutionary purpose to facilitate the fight or flight response.

In addition to modulating the acute stress response the HPA axis releases corticosteroids in a diurnal rhythm. In healthy individuals, cortisol rises rapidly after waking, then gradually falls over the course of the behavioral day. There is a brief rise again in the late afternoon, falling again with a trough during the behavioral night (Oster et al. 2016).

Corticosteroids operate in the stress system through the mineralocorticoid and glucocorticoid receptor (MR and GR) on neurons of limbic structures. During an acute stress response MR activation is implicated in the onset of the stress response, and GR activation mobilizes energy stores for the purpose of facilitating recovery (Herman et al. 2016). GR also participates in the control of energy metabolism, from appetite and macronutrient choice to energy disposition and storage.

The effect of repeated stress exposure has been well-characterized in the brain and leads to hippocampal dysfunction. In the short term, elevated adrenal steroids can suppress neuronal mechanisms that are involved in short term memory (Kirschbaum et al. 1996; B S McEwen and Sapolsky 1995). Repeated stressors cause atrophy of dendrites and after prolonged stress can

result in their death (B S McEwen and Sapolsky 1995; Uno et al. 1989). Indeed, psychosocial disorders related to unremitting stress (e.g. depression, post-traumatic stress disorder, and Cushing's syndrome) are associated with hippocampal atrophy as measured by magnetic resonance imaging (Bremner 2006).

Unremitting stress exposure results in adaptive changes in serum concentrations of corticosteroids over the course of the diurnal rhythm, after an acute stressor, and at baseline. People exposed to unremitting stress have a flat diurnal profile of cortisol release with lower morning peak cortisol and higher evening cortisol. Moreover, the acute stress response is often greater in individuals with high allostatic load and the concentration of corticosteroids takes longer to return to baseline (Herman et al. 2016). As a compensatory mechanism, unremitting exposure to stressors results in lower baseline corticosteroids (H Selye 1946). The mechanisms that regulate stress reactivity are unique from that of baseline corticosteroid concentrations (Sapolsky, Romero, and Munck 2000). Moreover, imposition of an acute stress and subsequent sampling of the blood or saliva can be used to experimentally measure corticosterone stress reactivity and response. These data, along with baseline concentrations of corticosteroids, can be used to infer long-term exposure to stress.

Other tissues in the body respond to allostatic load of unremittent stressors via direct and indirect mechanisms. Repeated blood pressure surges resulting from psychosocial stress are linked to atherosclerosis and myocardial infarction (Bairey Merz et al. 2002). Exposure to stress also results in alterations of brain-gut interactions. Aberrant CRH signaling increases permeability and alters motility of the gastrointestinal tract and therefore has been indicated in the pathology of inflammatory bowel disease, irritable bowel syndrome, and other gastrointestinal diseases (Taché, Kiank, and Stengel 2009). Metabolic disorders including diabetes and abdominal obesity are also influenced by stress exposure. While metabolic diseases are often multifactorial and polygenic, initiation of disease requires the combination of underlying genetic predisposition and

environmental factors (Morton 2010). There is an obvious link between metabolic disease, hypercaloric diet, and physical inactivity, but recent data has clearly established a link between metabolic syndrome, insulin resistance, and lifetime stress exposure including stress resulting from low socioeconomic status (Brunner, Chandola, and Marmot 2007; Mackenbach et al. 1997; B. McEwen and Mirsky 2002). Indeed, animal models of chronic stress link HPA hyperactivity with obesity, hyperphagia, and hypercholesterolemia. Elevated glucocorticoids stimulate gluconeogenesis in the liver, opposes insulin effects, and prevents insulin secretion (Sapolsky, Romero, and Munck 2000; Mary F. Dallman 2010; M F Dallman 1993).

Links between stress exposure and cancer are less clear. Experimental research in animals has determined that stressors are associated with increased initiation, growth, and metastasis of certain tumors; some of these mechanisms have been recapitulated in human subjects. However, prospective studies have shown mixed results. The lack of data is partly attributable to the practical difficulties in designing and implementing adequate studies. For example, there are large individual differences in the stress response (Ellis, Jackson, and Boyce 2006) and the effect of stress-related perturbations is likely tissue specific (Cohen, Janicki-Deverts, and Miller 2007).

Individual differences in the stress response depends not only on the environment (e.g. timing, intensity, and duration of stressor), but also the genetic predisposition of the individual. While the mechanisms of action have not been clearly elucidated, it has been hypothesized that there is a critical period during development in which the levels of stress hormones “calibrate” the HPA axis. For example, early-life exposure to moderate levels of corticosterone is associated with stress resilience in adult rats, whereas exposure to high doses is associated with stress vulnerability (Bruce S McEwen 2008). Recent data have also demonstrated that there is a genetic component to the stress response. There are single nucleotide polymorphisms that predict resiliency to environmental stressors (Zhou et al. 2008; Rodrigues et al. 2009). In fact, exposure

to environmental stressors such as prenatal stress and childhood abuse induce epigenetic variation in humans (Oberlander et al. 2008; Radtke et al. 2011; McGowan et al. 2009). Mechanistic studies in rodents have demonstrated that these epigenetic changes can be inherited and can cause behavioral changes in subsequent generations.

Despite individual differences in the stress response, psychosocial stress can be reproducibly modeled using rodents. Similar to women, female rodents are highly social animals that spend a significant amount time in physical contact. These interactions mediate the animal's response to daily stressors (Gretchen L Hermes et al. 2006; Hawkley et al. 2012). For rats, isolation is an atypical social condition that precludes natural behaviors such as cooperative grooming, feeding, and rearing offspring (Cohen 1988). The effect is a long-term suboptimal social environment, that prohibits the natural behavioral mechanisms of coping with stressful life events. For rodents in a laboratory setting, these stressors include those of animal husbandry, temperature, noise, and experimental procedures (S. A Cavigelli et al. 2005). After repeated exposure to stressors without a social support system, most rats recapitulate the elevated stress response, lower baseline, and flattened diurnal rhythm of glucocorticoids observed in women exposed to unremittent stress. Therefore, social isolation of female rats models chronic stressors in humans on a physiological level.

Health disparities are multi-factorial and result from a variety of factors including: economic determinants, education, neighborhood, environment, access to care, and provider bias (Djuric et al. 2008). Racial and ethnic minorities have a greater negative burden of these factors and therefore poorer health outcomes than that of white Americans.

The physiologic response to chronic stress may also contribute to the disparities in health outcomes. Chronic stressors associated with health disparities include perceived discrimination, neighborhood stress, daily stress, family stress, acculturative stress, environmental stress (such as low socioeconomic status) and maternal stress (Djuric et al. 2008; McCabe et al. 2010; Lehavot

and Simoni 2011). Health disparities associated with chronic stress are particularly associated with metabolic, immune, and reproductive health disorders (Jang et al. 2010; Mezuk et al. 2010; D. R. Williams and Neighbors 2001; Kaholokula, Iwane, and Nacapoy 2010; B S McEwen and Sapolsky 1995).

Minorities generally report more stress than white Americans which is additionally increased by lower socioeconomic status (SES) in the aforementioned group (Cohen and Janicki-Deverts 2012). Linking reported chronic stress in racial/ethnic minorities and people with low SES to hormonal symptoms of chronic stress exposure has shown mixed results. However, this is likely due to differences in collecting and analyzing cortisol and individual differences in the manifestation of allostatic load over the life-course. Nonetheless, low SES has been consistently associated with a blunted pattern of diurnal cortisol secretion (Dowd, Simanek, and Aiello 2009). This pattern that has also been described in racial and ethnic minorities. Moreover, this disruption in cortisol rhythm is particularly pronounced in adolescents and women (DeSantis et al. 2007; Cohen and Janicki-Deverts 2012; McCallum et al. 2006; Franco Suglia et al. 2010).

While prospective studies on stress and breast cancer risk have shown mixed results, women exposed to the chronic stressor of racial discrimination and low socioeconomic status have a greater breast cancer incidence of aggressive subtypes such as TNBC (Vona-Davis and Rose 2009). Moreover, women particularly stressed by diagnostic uncertainty while awaiting biopsy results also have a similarly increased risk of having aggressive breast cancer (Taylor et al. 2007; Vona-Davis and Rose 2009; Liao et al. 2008).

The association between psychosocial stress and breast cancer risk has been successfully modeled using rodents. Sprague Dawley rats, which are genetically predisposed to develop a wide variety of malignant and non-malignant mammary tumors, develop a greater proportion of malignant neoplasms when exposed to the stressor of social isolation over the life course (G. L. Hermes et al. 2009). As described earlier in the section, simply housing female

rodents on a rack of single cages in a colony room (social isolation) is enough to induce a hypervigilant state and heighten glucocorticoid reactivity to everyday husbandry and mild experimental stressors even though they can still see, hear, and smell their group-housed counterparts (Hawkley et al. 2012; LeFevre and McClintock 1991; Gretchen L. Hermes and McClintock 2008). As early as 8 weeks after the imposition of social isolation, isolated rats have increased stress reactivity and delayed stress recovery (G. L. Hermes et al. 2009). Rats with higher stress reactivity are more likely to die with mammary tumors and a rat's social role within a group of rats also predicts tumor burden (Sonia A. Cavigelli, Yee, and McClintock 2006; Yee et al. 2008). Thus, it is high or low glucocorticoid stress reactivity that is the key predictor of mammary tumor development, even though it may be caused by different psychological conditions among rats.

Social isolation is also associated more aggressive tumor biology in mouse models of breast cancer. The C3(1) SV40 Large T Antigen (Tag) mouse develops mammary tumors that mimic the histology and genetic expression of TNBC (Green et al. 2000). In this model, social isolation is associated with an increase in size and grade of mammary tumors (J. B. Williams et al. 2009). The changes in tumor biology correlates with metabolic changes specifically to the mammary adipose tissue prior to the presence of invasive cancer (Volden et al. 2013). Indeed, the mammary adipose tissue secretome of isolated mice increases the proliferation of pre-malignant epithelial cells more than the adipose tissue secretome from mice living in groups (Volden et al. 2013; Volden et al. 2016). Therefore, it is likely that the association between psychosocial stressors and mammary cancer progression is mediated by molecular changes in the mammary adipose tissue in addition to direct GR activation of mammary epithelium.

The effect of GR expression on breast cancer progression depends on the molecular subtype (Pan, Kocherginsky, and Conzen 2011). As a nuclear receptor, GR has chromatin remodeling activity which is affected by the presence or absence of other nuclear receptors (West

et al. 2016; Yang et al. 2017). In ER+ breast cancer, high GR mRNA expression is associated with improved relapse-free survival presumably due to upregulation of transcripts that encode pro-differentiating pathways. The opposite association exists in ER- breast cancer in which high GR mRNA expression is associated with poorer prognosis (West et al. 2016; Yang et al. 2017).

While current studies are investigating the effect of GR activation on breast cancer progression, the involvement of GR in mammary gland development remains understudied and have shown contradictory results (Whirledge and Cidlowski 2017). GR activation of normal mammary epithelium *in vitro* results in decreased apoptosis with no difference in proliferation (Wu et al. 2014). However, female rats fed a corticosterone-supplemented diet and between four and eight weeks of age exhibit decreased mammary ductal extension and area (Zhu, Jiang, and Thompson 1998). It is plausible that GR activation of the mammary epithelium may prevent the necessary apoptosis for the clearing of the ductal lumen necessary for ductal extension (Gjorevski and Nelson 2011; Humphreys et al. 1997). In contrast, mice with functionally impaired GR of the mammary epithelium (GR^{dim}) also display decreased ductal extension (Reichardt et al. 2001). However, further studies have suggested that MR may compensate reduced GR signaling and thus the effects of GR may still be unknown (Kingsley-Kallesen et al. 2002).

While mice undergo limited nulliparous alveolobular development (described in Section 1.2), data in pregnant mice may provide clues as to the role of GR in ductal differentiation. For example, transgenic mice with mammary epithelial cell-specific deletion of the gene encoding GR, specifically late in pregnancy, exhibit defects in alveolobular development due to reduced cell proliferation (Wintermantel et al. 2005). Together these data suggest that GR activation of the mammary epithelium may differentially regulate mammary gland development based on timing and concentration.

Chapter 2: Psychosocial stress exposure disrupts mammary gland development

2.1: Abstract

Exposure to psychosocial stressors and ensuing stress physiology have been associated with invasive mammary tumors in rat models of human breast cancer. Many physiologic and environmental exposures that influence breast cancer risk occur during mammary gland development. However, the effect of psychosocial stress exposure on mammary gland development remains unknown. In a nulliparous Sprague-Dawley rat model of breast cancer, we demonstrate that social isolation increased glucocorticoid reactivity to everyday stressors and reduced the size of the mammary gland ductal tree and its alveolobular differentiation during late puberty and young adulthood. There was a marked spatial gradient in mammary gland differentiation, yet isolation blunted alveolobular differentiation in the central mammary gland. Surprisingly, given their vulnerability to carcinogens, terminal end bud (TEB) number was not associated with isolation or blunted mammary gland growth or differentiation. They were associated with an increased glucocorticoid stress response and accelerated loss of glucocorticoid receptor expression. Social isolation enhanced corpora luteal function and only reduced estrogenization in early adulthood, a pattern that precludes modulated ovarian function as a sufficient mechanism for the effects of isolation. Our findings provide an integrated view of mammary gland development throughout puberty and early adulthood and highlight the importance of glucocorticoids in nulliparous ductal growth alveolobular differentiation. Moreover, we demonstrate that the exposure to psychosocial stress disrupts both mammary gland growth and differentiation, potentially contributing to a later increase in mammary cancer risk.

2.2: Introduction

Social isolation is a psychosocial stressor for rats, a social species. Exposure to social isolation beginning in puberty is associated with early onset of large and malignant mammary tumors in Sprague-Dawley rats (G. L. Hermes et al. 2009). Because mammary cancer risk is increased when the mammary gland fails to fully differentiate (Jose Russo et al. 2005; MacMahon et al. 1970; Colditz and Frazier 1995; Osborne, Rudel, and Schwarzman 2015), we hypothesized that rats exposed to social isolation during mammary gland development would have less differentiated mammary glands. Indeed, isolated rats have less developed nipples than their grouped counterparts, an external indicator of delayed mammary gland development (Gretchen L. Hermes and McClintock 2008; Cathrin Brisken 2002).

Sexual differentiation and growth of the mammary gland begins as the ovary matures between four and eight weeks of age (Russell C. Hovey, Trott, and Vonderhaar 2002). Ductal extension is led by the penetration of terminal end buds (TEBs) into the mammary fat pad (Andrechek et al. 2008; Young 2013; Paine and Lewis 2017). Due to their high proliferative index, TEBs are more susceptible to DNA-damage from carcinogens than other ductal structures, and thus have been the primary focus of studies of environmental causes of mammary cancer (Frenkel, Wei, and Wei 1995; Abba et al. 2016). Exposure to carcinogens induces malignant transformation of the ductal epithelium likely through impaired DNA-repair and/or epigenetic alterations (I. H. Russo and Russo 1978; Cavalieri and Rogan 1978). Moreover, women exposed to carcinogens or radiation during the time at which they have terminal ductal lobular units, the human equivalent to TEBs, have significantly higher risk of developing breast cancer later in life (Colditz and Frazier 1995; Goodman et al. 1997). Therefore, we hypothesized that social isolation, a psychosocial stressor, would increase TEB number and thereby increase mammary cancer risk.

In addition, rodent experiments and human epidemiological studies indicate that reducing the later stages of mammary gland differentiation also increases breast cancer risk. As the ducts

reach the end of the mammary fat pad, the TEBs cavitate, creating terminal ducts (TDs). Some of the ducts differentiate into alveolar buds (AB) and then lobules (L), which displace the mammary fat and take up progressively more space in the developing mammary gland (J Russo et al. 1990). This process that lasts throughout early adulthood (P Schedin, Mitrenga, and Kaeck 2000; Y. N. Sinha and Tucker 1966). Alveolobular differentiation is also dynamic during each ovarian cycle and it is at its greatest during the luteal phase of the estrous cycle when luteal progesterone is high (P Schedin, Mitrenga, and Kaeck 2000; Joshi et al. 2010). Final differentiation occurs with full-term pregnancy, while a mammary gland that remains relatively undifferentiated is at greater risk for developing most types of breast cancer (Bernstein 2002; MacMahon et al. 1970; D. K. Sinha, Pazik, and Dao 1988; Hilakivi-Clarke et al. 2006). Therefore, we hypothesized that social isolation might also limit the degree of ductal extension and alveolobular differentiation.

Mammary gland development is under the control of ovarian steroids (Astwood, Geschickter, and Rausch 1937; Smith 1955), which may mediate the effect of social isolation on mammary gland development. Estrogen is the primary driver of ductal extension and its proliferative effects are at least in part mediated by paracrine activity via adipocytes and other stromal cells (Mallepell et al. 2006). Estrogen signaling also upregulates progesterone receptor (PR) expression in the developing mammary gland and allows mammary epithelial cells to respond to circulating progesterone (Haslam and Shyamala 1979). Progesterone signaling occurs through both direct and paracrine mechanisms and is involved in ductal branching and alveolobular development (reviewed in: (Cathrin Brisken and O'Malley 2010a)). Variation in estrogen and progesterone throughout the ovarian cycle results in alternating proliferation (driven by the peaking of these hormones during the luteal phase) and apoptosis (caused by their nadir during follicular phase) (P Schedin, Mitrenga, and Kaeck 2000; Joshi et al. 2010). However, both socially isolated rats and women undergoing psychosocial stress have accelerated ovarian

development in early puberty, which could accelerate, not delay mammary gland development (Surbey 1990; Sung et al. 2016; Fenster et al. 1999; Gretchen L. Hermes and McClintock 2008). We evaluated the effects of social isolation on ovarian function throughout late puberty and early adulthood to determine whether ovarian function could mediate the effects of isolation.

Social isolation is a mild unremitting psychostressor that increases anxiety and induces a hypervigilant state (G. L. Hermes et al. 2009). Rats exposed to social isolation acquire heightened glucocorticoid (GC) reactivity to everyday stressors compared to grouped counterparts, who have a social buffer ameliorating their stress response (G. L. Hermes et al. 2009; Yee et al. 2008). Our lab and others have demonstrated that GCs inhibit estrogen-induced proliferation in the mammary epithelium (West et al. 2016; Yang et al. 2017). Indeed administering GC decreases the size of the mammary ductal tree (Zhu, Jiang, and Thompson 1998). Although the rat model of socially isolated housing is not directly applicable to humans, it is a model for psychosocial situations that heighten GC reactivity, particularly to everyday stressors. Dysphoric adolescent women and young adults exhibit similar heightened GC reactivity if they have limited social supports, low self-esteem, and/or or low social economic status (Hankin et al. 2010; Decker 2000; Harkness, Stewart, and Wynne-Edwards 2011; Pilgrim, Marin, and Lupien 2010). Similar to our experimental model of social isolation, women exposed to these unremitting stressors have a higher risk of developing aggressive breast cancers, such as the triple-negative subtype (Vona-Davis and Rose 2009; Taylor et al. 2007; Liao et al. 2008). Using a longitudinal study of natural development to identify endocrine mechanisms by which social isolation affects mammary gland development and in turn increases breast cancer risk will provide direction for cellular studies and underscore the importance of prevention strategies during late puberty and early adulthood.

In this longitudinal study of natural development, we determined the effect of social isolation on rat mammary gland development by examining mammary gland architecture and histology at four week intervals throughout puberty and early adulthood. Morphology of the

mammary gland including ductal extension and structures (TEB, TD, AB and L) were assessed using whole mounts. We also used histologic measurements to quantify the degree of alveolobular development after ductal extension and the composition of the mammary gland. A non-invasive ovarian hormone bioassay (vaginal cytology) was used to determine relative systemic exposure to estrogen and progesterone throughout mammary gland development (Cora, Kooistra, and Travlos 2015). Serum GC levels before, during, and after an acute restraint stressor was also measured to assess GC reactivity to daily stressors associated with routine animal husbandry (G. L. Hermes et al. 2009; Sharp et al. 2003), along with behavioral assessment of hypervigilance and evidence of adrenal fatigue. Finally, ER, PR and GR expression in the mammary glands of isolated and grouped rats were assessed with immunohistochemical staining.

2.3 Methods

2.3.1: Sprague-Dawley rats

Female Sprague-Dawley IGS rats were bred at Charles River Laboratories, Inc. (Portage, MI and Kingston, NY) weaned into all-female groups at three weeks of age and shipped in a series of cohorts to our laboratory at four weeks of age. Upon arrival, they were housed in groups, and at 4.5 weeks of age transferred to either single or uncrowded grouped housing of five rats, balanced for vigilant temperament as measured during an exploration stressor (described below;(Sonia A. Cavigelli, Yee, and McClintock 2006)). Uncrowded caging provided single and grouped rats the same area per animal (20 x 24 x 18cm and 46 x 61x 36 cm). All were housed in solid stainless steel cages hung on shared racks in the same colony room (21 $\pm$ 1°C), with wire covering the front and floor over shared bedding (Harlan Teklad Aspen Sani-Chips, 7090A) pans. Thereby, rats could see, hear and smell each other, but could interact socially only in the group housing cages. Rats were housed under a reversed L:D cycle (14:10, dark onset 08:00 h) so that all experimental manipulations were performed during rats' behavioral day. Food (Harlan Teklad

Rodent Diet, 8604) and water were provided *ad libitum*. Rats were routinely handled during cage changes and health inspections. National Institutes of Health and University of Chicago Animal Care Guidelines were followed for all studies.

2.3.2: Experimental design

Mammary glands were harvested during puberty (8 and 13 wks of age) and early adulthood (17, 21 and 25 weeks of age). Ovarian cyclicity was quantified in the two weeks prior to sacrifice and behavioral stress reactivity one week prior. In addition, GC stress reactivity was measured at 17 weeks of age. Rats were sacrificed in proestrus/estrus to minimize ovarian cycle variability in mammary morphology (S. A Cavigelli et al. 2005; P Schedin, Mitrenga, and Kaeck 2000). All ovarian and stress measures were standardized at 5-6 hours into their behavioral day, when GC rhythm is at its nadir. The total ovarian weight and total adrenal weight were adjusted for body weight (mg/100g body weight).

2.3.3: Mammary whole mount preparation and analysis

Upon sacrifice, the right inguinal mammary gland quadrant was removed, spread onto a large microscope slide (2”x3” or 3”x4”) and fixed with Carnoy’s fixative. The mammary gland was then stained with carmine alum and the lipid removed using xylene (de Assis et al. 2010). An image of the cover slipped mammary gland whole mount slide was created with a 3600 dpi flatbed scanner (optimal for double blind anatomical measurements on the digital images with ImageJ software).

Ductal extension was measured from the top of the lymph node (LN) to the most distal ductal structure (TEB or TD) at the leading edge of the ductal tree (Fig. 1a). Because we measured ductal extension in the whole mount, we standardized gland size to body size. While there was a range in body weight among all rats, there were no significant difference in body weight between rats of each housing condition. Distal mammary gland ductal area was the area

between a horizontal line transecting the LN and the traced perimeter of the mammary whole mount (zones B and C). Each measure was taken as a single measure and therefore required a minimum of 9 rats in each housing condition for statistical analysis.

Spatial differences in mammary gland development were measured within four equal sectors of distal ductal area of the mammary gland: adjacent to the nipple (S1), middle of mammary gland along the dorsal fat ridge (S2), middle of mammary gland thin ventral fat pad (S3), and at the distal end, furthest from the nipple (S4). The prevalence of all mammary gland ductal structures, including TEBs (103 ± 16.7 μm), TDs, alveolar buds (AB), and lobules, were quantified in the outer 1 mm annulus (perimeter) of each section (Young 2013). As a context for interpreting these results, we made similar mammary gland whole mount measurement of 84 wk old nulliparous rats.

2.3.4: H&E staining of ductal structures (TEB, TD, AB, and Lobules), fat, and stroma

The development of ducts, alveolobular units, stroma and adipose tissue was quantified in H&E stained tissue from the left inguinal mammary gland, standardized immediately rostral to the LN. The tissue was fixed, paraffin embedded, sectioned, and H&E stained. We performed whole slide scanning (CRi Pannoramic Scan Whole Slide Scanner). The slide was parsed into 1000 x 1000 pixel images. The tissue structures on each parsed image were digitally separated using color deconvolution (Ruifrok and Johnston 2001). This analysis consisted of multiple measures per slide that required relatively few rats for high statistical power.

Pink (eosin) stained tissue is representative of stroma and blood vessels, purple (hematoxylin) stained tissue is ductal structures (DS) and unstained white is adipose tissue. Only ductal structure-associated stroma was included in the analysis. Excluded stroma included muscle and other non-adipocyte stroma that did not directly support the growing ductal structures.

2.3.5: Immunohistochemistry

Antibodies for GC, estrogen, and progesterone nuclear receptors (GR, ER-alpha, and PR) were: GR-XP (Cell Signaling 3660), ER-alpha (Novocastra clone 6F11), and PR (Thermo-Fisher RM-9102). Following standard protocols, the slides were stained for GR and ER using the Leica Bond RX automatic stainer. Epitope retrieval solution II (Leica Biosystems, AR9640) was used for 20 minutes. Anti-GR antibody (1:1500) was applied for 25 minutes and anti-ER-alpha antibody (1:160) was applied for one hour. Antigen-antibody binding was detected with Bond polymer refine detection (Leica Biosystems, DS9800). For PR, the tissue sections were deparafinized and rehydrated using xylene and serial dilutions of EtOH to distilled water. They were incubated in antigen retrieval buffer (DAKO, S1699) and heated in a steamer over 97°C for 20 minutes. Anti-PR (1:50) antibody was applied on tissue section for one hour at room temperature in a humidity chamber. Following TBS washing, the antigen-antibody binding was detected with Envision+ anti-rabbit system (DAKO K4003) and DAB+ chromogen (DAKO, K3468). For all three stains, tissue sections were briefly immersed in hematoxylin for nuclear counterstaining and were covered with cover glass.

Representative slides of mammary glands from rats of each age (13, 17, 21, 25 weeks) and housing condition (grouped and isolated) were scanned using CRi Pannoramic Scan Whole Slide Scanner. Visual inspection detected a difference in developmental rate at 21 weeks. Therefore, expression of all three receptors (% ER+, PR+ and GR+) were measured in the luminal and basal ductal epithelial cells of five grouped and five isolated rats at this age blinded to housing condition.

2.3.6: Ovarian steroid exposure

Estrogen and progesterone exposure during successive ovarian cycles was assessed with daily vaginal saline lavage, a well-established bioassay of ovarian function enabling non-invasive

repeated measures of cycles (Cora, Kooistra, and Travlos 2015). Samples were collected and analyzed 2 to 5 hours into the behavioral day. We quantified the changing proportion of cornified epithelial cells, nucleated epithelial cells, and leukocytes as reliable indicators of ovarian cycle phase (Cora, Kooistra, and Travlos 2015). Duration of estrogen exposure was quantified by the percent of the 2 week epoch with only nucleated or cornified vaginal epithelial cells, a well-established bioassay of estrogenization (Cora, Kooistra, and Travlos 2015). Luteal progesterone exposure was estimated by the percent of epochs with primarily leukocytes during metestrus.

2.3.7: Corticosterone stress reactivity

Serum concentrations of baseline, reactive and recovery corticosterone (in response to a mild stressor) were performed in a subset of 17-week-old rats (n=9) using a mild restraint test (restraint tube, Harvard Apparatus)(G. L. Hermes et al. 2009). One handler removed rats, one at a time, from the housing room, alternating housing conditions and carried them into an adjacent procedure room where two handlers performed the restraint stress procedure. Baseline blood samples were obtained by tail nick and subsequent samples collected by gently disturbing the clot. Blood was collected into microcentrifuge tubes at baseline, after 30 min (16:30 h) of imposed stress and during recovery, 1 (17:00 h) and 2 h (18:00h). In between sample collection, animals were placed into clean recovery cages in a room adjacent to the procedure room. Samples were kept on ice during blood collection and stored overnight in at 4°C until centrifuge and serum extraction the next day. Serum samplewere frozen at -30°C until corticosterone ELISA (IBL America B79175) assay. Samples were run in duplicate on the same assay (intra-assay coefficient of variance 9.4%; inter-assay variance 8;(G. L. Hermes et al. 2009; J. B. Williams et al. 2009)). Serum corticosterone reactivity and recovery was standardized to baseline to control for individual differences in baseline corticosterone and slight variation in time of day of sample collection

2.3.8: Behavioral response to exploration stressor

Exploration in an unfamiliar environment is a classic stressor for rodents. We measured stress behavior twice during the lifespan with an exploration stressor at 4.5 weeks (3'x3') and again 2 weeks before sacrifice (4'x4') (G. L. Hermes et al. 2009; S A Cavigelli and McClintock 2003). The exploration arena was made of opaque Plexiglas walls, with a home base enclosure in a corner, scented with familiar odors (handful of soiled bedding from the subject rat's bedding pan and, in the center, a small overturned ceramic bowl. The rat was brought from its home cage in the adjacent colony room and gently placed into the home base. Vigilance was measured as the latency to emerge from home base within five minutes. The same handler was used throughout, and the open field base was wiped down with cavi-wipes between animals to remove stress odors.

2.3.9: Statistical analysis

Statistical analyses were conducted with *R* (NS = not significant (P > 0.05, two-tailed tests), all means ± SEM)(Biffi and Tuissi 2017). Arcsine transformed arcsine [sqrt (p)] percentages met parametric distribution requirements. To account for genetic or significant variation among this non-syngeneic Charles River rat strain, we included cohort as a variable in the multivariate analyses.

2.5: Results

2.4.1: Mammary ductal extension and not TEB Number is altered by social isolation

Terminal end buds. Social isolation had not altered TEB number in the mammary tree perimeter by 8 weeks of age, when carcinogens exert their malignant effects (I. H. Russo and Russo 1978) (Figs. 1a and b; 27 ± 7 TEBs in grouped versus 28 ± 7 TEBs in isolated rats, P = 0.68, *Students*

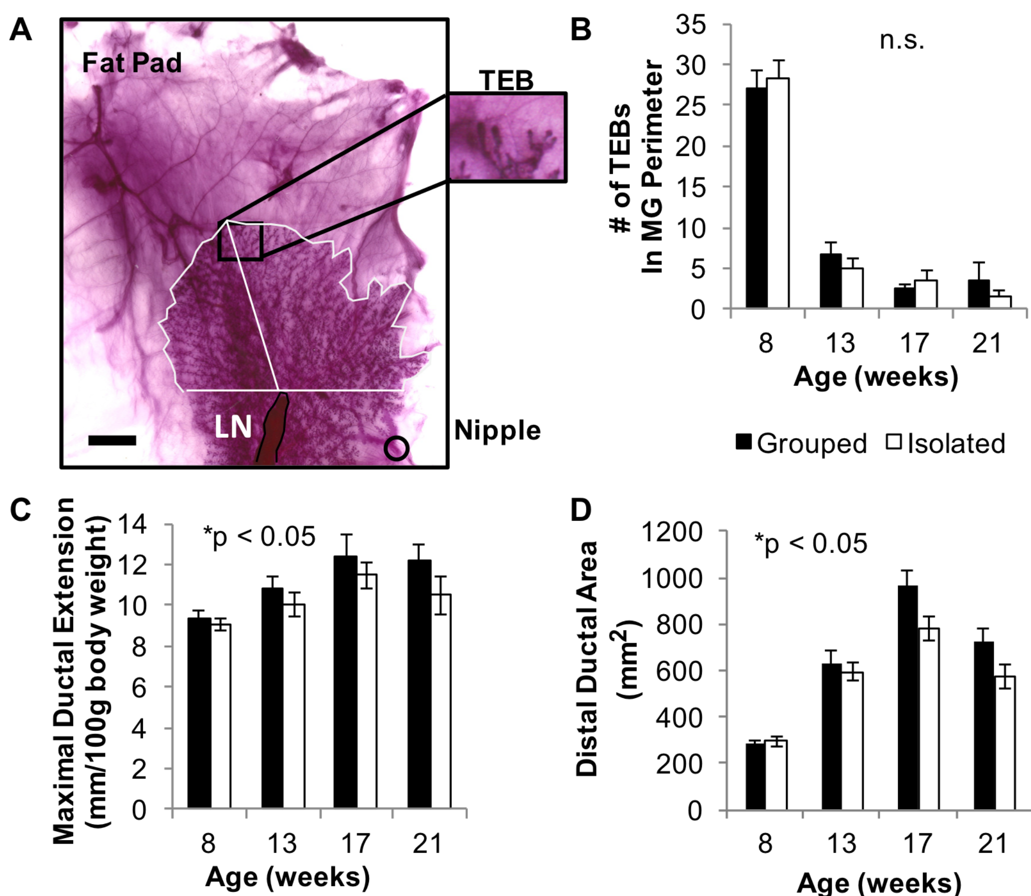
T-test). By this age, the TEBs have led the mammary ductal tree from the nipple, past the lymph node (LN) and into the mammary fat pad, beginning to wrap around the flanks of the rat (Supp. Fig. 1). By 13 weeks of age, TEB number had dropped precipitously at the leading edge of the ductal tree in both social conditions, and remained low as the ductal tree continued to extend. Social isolation had no effect on TEB number at any age (social condition: $F(1, 71) = 0.2$, $P = 0.68$, age: $F(3, 71) = 127.6$, $P < 0.0001$, interaction: $F(1,71) = 0.9$, $P = 0.46$).

Social isolation also did not disrupt the average size of TEBs at 8 weeks of age when they are most abundant (107.9 ± 1.2 μm in grouped versus 106.2 ± 1.5 μm in isolated rats , $P = 0.366$). TEB size is a result of proliferative index, and because we observe no difference in size, we would not expect to see a significant difference in proliferative index (Paine and Lewis 2017). Moreover, there was not a greater density of TEBs in the isolated rats (10 ± 1 TEBs per mm^2 in both grouped and isolated rats. $P = 0.70$, *Students T*-Test).

Ductal extension. Ductal extension continued throughout puberty and young adulthood, reaching a maximum at 17 weeks of age (Fig. 1c; Supp. Fig. 1). Throughout late puberty and young adulthood, ductal extension in social isolates was significantly shorter than in rats living in groups

Figure 2.1: Effect of social environment on mammary gland development of terminal end buds, ductal extension, and distal ductal area (measured in mammary gland whole mounts).

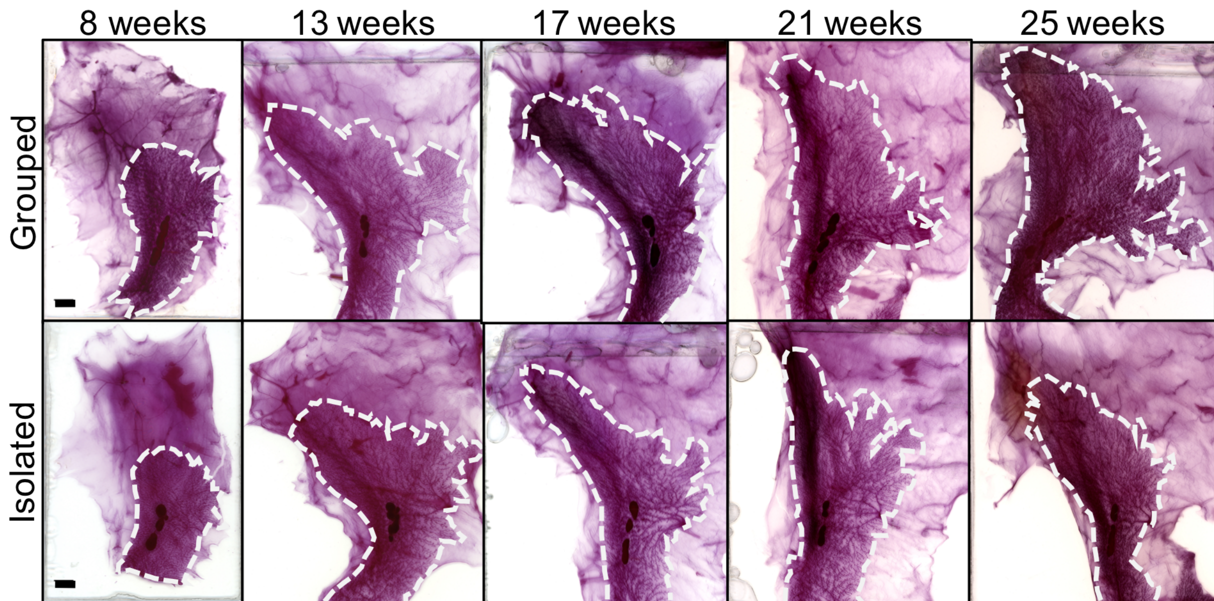
A. a representative digital image of a mammary gland from a group-housed Sprague-Dawley rat (8 weeks of age) stained with carmine alum, indicating the fat pad, lymph node (LN) terminal end buds (TEBs), and nipple. Scale bar = 5 mm. **B, C, D.** Effects of social condition on mammary gland structures from puberty to early adulthood (8, 13, 17, and 21 weeks of age), measuring **(B)** TEB number at the periphery, **(C)** maximal ductal extension, and **(D)** distal ductal area; mean ± SEM; ≥ nine rats per age and social condition. $^*P < 0.05$, 3-factor ANOVA (housing, age, and cohort).



(Fig. 1c, Supp. Fig. 1; age: $F(3, 72) = 9.96$, $P < 0.0001$. social condition: $F(1, 72) = 7.1$, $P = 0.01$, interaction: $F(3, 72) = 0.6$, $P = 0.6$, cohort $F(1, 72) = 17.7$, $P < 0.0001$).

Mammary Gland Distal Ductal Area. As ductal extension increased throughout puberty and young adulthood (until 17 weeks), the ductal tree expanded (distal ductal area) in both social conditions

Supplementary Figure 2.S1: Mammary gland whole mounts.
Representative digital images of right inguinal mammary gland of rats aged 8, 13, 17, 21, and 25 weeks, after exposure to grouped or isolated social condition since weaning. Dashed line indicates perimeter of mammary ductal tree. Scale bars, 5 mm.



(Fig. 1d; Supp. Fig. 1). Social isolates had significantly smaller distal ductal area compared to grouped rats at all ages (8-21 weeks; social condition: $F(1, 90) = 4.9$, $P = 0.03$, age: $F(3, 90) = 127.8$, $P \leq 0.0001$, interaction: $F(3, 100) = 1.2$, $P = 0.30$). The observed decrease in ductal extension and distal ductal area in social isolates does not manifest until 13-17 weeks as demonstrated by the error bars in Fig. 1c-d

Supp. Fig 1). Social isolates had significantly smaller distal ductal area compared to grouped rats at all ages (8-21 weeks; social condition: $F(1, 90) = 4.9$, $P = 0.03$, age: $F(3, 90) = 127.8$, $P \leq 0.0001$, interaction: $F(3, 100) = 1.2$, $P = 0.30$).

2.4.2: Spatiotemporal gradient of differentiation exists in the pubertal mammary gland

Removing fat from whole mounts revealed structural details of mammary tree differentiation. Ductal differentiation into alveolobular units had begun close to the nipple (Sector 1 (S1)) as early as 8 weeks of age when TEBs were still extending through the distal fat pad (Sector 4 (S4), Fig. 1a, Fig. 2a; Supp. Fig. 1). Therefore, the proximal portion of the mammary ductal tree (S1) had more time to differentiate than did the distal portion (S4). This created a spatial gradient of differentiation, with the most differentiated mammary gland in S1 near the nipple, followed by adjacent S2, S3 opposite the nipple and finally S4, the most distal sector (Fig. 2). During 13-25 weeks of age, differentiation progressed in all sectors. Nonetheless, in the most distal sector (S4), most rats still had predominantly undifferentiated TDs in the perimeter, either accompanied by TEB, with the capacity for continued development, or AB; only 10% had predominantly AB, and none developed lobules. Close the nipple, 90% of rats had TDs with AB and 10% had lobules. The middle sectors were intermediate in differentiation, sustaining the spatial gradient despite all being exposed to the same hormonal environment. Indeed, growth and differentiation in these nulliparous animals continued through 84 weeks of age, retaining the spatial gradient of predominantly undifferentiated TD with TEBs (S1 0% to S4 20% of rats) versus lobules as the primary structures (S1 50% to S4 33% of rats). Overall, location in the mammary gland (S1-S4) had a bigger effect on variation in mammary gland differentiation than did chronological age. Moreover, the effect of age was different in the various sectors. In the least mature sector (S4), development was characterized by the loss of TEBs as the primary or secondary structures, whereas in the most mature sector (S1), ABs and lobules were surpassed by TDs as the most

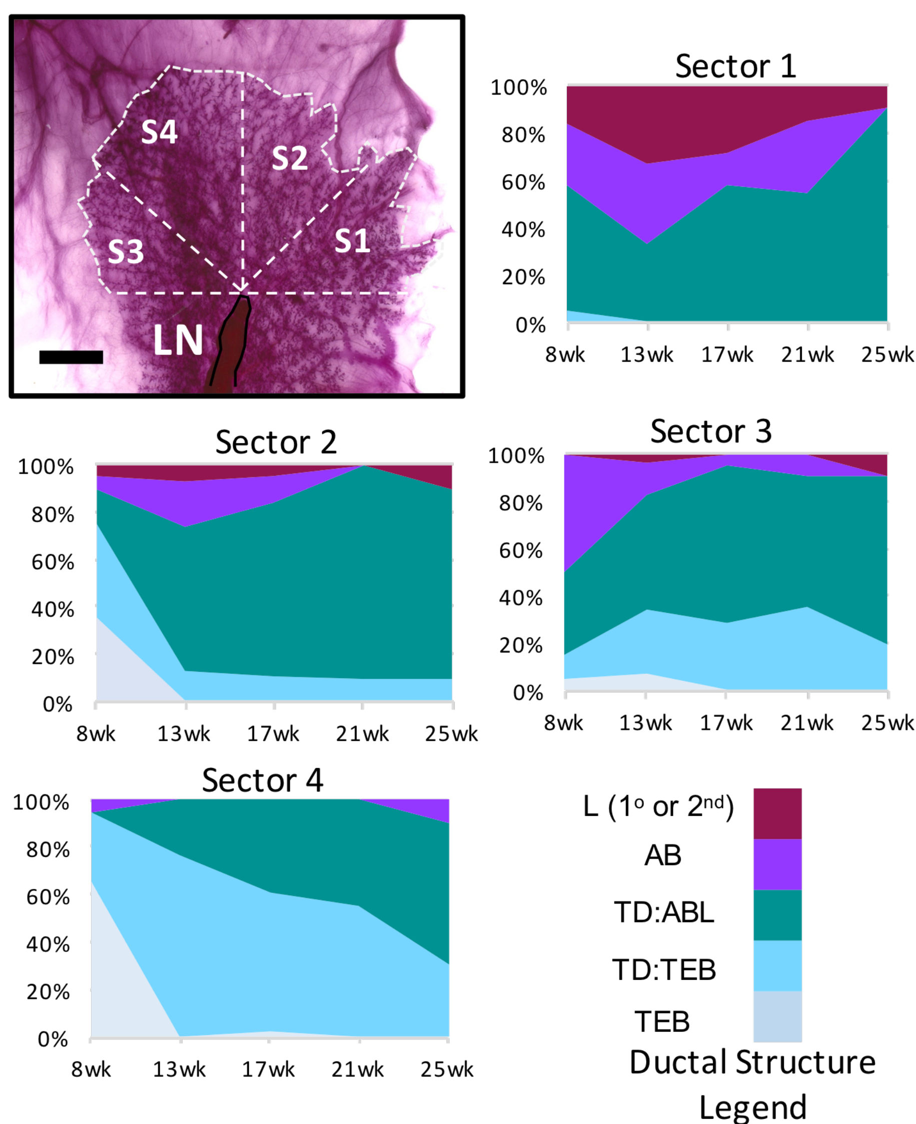
prevalent structures. (Fig 2. Sector $F(3, 337) = 6.0$, $P = 0.00001$, age: $F(4, 113) = 1.4$, $P = 0.17$, interaction: $F(12, 337) = 2.7$, $P = 0.002$). To standardize mammary gland tissue collection for analysis of mammary gland composition, a strip was taken at the intersection of the four sectors, just distal to the LN.

2.4.3: Social isolation alters central mammary gland composition: ducts, stroma, and fat

Ductal Differentiation. Analysis of H&E stained central mammary gland tissue, just rostral to the LN, was consistent with the differences seen in peripheral ductal structures of mammary gland whole mounts (Supplementary Figure 2.S2) . After maximal ductal extension (17 weeks), the proportion of the mammary gland consisting of ductal structures (ducts, TDs, alveoli and lobules; see Fig. 3) continued to increase through 25 weeks, reflecting continued arborization and differentiation of the mammary gland tree. However, socially isolated rats had significantly smaller and less elaborated ductal structures (e.g. fewer alveoli and lobules) compared to their grouped counterparts (Fig. 3a-c; social condition: $F(1, 23) = 5.3$ $P = 0.03$, age: $F(2, 23) = 5.6$, $P = 0.01$, interaction: $F(2, 24) = 1.2$, $P = 0.10$). In isolated rats, alveolobular maturation stopped by 21 weeks, whereas it continued through 25 weeks in the grouped rats, evidencing stunted maturation of ductal structures in isolated rats.

Stroma. Stroma continued to develop only in group housed rats (17 – 25 weeks), not in the isolates (Fig. 3c, social condition: $F(1, 23) = 4.0$ $P = 0.06$, age: $F(2, 23) = 3.8$, $P = 0.04$, interaction: $F(2, 23) = 4.0$, $P = 0.03$). Overall, more surrounding stroma was associated with larger ductal structures in both grouped and isolated rats (Supp. Fig. 3, $R = 0.70$, $P < 0.001$), consistent with previous reports that stromal growth follows ductal structure differentiation and its invasion into the fat pad (R C Hovey, McFadden, and Akers 1999; Gjorevski and Nelson 2011). The stunted

Figure 2.2: Differentiation of ductal structures in four different sectors of the distal rat mammary gland (S1 nearest the nipple to S4 most distal).
A representative digital image of a mammary gland from a group-housed Sprague-Dawley rat (8 weeks of age) stained with carmine alum. White dashed lines depict maximal ductal extension, the perimeter and four equal sectors (S1-S4) within the distal ductal area. Scale bar = 5 mm. TEB (terminal end bud), TD (terminal duct), AB (alveolar bud), L (lobule). The most prevalent structure is listed first, followed by the secondary structure: Individual variation is indicated by the % of rats at each age that had a specific pattern of primary and secondary structures. The spatial gradient in mammary gland differentiation was greater than differences across chronological age.



stromal development in isolates indicates impairment of this process.

Fat. In isolates, the proportion of mammary gland adipose tissue was sustained 17-25 weeks of age, whereas it decreased in grouped rats, particularly at older ages (Fig. 3c, social condition: $F(1, 23) = 5.6$, $P = 0.03$, age: $F(2, 23) = 5.4$, $P = 0.01$, interaction: $F(2, 24) = 4.5$, $P = 0.02$). In grouped rats, the fat occupied a smaller proportion of the mammary gland as it was displaced by the expanding ductal and stromal structures. Therefore, the fat to duct ratio was much higher in the isolates as fat was maintained with only a minimal expansion of ducts or stroma. Thereby, in the isolates, fully 80% of the mammary gland was comprised of fat throughout early adulthood, versus 65% in grouped rats.

Supplementary Figure 2.S2: Color deconvolution for quantification of mammary gland structures. ductal structures, stroma, and adipose tissue.
A representative section of a left inguinal mammary gland (H&E stain), hematoxylin stains ductal structures purple, eosin stains stroma and blood vessels pink, and unstained white adipose tissue.

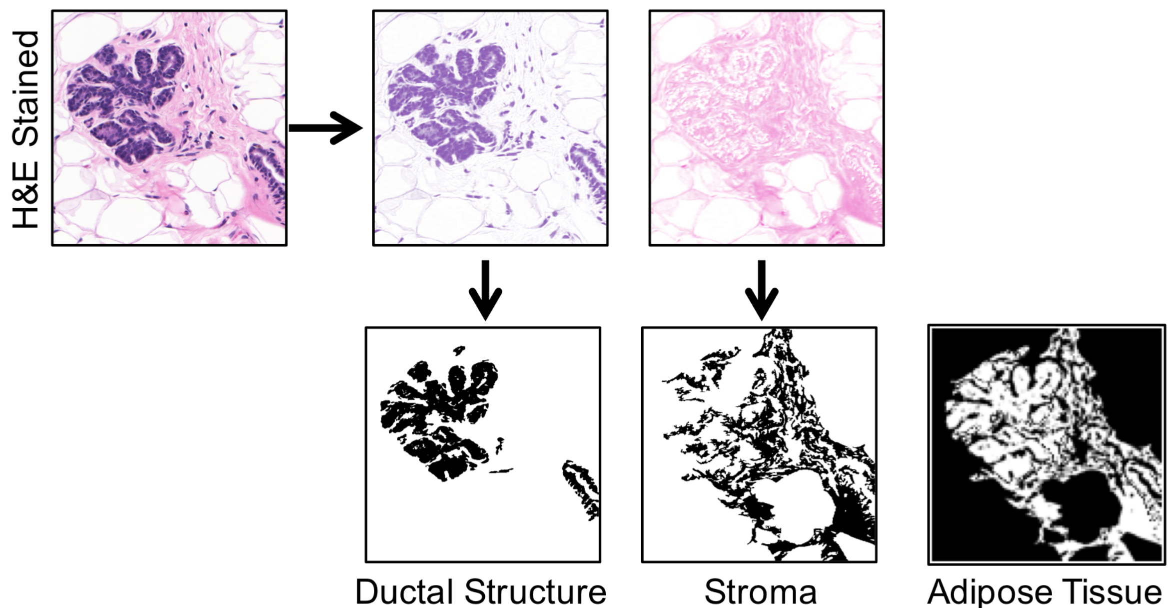
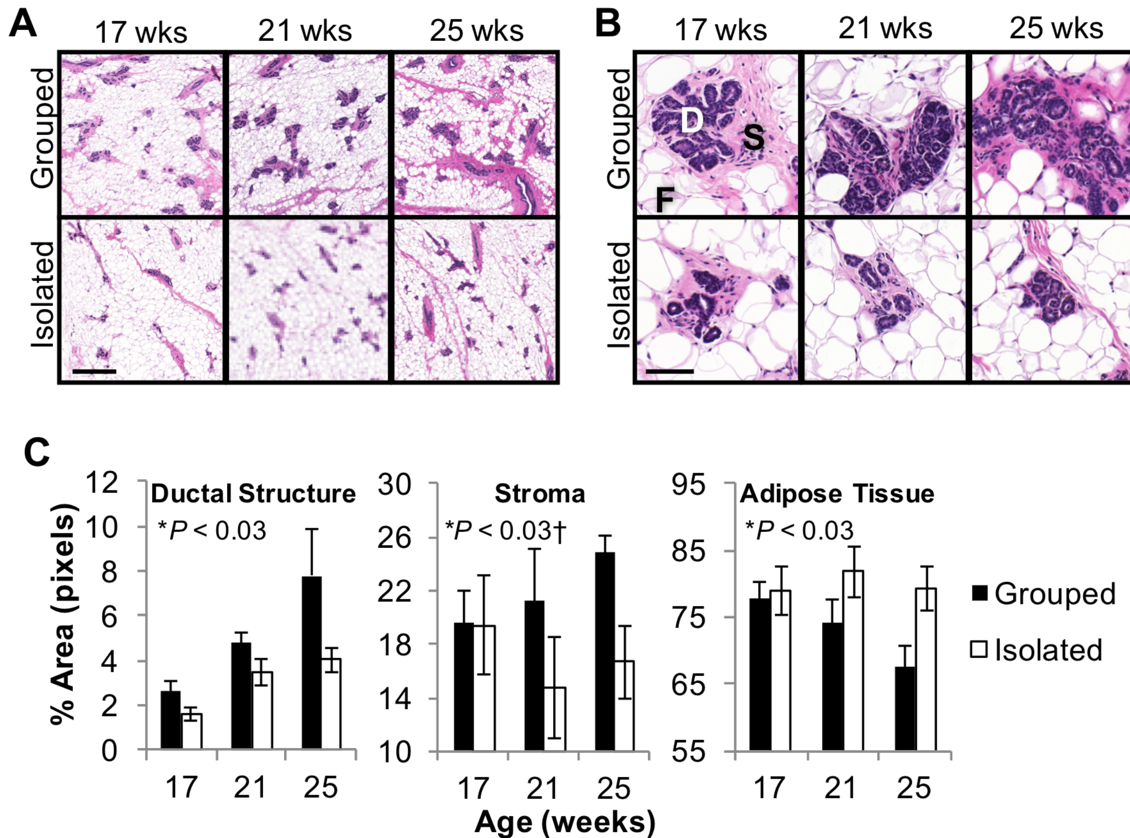


Figure 2.3 Characterization of mammary gland composition.
Representative H&E stained cross-sections rostral to the lymph node of the left inguinal mammary gland, at 17, 21, and 25 weeks of age **(A)** Ductal Structures (D), fat (F), and stroma (S), 5X magnification, scale bar = 500 μm and **(B)** 20X magnification, scale bar = 100 μm. **C.** Effect of social condition on % mammary gland area occupied by ducts, stroma, and fat (mean ± SEM, ≥ four rats per age and social condition; 2-factor ANOVA (social condition and age), * *P* < 0.05, ł interaction).

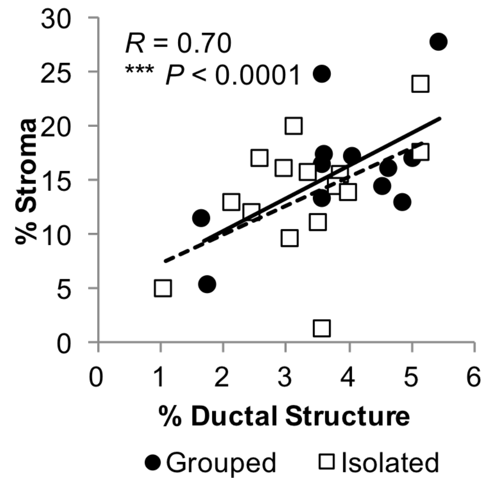


2.4.4: Ovarian steroid exposure is altered by social isolation

Luteal Progesterone Exposure. Between 13 and 25 weeks of age, isolated rats sustained more mature ovarian cycles than grouped rats and were more likely to be exposed to luteal progesterone after ovulation (Fig. 4a; social condition: $F(1, 79) = 11.4$, $P = 0.001$, age: $F(3, 79) = 0.5$, $P = 0.65$, interaction: $F(3, 79) = 0.6$, $P = 0.59$). Luteal progesterone is indicated by

predominance of leukocytes in vaginal cytology on estrus through diestrus (Cora, Kooistra, and Travlos 2015).

Supplementary Figure 2.S3: Association between stroma and ductal structures in grouped and isolated rats.
Meaured at at 17, 21, and 25 weeks of age (R = 0.70, *** $P < 0.001$, n = 26).

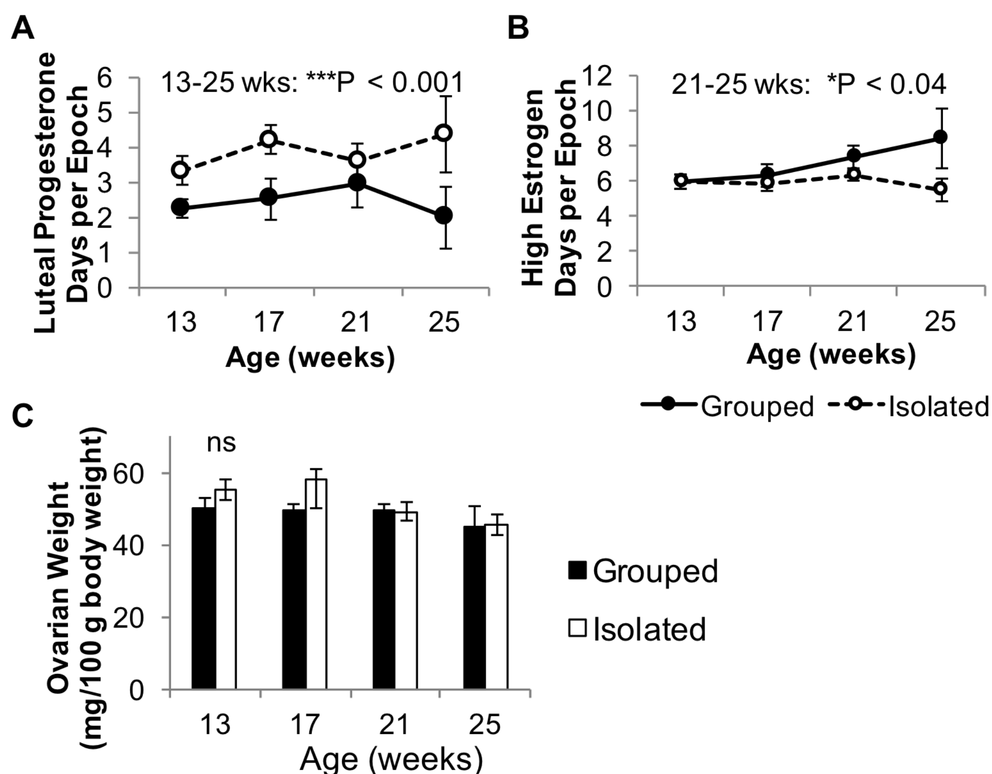


Estrogenization. At 13 and 17 weeks of age, isolation was not associated with lower estrogenization (Fig. 4b; social condition: $F(1, 44) = 0.7$, $P = 0.40$, age: $F(1, 44) = 1.3$, $P = 0.27$, interaction: $F(1, 44) = 1.0$, $P = 0.33$). Estrogenization is quantified by nucleated and cornified epithelial cells in vaginal cytology without leukocytes (Cora, Kooistra, and Travlos 2015). Nonetheless, they did have lower estrogenization at the ages when isolated rats had detectable stunted alveolobular and stromal differentiation (Fig. 4b, 21 and 25 weeks of age; social condition $F(1, 26) = 4.91$ $P = 0.04$, age: $F(1, 26) = 0.03$ $P = 0.87$, interaction: $F(1, 26) = 1.5$ $P = 0.24$).

Ovarian Weight. The ovarian weights of grouped and isolated rats were not significantly different (Fig. 4c; social condition: $F(1, 30) = 2.2$, $P = 0.15$, age: $F(1, 30) = 3.5$, $P = 0.07$), interaction: $F(1,30) = 1.0$, $P = 0.32$, cohort $F(2, 30) = 4.1$, $P = 0.03$; mg/100g body weight).

Figure 2.4: Ovarian steroid exposure (measured by vaginal cytology and ovarian weight).

A. Number of days of luteal progesterone exposure per 14-day epoch. **B**. Number of days of high estrogenization per 14-day epoch. **C.** Average ovarian weight (mg/100g body weight). Mean ± SEM; ≥ nine rats per age and social condition. *P < 0.02, ***P < 0.001 3-factor ANOVA (housing, age, and cohort).



2.4.5: Increased vigilance and glucocorticoid stress reactivity in isolated rats

During the stressor of exploring a novel environment, isolated rats, compared to those in social groups, exhibited greater vigilance throughout puberty and early adulthood. When placed in the home base of a novel exploration arena, isolated rats remained for prolonged times (Fig. 5a; 13-25 weeks of age, latency to emerge = 128.4 ± 17 secs vs 55.2 ± 10 secs, Log rank Mantle-Cox test X^2 = 14.18 P < 0.001). Longer latency to emerge is indicative of greater vigilance, a behavioral trait that is associated with increased GC reactivity and also with mammary cancer risk (Roth and Katz 1979; Gretchen L. Hermes and McClintock 2008; Yee et al. 2008; Sonia A. Cavigelli, Yee, and McClintock 2006).

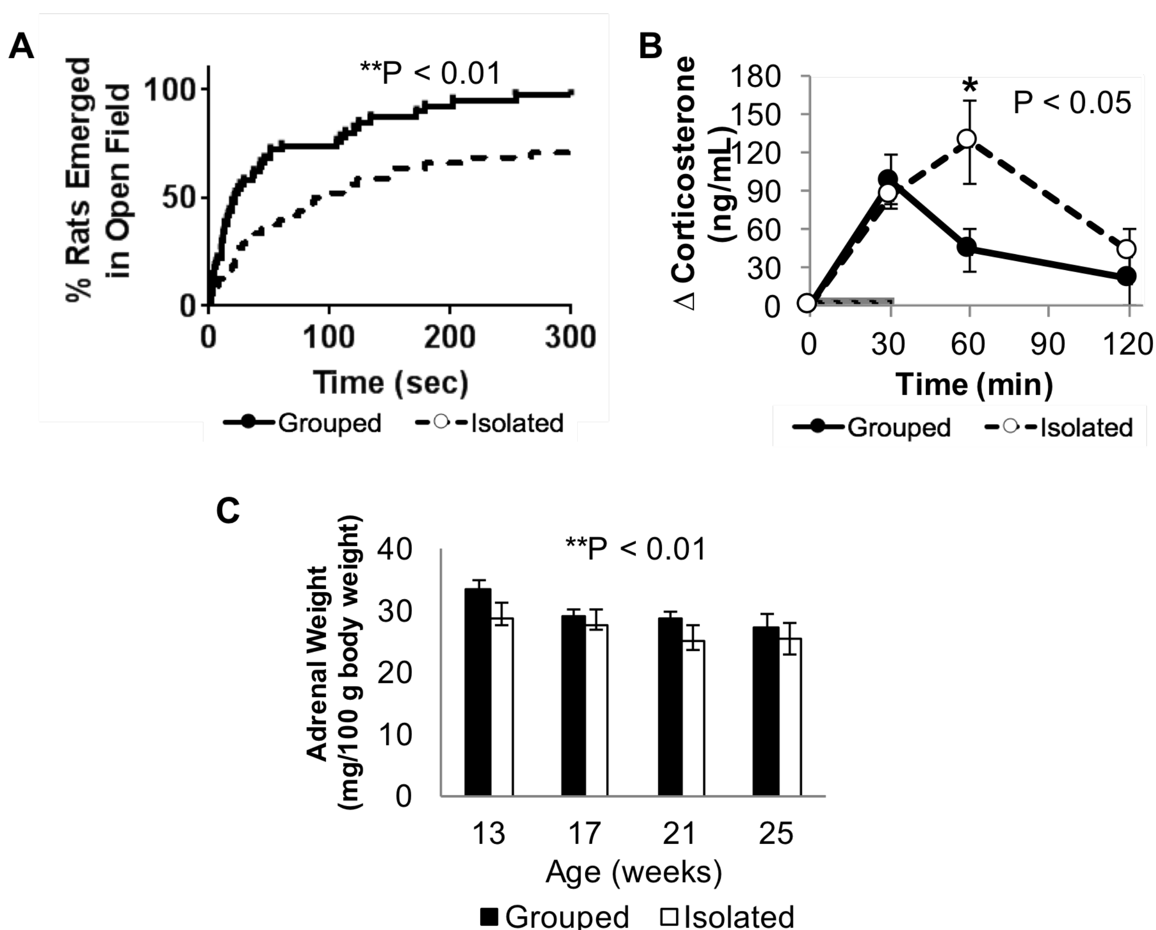
We next determined whether isolates indeed had more robust GC stress reactivity by imposing a restraint test on isolated and grouped rats at 17 weeks of age. After the stressor ended, the serum corticosterone levels of isolated rats continued to rise while the grouped rats immediately began to recover (Fig. 5b; 127.8 ± 32.5 ng/mL versus 44.3 ± 16.7 ng/mL, $P = 0.05$, *Students T*-test). Total exposure to corticosterone following the restraint stress challenge was twice as high in isolated versus grouped rats (8351.1 ± 1108.7 ng/mL/90 min versus 4118.3 ± 1585.0 ng/ml/120 min, $P = 0.08$, *Students T-Test).*

Finally, we looked for evidence that exposure to repeated everyday stressors produced adrenal fatigue (Hans Selye 1952). Throughout 13-25 weeks, the isolated rats had lower total adrenal weights (mammary gland/100g body weight), which is consistent with adrenal fatigue (Fig. 5c; social condition: $F(1, 59) = 6.88$, $P = 0.01$, age: $F(3, 59) = 3.91$, $P = 0.01$), interaction: $F(3,59) = 0.72$, $P = 0.54$, cohort $F(4, 59) = 0.36$, $P = 0.84$). Moreover, the isolated rats had lower baseline corticosterone at compared to their grouped counterparts (32.5 ± 8.5 ng/mL versus 65.3 ± 5.8 ng/mL, $P = 0.0006$, *Students T*-test) consistent with repeated exposure to stressors (S. A Cavigelli et al. 2005).

2.4.6: Expression of glucocorticoid (but not estrogen or progesterone) receptors is reduced in isolated rats

Estrogen Receptor (ER). Ductal cells (including both the inner layer of luminal ductal cells and the outer myoepithelial cells) expressed ER-α throughout late puberty and early adulthood (13-25 weeks), with maximal expression at younger ages and less expression at older ages (Fig. 6a). ER was also expressed in mammary fat, and stroma. At 21 weeks, there was no significant difference in ER expression between rats of either social condition (67.0% ± 2 versus 63.3% ± 1, $P = 0.69$, Mann Whitney U).

Figure 2.5: Vigilance testing, glucocorticoid stress reactivity, and adrenal weights. **A.** Percentage of rats emerging from home base in an open-field test (13-25 weeks of age). Log rank Mantle-Cox test, ****P* < 0.001. **B.** corticosterone stress reactivity and recovery during and after a passive restraint stressor (Lined bar; 17 weeks; 30 minutes post restraint **P* < 0.05 Students *T*-Test). **C.** total adrenal gland weight (mg/100g body weight; mean ± SEM. **P <0.01, 3-factor ANOVA (housing, age, and cohort).



Progesterone Receptor (PR). PR was primarily expressed in ductal tissue and only weakly expressed in stroma at the oldest age (25 weeks, Fig. 6b). PR was not observed in the adipose tissue. The PR expression in the ductal cells was in a salt-and-pepper pattern throughout development, typically in luminal cells, but some PR+ myoepithelial cells were observed. The strength and pattern of PR expression in the ducts remained unchanged between 13 and 25

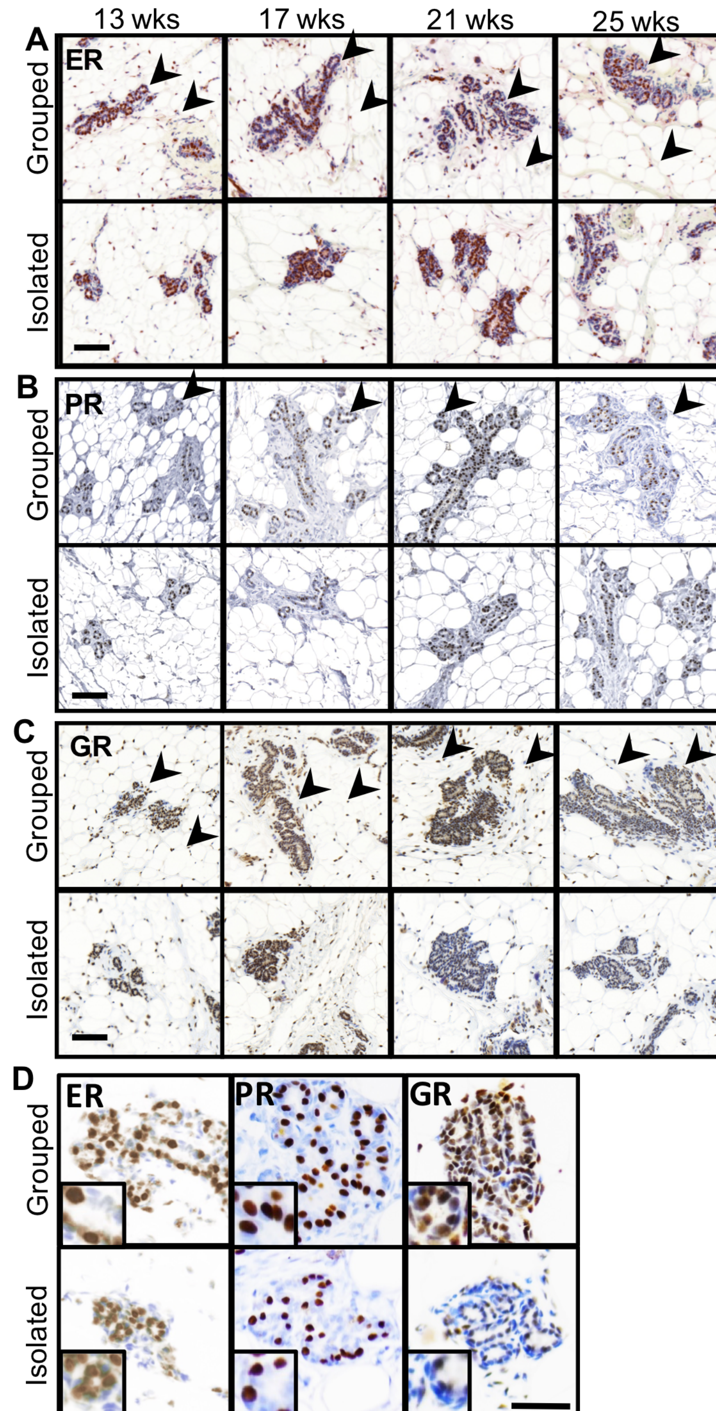
weeks of age and did not differ between grouped and isolated rats at 21 weeks of age (27.5% ± 3 versus 28.5% ± 3, P = 0.83, Mann Whitney U).

Glucocorticoid Receptor (GR). Ductal cells (including both the inner layer of luminal cells and the outer myoepithelial cells) stroma and mammary fat expressed GR throughout late puberty and early adulthood (13-25 weeks; Fig. 6c). Although isolates and groups had a similar developmental pattern in ductal GR expression, the isolate's developmental pattern was accelerated, creating differences by 21 weeks (Fig. 6c). Initially, GR was highly expressed in all of the ductal cells in the ducts, alveoli, and lobules, which continued in grouped rats until 25 weeks of age, whereupon GR expression decreased compared to the earlier ages, presenting a salt-and-pepper pattern in the developing alveoli and lobules. In contrast, isolates lost GR expression 4 weeks earlier, at 21 weeks of age, despite having less alveolobular elaboration (77.4% ± 3 versus 89.8% ± 2, P = 0.04, Mann Whitney U).

2.5: Discussion

We have previously demonstrated that socially isolated rats have a higher risk for spontaneously developing invasive cancer (G. L. Hermes et al. 2009). Here we show that isolation stunted mammary gland differentiation (Fig. 1, 3, and 4; (Gretchen L. Hermes and McClintock 2008)), a well- known developmental risk factor for many types of breast cancer (Bernstein 2002; MacMahon et al. 1970; D. K. Sinha, Pazik, and Dao 1988; Hilakivi-Clarke et al. 2006). It also reduced the size of the mammary ductal tree by shortening ductal extension and thereby the area of the mammary ductal tree (Fig. 1, 3, and 4; (Gretchen L. Hermes and McClintock 2008)). It is noteworthy that isolation did not change the number of TEBs, which extensive work has shown mediate the malignant effects of carcinogens (I. H. Russo and Russo 1978; Young 2013). The action of carcinogens is greatest in early puberty, before the ovary and adrenal fully mature

Figure 2.6: Characterization estrogen, progesterone, and glucocorticoid receptors. Representative cross-sections rostral to the lymph node of the left inguinal mammary gland at 13, 17, 21, and 25 weeks of age stained for **(A)** estrogen receptor (red), **(B)** progesterone receptor (brown), and **(C)** glucocorticoid receptor (brown). Arrows indicate positive receptor expression staining; for **A-C**, 20X magnification, scale bar = 100 µm. **D**. high resolution images of each receptor (ER, PR, and GR) expression at 21 weeks of age, scale bar = 50 µm. For all slides **A-D**, Hematoxylin (blue) was used for a counterstain.



(Young 2013; Zehr, Gans, and McClintock 2001; R. D. Romeo 2010), and a period on which carcinogen-based studies have keenly focused as a key window of exposure that indicates later cancer risk. However, our results suggest that impaired development later in life is more important for mediating cancer risk in socially isolated rats. Thus, our results provide evidence that measurements of structures beyond TEBs are necessary to fully understand how environmental stressors can impact mammary gland development and potentially increase mammary cancer risk.

Our longitudinal study extends the bountiful data from Russo and others that mammary gland development continues after TEBs cavitate in early puberty (I. H. Russo and Russo 1978; Young 2013; I. H. Russo and Russo 1996; Paine and Lewis 2017). It clarifies two fundamental phases of mammary gland development that progress during late puberty and young adulthood: growth (ductal extension and expansion of the ductal tree through undifferentiated structures) and differentiation of the ductal tree morphology from ABs to lobules. While individual differences exist in the morphologic make-up of each sector of the mammary gland, there is a common spatial gradient in mammary ductal development that is sustained throughout adulthood (Fig. 2). During late puberty, in the most distal part of the ductal tree (S4), TEBs are continuing to extend the ductal tree while closer to the nipple (S1), maximal ductal extension has been reached and alveolobular development has already begun. Even in young adulthood, TDs are the predominant structure in the distal sector (S4), while closer to the nipple (S1) the differentiated ABs and Ls are primary or secondary having had the longest opportunity to differentiate. This is consistent with clinical reports that breast cancer is less likely to arise close to the nipple (Sacchini et al. 2006).

By 13 weeks, late puberty, the adrenal and ovarian axes are maturing and regular ovarian cycles become more common (Zehr, Gans, and McClintock 2001; Gans and McClintock 1993; Gretchen L. Hermes and McClintock 2008; R. D. Romeo 2010). The ductal tree has extended further, and TEB number has significantly dropped. While TEBs are largely considered to be the

drivers of ductal extension, TDs are the most abundant structures in the distal ductal areas (S2-4), and growth of the ductal tree continues through 17 weeks. Therefore, TDs likely retain some proliferative capacity and are likely driving ductal growth under the instructive signals from the ovary, as ovarian cycles regularize.

By 17 weeks of age, early adulthood, maximal ductal extension and mammary gland area has been reached and a shift occurs from primarily ductal growth to ductal differentiation. During this period, both the ovarian and adrenal axes are fully mature (LeFevre and McClintock 1991; Gans and McClintock 1993; Gretchen L. Hermes and McClintock 2008; R. D. Romeo 2010). During the subsequent ovarian cycles, now with regular exposure to progesterone from the corpus luteum, the ductal tree differentiates into ABs and then lobules. mammary gland differentiation continues through 25 weeks of age, although TD are still the predominant structures, particularly in the distal areas (S2-S4; Fig. 2). Much of the extant literature on physiological, cellular and genetic mechanisms of mammary gland development uses rats that are just 8 weeks of age, still in early puberty. Yet, the mammary gland continues to develop through late puberty into early adulthood while maturation of the ovarian and adrenal axes creates a changing hormonal milieu. Given that human breast development also continues into young adulthood, future mechanistic studies of mammary gland development as a cancer risk factor, can use this rat model, and capitalize on the striking gradient that lays out the developmental processes in a spatial gradient.

Social isolation slows growth of the mammary ductal tree primarily during late puberty, and also impairs its differentiation, primarily during early adulthood. Because both mammary gland growth and differentiation are promoted by progesterone and estrogen (Andrechek et al. 2008; Young 2013; Paine and Lewis 2017; Joshi et al. 2010; Smith 1955; C Brisken et al. 1998; Beleut et al. 2010; Javed and Lteif 2013; Haslam and Shyamala 1980; Sharma et al. 2011; Shyamala 1999; Russell D Romeo, Lee, and McEwen 2004; Lee et al. 2013), one would expect that isolated rats would have less luteal progesterone and less estrogenized ovarian cycles if ovarian function

were sufficient to mediate these effects of social isolation. However, the opposite was observed; isolates had more not less luteal progesterone (confirming prior results (Gretchen L. Hermes and McClintock 2008; McClintock 1981; Aron 1979)) and did not manifest less estrogenization until young adulthood (Fig 4b;13-17 weeks). Moreover, estrogen signaling increases PR expression in the developing ductal epithelium (Read et al. 1988) . Despite the decrease in circulating estrogen in isolates, mammary gland epithelial PR expression remained the same between rats of both social conditions (Fig. 6b). Thus, although progesterone and estrogen clearly are necessary for mammary gland growth and differentiation, they alone cannot explain the difference between isolated and grouped rats in mammary gland development.

Glucocorticoids are a more plausible candidate for mediating the effects of social isolation on stunted mammary gland growth and differentiation. Elevated GC reactivity resulting from social isolation is detectable as early as 13 weeks of age, sustained throughout adulthood (Fig. 5b; (Gretchen L. Hermes and McClintock 2008; G. L. Hermes et al. 2009)) and is associated with adrenal fatigue, evidenced by smaller adrenals. GC receptors are present in all ductal structures. Indeed, female rats fed corticosterone-supplemented diet between four and eight weeks of age had decreased mammary ductal extension and area (Zhu, Jiang, and Thompson 1998). Similar to ductal extension, alveolobular differentiation requires cycles of proliferation and apoptosis to form alveoli and clear the developing lumen (reviewed in (Gjorevski and Nelson 2011)). It is plausible that elevated GR activation in the mammary gland of the isolated rats could prevent the proper clearing of the alveolar lumen and prevent further proliferation and alveolobular development (Wu et al. 2004); this by definition would preserve a greater proportion of progenitor cells to differentiated epithelial cells in the adult mammary gland and may contribute to later cancer risk (Ginestier and Wicha 2007). Moreover, our lab and others have demonstrated that GCs inhibit estrogen-induced proliferation in the mammary epithelium (West et al. 2016; Yang et al. 2017). Increased epithelial cell GR activation provides a possible mechanism by which social

isolation could decrease estradiol-dependent mammary ductal extension despite no difference in estrogenization. Future experiments utilizing laser capture microdissection would allow us to analyze downstream targets for GR to confirm this hypothesis.

There was a significant decrease in GR expression in the mammary ductal tree of isolated rats at 21 weeks of age, four weeks earlier than a similar decrease was observed in grouped rats (25 weeks). After the decrease in GR expression there was no further alveolobular differentiation in the isolates (Fig. 6c; 21-25 weeks), whereas it continued through 25 weeks in the grouped rats. The decrease in GR expression could prevent further alveolobular differentiation. Indeed, inhibition of GR DNA-binding decreases mammary ductal proliferation in virgin mice (Reichardt et al. 2001). However, these data also underscore that the role of aberrant GR activation on nulliparous mammary gland development, particularly alveolobular differentiation, remains largely unknown (Whirledge and Cidlowski 2017).

The decrease in alveolobular differentiation in the isolates was associated with a decrease in stroma and a relative increase in mammary adipose tissue (Fig. 2c). Stromal area correlated highly with ductal structure area in all rats (Supp. Fig. 3), it may be the impaired alveolobular development was driving the decrease in stroma. However, it is unclear whether the increase in mammary adipose tissue is solely the result of a decrease in the proportion of ductal structures or an additional increase in fat deposition. The latter is supported by previous data that prolonged exposure of GCs, such as that observed in the isolates, promotes expansion of fat depots including in the mammary gland (Volden et al. 2013). Moreover, previous studies from our lab demonstrate that there are significant changes in the mammary adipose secretome in response to social isolation that can increase cancer cell proliferation and survival (Volden et al. 2013; Volden et al. 2016). How these secreted factors influence cells in mammary gland development is the subject of future research.

Previous work from our lab demonstrates that exposure to psychosocial stress is associated with increase in invasive mammary cancer (G. L. Hermes et al. 2009). Here, we present a developmental mechanism of increased breast cancer risk that operates during puberty and young adulthood. This association between decreased differentiation and increased mammary cancer risk may be counter to bountiful data that decreased mammographic density is protective (Vachon et al. 2007). Instead, we hypothesize that observed decrease in ductal differentiation is a result of the inability of mammary ductal progenitor cells to produce differentiated daughter cells. Indeed, recent data suggests that women at high-risk for developing breast cancer have less differentiated ductal trees and have a relative increase in mammary progenitor cells (Honeth et al. 2015). Moreover, the relationship between mammary gland composition early in life and mammographic density has largely focused on differences in body mass index and may not be applicable to our social isolation model (Harris et al. 2011; Bertrand et al. 2015).

The public health implications of this work are broad. Our longitudinal study of natural mammary gland development in nulliparous rats connects a long-known risk factor for breast cancer, decreased differentiation of the mammary ductal tree, with glucocorticoid stress reactivity heighted by social isolation. We demonstrate that psychosocial stress can significantly alter mammary gland development spanning puberty and early adulthood. The developing mammary gland is susceptible to environmental insult for a longer portion of the lifespan than previously appreciated. Moreover, we provide evidence that variation in the ovarian cycles observed in the isolates, including increased luteal progesterone and later decreased estrogenization, is not sufficient to mediate decreased mammary gland development by social isolation. We propose that decreased ductal differentiation and accelerated luteal function in the isolated female rats are both a result of elevated GC signaling and contribute to decreased alveolobular development. Understanding the exact mechanisms by which increased GC exposure decreases ductal growth

and differentiation in association with increased mammary cancer risk is likely to reveal important mechanisms of breast cancer etiology.

Chapter 3: Glucocorticoid Receptor Activation Inhibits Proliferation and Promotes Survival of Mammary Stem and Progenitor Cells

3.1: Introduction

Psychosocial stress exposure is associated with an elevated glucocorticoid stress response, decreased mammary gland development, and increased mammary cancer risk in female Sprague-Dawley rats (G. L. Hermes et al. 2009; Gretchen L. Hermes and McClintock 2008). Previous work from our lab and others have suggested a role for glucocorticoid receptor in breast cancer progression (Volden and Conzen 2013; West et al. 2016; Pan, Kocherginsky, and Conzen 2011), yet its role in mammary gland development, particularly in normal mammary epithelial differentiation, remains undefined (Whirledge and Cidlowski 2017). During mammary gland development, mammary stem cells and lineage-restricted progenitor cells populate mammary ductal tree of multiple phenotypically and functionally distinct cell populations including terminally differentiated luminal and basal epithelial cells (Visvader and Stingl 2014). Because these stem and progenitor cell populations are dynamic through the lifetime and are a long-lived target for errors, they provide an attractive but poorly understood link between psychosocial stress exposure, mammary gland development, and breast cancer risk. We hypothesize that the elevated glucocorticoid stress response induced by psychosocial stress inhibits morphological differentiation of the mammary ductal tree through inhibiting the differentiation of mammary progenitor cells.

Life events that promote differentiation of the mammary ductal tree protect against breast cancer. For example, the mammary gland is more susceptible to DNA-damage early in pubertal development as compared to in adulthood (Jose Russo et al. 2005; J Russo, Wilgus, and Russo 1979). Moreover, childbearing increases differentiation of the mammary ductal tree and reduces the risk of most types of breast cancer (Ewertz et al. 1990; MacMahon et al. 1970); the younger the age at first pregnancy, the lower the risk.

It has been hypothesized that differentiation of the mammary ductal tree protects against breast cancer risk through reduction of mammary stem cell number. Indeed nulliparous women and women with the BRCA1 mutation, who are at higher risk of developing breast cancer, have less differentiated mammary ductal trees with a greater proportion of ALDH1A1+ mammary stem cells to differentiated cell types (Honeth et al. 2015). However, mouse models studying the effect of parity-induced differentiation on mammary stem cell number have shown mixed results. Several studies have identified a decrease in MRU activity in unfractionated mammary epithelial cells from parous mice, while others have found no decrease (Siwko et al. 2008; Meier-Abt et al. 2013; Britt et al. 2009). Others have suggested that a decrease in luminal progenitors, rather than mammary stem cells is responsible for differentiation-induced breast cancer protection.

Studies on mammary differentiation, mammary stem cell number, and breast cancer risk have largely utilized mouse models, and therefore have studied parity-induced differentiation rather than nulliparous mammary ductal differentiation. While overlapping, the mechanism by which parity-induced differentiation of the ductal tree decreases breast cancer risk may be different than that of nulliparous differentiation. We hypothesize that rats exposed to psychosocial stress, which have less differentiated ductal trees and are at higher risk of developing invasive mammary cancer, will have a greater proportion of mammary stem cells to differentiated epithelial cells as compared to their less stressed counterparts.

A variety of systemic and locally derived factors influence the proliferation of stem and progenitor cells and also influence their renewal capacity (R. M. Esper et al. 2015; Visvader and Stingl 2014; Joshi et al. 2010; Asselin-Labat et al. 2010). The signaling of reproductive steroid hormones, estrogen and progesterone, on increasing mammary stem and progenitor cell population has been well-characterized and largely operates through PR and ER activation of more differentiated accessory cells (Visvader and Stingl 2014). Our lab has reported that exposure to psychosocial stress is associated with an altered reproductive hormone profile

including elevated exposure to luteal progesterone. We hypothesize stress-induced changes in the reproductive hormone profile may influence the size of the mammary stem and progenitor pool.

It is plausible that elevated glucocorticoids induced by psychosocial stress may also increase the mammary stem and progenitor cells population independent of the effect of stress on estrogen and progesterone. Previous studies have demonstrated that pretreating mammary precancerous epithelial cells (MCF10A-ras) with glucocorticoids increases their mammosphere forming potential (Sorrentino et al. 2017). However, the role of glucocorticoids in biology of untransformed mammary stem and progenitor cells has not been examined.

Mammary adipose tissue can also influence mammary stem cell biology. Mammary adipose tissue-derived proteins (e.g. leptin and adiponectin) can respectively increase and decrease self-renewal of mammary stem cells (Raymond M. Esper et al. 2015). Moreover genes involved in lipid metabolism are also indicated in cancer stem cell biology and are increased with psychosocial stress exposure (Volden et al. 2013; Corominas-Faja et al. 2014). Our lab has clearly demonstrated that psychosocial stress alters the mammary adipose tissue secretome and increases the proliferation and invasion of pre-invasive mammary epithelial cells (Volden et al. 2013; Volden et al. 2016). However, the effect of psychosocial stress the mammary adipose tissue secretome during mammary gland development remains unknown. Together, these data suggest that the mammary adipose tissue secretome in rats exposed to psychosocial stress could also differentially regulate mammary stem cell biology.

There are several methods to determine the size and composition of the mammary stem and progenitor cell population. In mouse and human studies, flow cytometry is utilized to quantify the relative proportion of mammary stem cells to epithelial cells. While still being defined, the basal cells with high expression of CD49f and low expression of luminal markers include the purported mammary stem cell population, while cells expressing CD49f and high expression of

luminal markers are considered lineage restricted progenitors. Recent data suggests that rat mammary stem/progenitor cells share the same $CD24^{+}CD49f^{+}$ cell surface signature as mice and can also be further delineated into predominantly basal and luminal progenitors by the intensity of CD49f expression (Sharma et al. 2011; Vela and Escrich 2016). However, the ability of primary rat cells enriched for these markers to survive and produce colonies in culture has not been demonstrated.

The propagation potential, or the ability of an epithelial cell to produce progeny, is influenced by its status in the stem cell hierarchy and its ability to proliferate and differentiate. 3D-culture can be used as an *in vitro* method to determine the propagation potential of cells. When grown under proliferative conditions, the more undifferentiated a cell, the greater its ability to form a clonal colony (Bouras et al. 2008). This clonal colony will be hereafter referred to as a spheroid. Moreover, 3D culture can be used as a tool to determine what exogenous factors influence propagation.

In this study, we determined the effect of elevated glucocorticoids on the proliferation of mammary stem and progenitor cells. First, we examined the mammary progenitor population during mammary gland development in rats exposed to social isolation-induced psychosocial stress compared to those living in groups. Second, we correlated those populations in individual rats to previous exposure to reproductive and stress steroids. We next determined the effects of glucocorticoid receptor activation on mammary progenitor cell proliferation. Finally, we examined the effect of the mammary adipose tissue secretome on mammary stem cell survival. Our findings underscore an integrative and underappreciated role of glucocorticoid receptor activation on mammary epithelial differentiation in the context of its microenvironment.

3.2: Materials & Methods

3.2.1: Sprague-Dawley rats

To study the effect of social isolation on mammary stem cell populations, female Sprague-Dawley IGS rats were bred at Charles River Laboratories, Inc. (Kingston, NY) weaned into all-female groups at three weeks of age and shipped in a series of cohorts to our laboratory at four weeks of age. Upon arrival, they were housed in groups of five, and at 4.5 weeks of age transferred to either single or uncrowded grouped housing of five rats, balanced for vigilant temperament as measured during an exploration stressor (as described in (Sonia A. Cavigelli, Yee, and McClintock 2006). To study the effect of corticosterone exposure mammary cells that were not exposed to high levels of stress hormone, group-housed rats were used. Female rats were shipped at 15 weeks of age and housed in groups of five for two weeks. All rats were housed under a reversed L:D cycle (14:10, dark onset 08:00 h) upon arrival to the animal facility so that all experimental manipulations were performed during rats' behavioral day. Rats were routinely handled during cage changes and health inspections. National Institutes of Health and University of Chicago Animal Care Guidelines were followed for all studies.

3.2.2: Experimental design

Mammary glands were harvested during puberty at 17 weeks of age. Ovarian cyclicity was quantified in the two weeks prior to sacrifice and behavioral stress reactivity one week prior as previously described for the social isolation study. Rats were sacrificed in proestrus/estrus to minimize ovarian cycle variability in mammary stem cell biology (S. A Cavigelli et al. 2005; P Schedin, Mitrenga, and Kaeck 2000; Joshi et al. 2010). All ovarian and stress measures were standardized at 5-6 hours into their behavioral day, when GC rhythm is at its nadir.

3.2.3: Mammary cell isolation

Upon sacrifice, the right inguinal quadrant of mammary glands were removed using the most caudal rib as an anatomical landmark. The mammary gland was cut into three approximately

equal pieces and placed in serum-free media supplemented with 1% penicillin/streptomycin (P/S), 1% fatty acid free bovine serum albumin (BSA), and 1% collagenase for 15 hours at 37 degrees as previously optimized. At that time, individual mammary cells (including MaSCs, epithelial, endothelial, and immune cells) were separated from the mammary adipocytes through centrifugation. The cell pellet underwent sequential treatment with NH_4Cl to lyse the red blood cells and then DNAse/dispase prior to organoid plating and antibody staining. These cells will be referred to as the harvested mammary cell suspension.

3.2.4: MaSC classification & quantification.

The harvested mammary cell suspension was blocked with unconjugated Fc antibody and then stained with anti- CD31 (Abcam 33858), CD45 (Abcam 33916), CD24 (BD Biosciences 562104), and CD49f (AbD Serotec MCA2034A647) antibodies. The $CD31^{+}CD45^{+}$ hematopoietic and endothelial populations were excluded by flow cytometry gating, and the remaining $CD24^{+}$ and $CD49f^{hi/lo}$ populations were quantified and collected using the BD FACSAria. The $CD24^{+}CD49f^{+}$ population is highly enriched for basal and luminal progenitor cells basal. Due to differences in collagenase efficiency between samples, proportion of progenitor to epithelial cells was reported rather than absolute number.

3.2.5: Three-dimensional cell culture of enriched mammary progenitor populations

Mammary cells enriched in basal and luminal progenitors ($CD31^{-}CD45^{-}CD24^{+}CD49^{hi}$ and $CD31^{-}CD45^{-}CD24^{+}CD49^{lo}$) were suspended, plated, and cultured in a Matrigel bead at a concentration of 5000 cells per 100 µL of Matrigel as previously described. To confirm that these enriched mammary epithelial cells include progenitors, the cells were propagated for 12 days in DMEM/F12 media containing, hydrocortisone, EGF, insulin, pen strep, B-27 and heparin

(sphereoid media) with 10% fetal bovine serum (FBS), as previously described (Liu et al. 2007). Resulting organoids were imaged using Axiovert 1000 Microscope.

3.2.6: Three-dimensional cell culture of mammary cells

Because of low yield of viable $CD24^{+}CD49f^{+}$ mammary stem and progenitor cells, mammary cell suspension was used for the three-dimensional cell culture of mammary cells as described in the introduction. 5000 cells were suspended, plated, and cultured in a 100 uL Matrigel bead (50% low growth factor Matrigel and 50% serum free media). Beads were treated with sphereoid media (described above), supplemented with 10% charcoal stripped serum and 4, 40, or 400 ng/mL corticosterone. Cells were plated in triplicate or quadruplicate and four biological replicates were used. After 5 days, spheroid colonies were scored and spheroids were trypsinized, washed, counted re-plated to form the second generation of spheroids.

In the second generation, all cells were grown spheroid media supplemented with fetal bovine serum (FBS) and assessed after an additional 5 days. This media induces proliferation of cells and therefore can be used to determine the capacity of the cells from the previous generation to produce progeny. Spheroid forming efficiency, the number of organoids, ≥50 μm in diameter divided by the original number of single cells seeded, is a measure of the percentage of cells plated that are mammary progenitor cells. An increase in forming efficiency indicates that treatment in the prior generation increased the proportion of progenitor cells to differentiated cells. Because of variance in organoid forming efficiency between biological replicates, organoid number and forming efficiency was reported as a percent of vehicle within biological replicates. Forming efficiency was calculated as the number of organoids, ≥50 μm in diameter divided by the original number of single cells seeded. All organoids were imaged using the Nikon Eclips Ti2.

3.2.7: Mammary adipose tissue conditioned media

The left inguinal mammary gland from was removed from each grouped and isolated rats, minced and spun down at 100 X g for 1 minute to enrich for mammary adipose tissue. For the conditioned media from grouped and isolated rats, the floating tissue was weighed and 1 g of tissue was incubated with 10 mL of serum free media, containing 1% BSA and 1% P/S at 37 °C for 8 hour. Media was harvested and sterile-filtered through a 0.22 μm syringe filter, aliquoted, and stored at -80 °C. We also tested the hypothesis that a pulse of corticosterone could increase the secretion of anti-apoptotic factors from mammary adipose tissue and thus promote the survival of mammary stem cells. Mammary fat from grouped rats was treated with 400 ng/mL corticosterone or vehicle (EtOH) for 10 minutes; 400 ng/mL is equivalent to what is observed in isolated rats exposed to an acute stressor. The tissue was subsequently washed with twice with PBS. The floating tissue was then weighed and was incubated in serum free media supplemented with BSA and P/S as described above. All media was thawed 4°C or on ice prior to use in experiments.

3.2.8 MCF-10A mammosphere formation and cell death.

The MCF-10A mammary cell line (ATCC) is an untransformed human mammary epithelial cell line. The cells have been authenticated by microscopic morphology and are negative for mycoplasma. Cells were seeded in non-adherent plates at 500 cells/well and were cultured with 100 μL of mammary adipose tissue culture media from each experimental condition (grouped versus isolated rats or grouped treated or untreated with corticosterone; described above). 0.04% methylcellulose and 1% YOYO-1 iodide was also added to each well to aid with imaging the mammospheres.

Mammosphere number and cell death were monitored using the Inucyte live content imaging system. The Incucyte system periodically captures phase-contrast and fluorescence images from individual wells of tissue culture plates. Accompanying software allows the user to mask phase and fluorescent images in order to quantify the number of cells above a certain size

and/or quantify fluorescence totals and object counts. YOYO-1 iodide, which fluorescently labels the nuclei of cells with compromised membranes, was used to quantify dead cells as previously described (Volden et al. 2016). Clusters of cells were counted as mammospheres if they were larger than 50 µm in diameter.

3.2.9 Quantitative RT-PCR

mRNA (1 µg) from each sample was reverse-transcribed using qScript cDNA synthesis kit (Quanta Biosciences). Q-RT-PCR was performed with the PerfeCTa SYBR Green FastMix (Quanta Biosciences). All reactions were performed in a Biorad iCycler iQ real-time PCR system. For Acca gene expression a T-test was performed on gene expression from grouped versus isolated animals after adjusting for the reference gene (beta-actin) as previously described (Volden et al. 2013).

3.2.10 Targeted analysis of lysophosphatidylcholine (LPC) and lysophosphatidic acid (LPA)

Analysis of LPC and LPA from mammary fat culture media was performed by the Wayne State Lipidomics Core Facility. Conditioned media was prepared as described above. Immediately after the media was harvested it was stored at -80 °C until it was shipped on dry ice to the Wayne State core facility. Samples were prepared by extraction with internal standards at the Wayne State facility. LPC and LPA analysis was performed using information dependent acquisition methods for quantitative LC-MS analysis with LPA and LPC analyzed in negative and positive mode respectively. For analysis, the three most differentially regulated lipid species between grouped and isolated rats were quantified and expressed as a percentage of lipid secretion from grouped rats.

3.3: Results

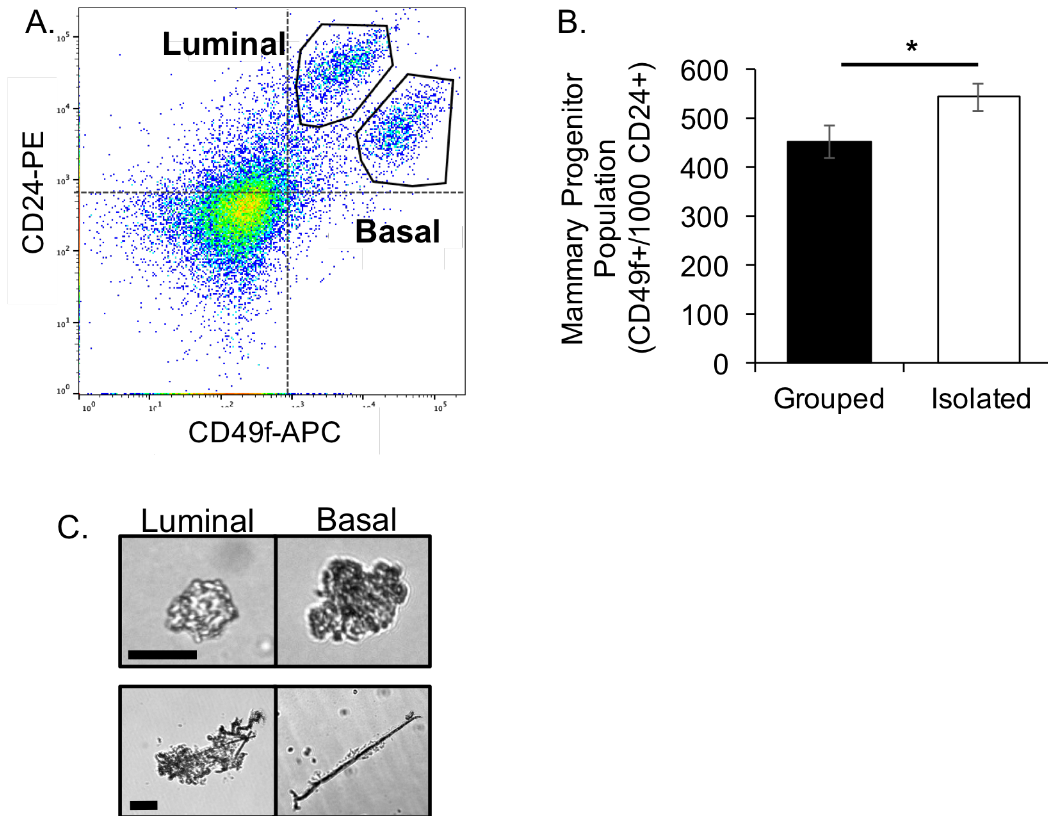
3.3.1: Psychosocial stress exposure increases the population of $CD24^{+}CD49f^{+}$ Cells

Rats exposed to post-weaning psychosocial stress exhibited an increase in the $CD24^{+}CD49f^{+}$ population enriched in mammary stem and progenitor cells (Fig 1a-b, 460 ± 30 $CD24^{+}CD49f^{+}$/1000 $CD24^{+}$ cells in grouped versus 530 ± 20 $CD24^{+}CD49f^{+}$/1000 $CD24^{+}$ cells in isolated rats, $P = 0.045$, n = 18, Students T-test). The difference in mammary stem cell population between grouped and isolated rats is appeared to be driven by expansion of the luminal progenitor population ($CD24^{+}CD49f^{lo}$) rather than the basal mammary stem cell population ($CD24^{+}CD49f^{hi}$ Supplemental 3.1B).

No difference in either the luminal or basal populations was observed at four weeks earlier at 13 weeks of age (405 ± 85 $CD24^{+}CD49f^{+}$/1000 $CD24^{+}$ cells in grouped versus 346 ± 75 cells in isolated rats, $P = 0.62$, n=10) or four weeks later at 21 weeks of age (667 ± 33 $CD24^{+}CD49f^{+}$/1000 $CD24^{+}$ cells in grouped versus 649 ± 89 cells in isolated rats, $P = 0.62$, n=10) (Supplemental Figure 3.1A). However, the sample size for both 13 and 21 week-old rats was half that of 17 week-old rats (n = 10 and n=9, respectively) and no conclusions can be made until more rats are sampled. However, there was a clear and highly significant increase in the proportion of stem/progenitor cells with increasing age (13-21 weeks; social condition: $F(1, 33) = 0.32$, $P = 0.58$, age: $F(1, 32) = 23.1$, $P \leq 0.0001$, interaction: $F(1, 33) = 0.15$, $P = 0.70$). Interestingly a similar expansion of basal stem cells and luminal progenitor cells has been described during pregnancy-associated alveolobular development (Asselin-Labat et al. 2010).

Both the basal stem cells ($CD24^{+}CD49f^{hi}$) and luminal ($CD24^{+}CD49f^{lo}$) progenitor population were capable of forming clonal colonies that ranged from acinar structures to branched formations (Figure 3.1C). These data confirm previous rat studies that these enriched populations contain mammary stem and progenitor cells (Vela and Escrich 2016).

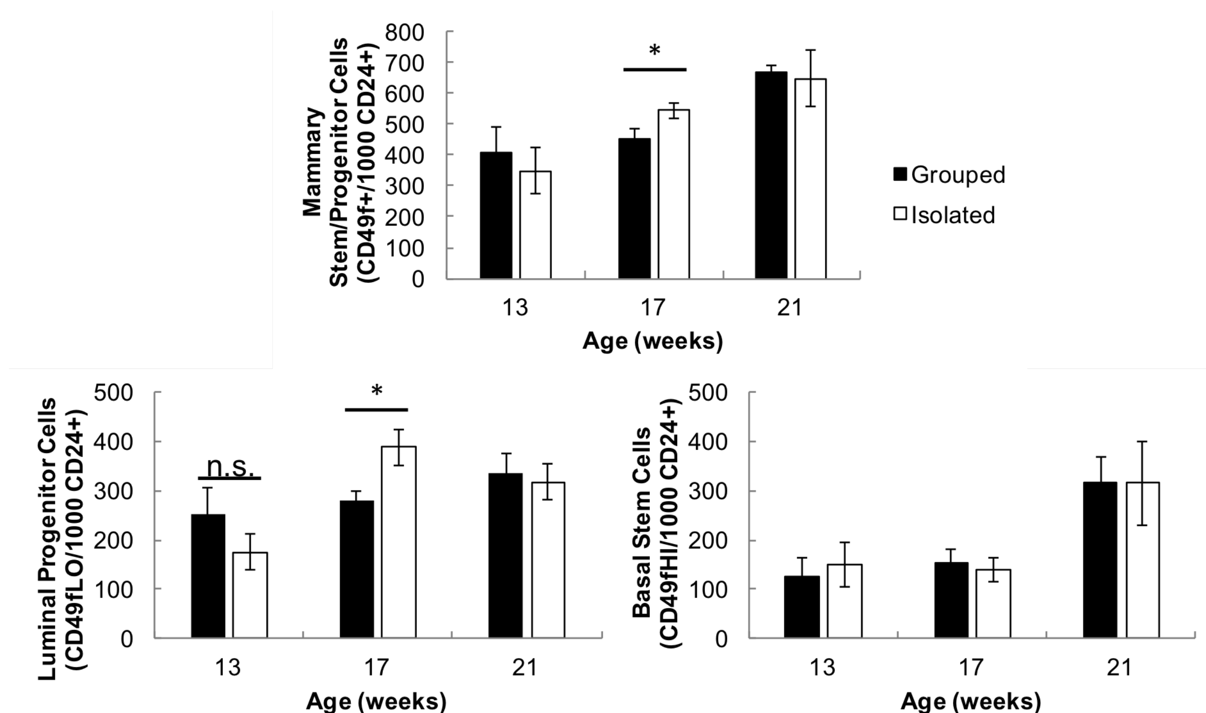
Figure 3.1: Cytometric analysis of rat mammary epithelial cell subtypes according to cell surface marker expression.
A. Representative flow cytometry plots of $CD24^{+}CD49f^{lo}$ (luminal progenitor) and $CD24^{+}C49f^{hi}$ (basal progenitor) cells in the $CD45^{-}CD31^{-}$ population within the mammary glands of 17-week socially-isolated and age-matched group-housed rats. **B,** Bar chart expressing the number of $CD24^{+}CD49f^{+}$ progenitor cells relative to the number of $CD24^{+}$ epithelial cells in the mammary gland for 17 week old socially-isolated and group-housed rats (mean ± sem). $^{*}P < 0.05$, *T*-test, n = 18. **C,** representative images of mammary progenitors grown in 3D culture producing acinar (top) and branched (bottom) colonies, scale bar = 50 µm.



3.3.2: Corticosterone reactivity but not progesterone and estrogen exposure correlates to $CD24^{+}CD49f^{+}$ population size

Previous reports and work in Chapter 2 demonstrate that psychosocial stress alters the reproductive hormonal mileu (G. L. Hermes et al. 2009; Gretchen L. Hermes and McClintock 2008). Isolated rats have increased exposure to luteal progesterone as early as 13 weeks of age and increased estrogen exposure during mammary ductal differentiation in early adulthood. Moreover, estrogen and progesterone have been implicated in mammary stem cell homeostasis (Joshi et al. 2010; Asselin-Labat et al. 2010). Therefore, we next looked for evidence that reproductive and stress steroid hormone exposure could be attributed to differences in these

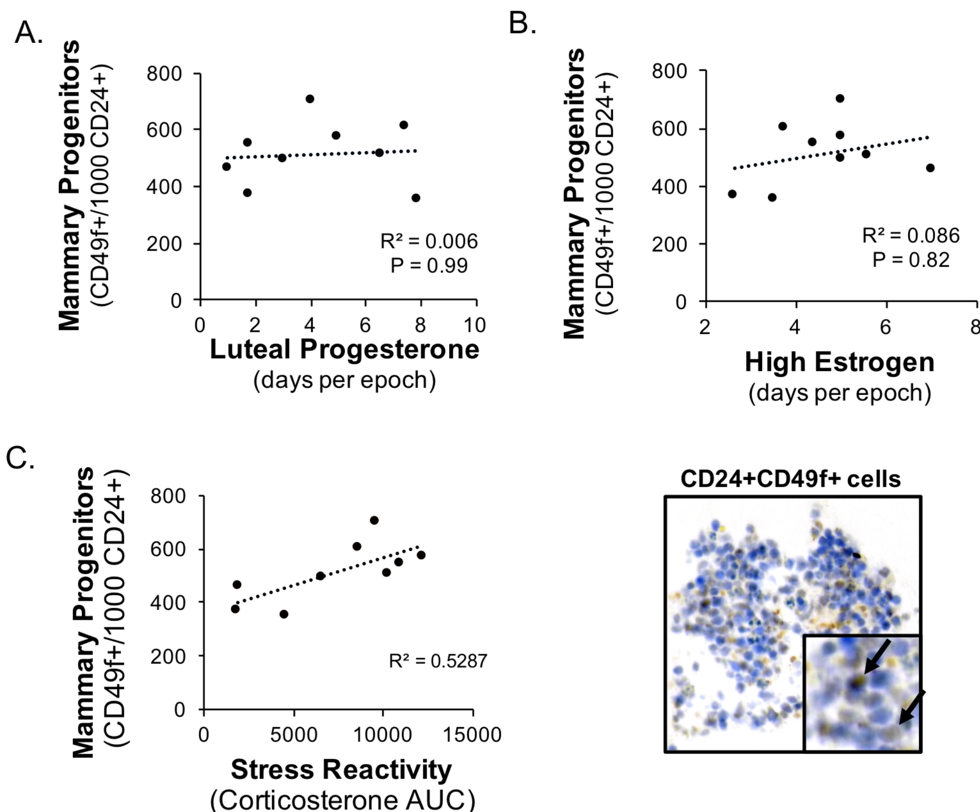
Figure 3.S1: Cytometric analysis of rat mammary epithelial cell subtypes at 13-17 weeks of age.
A. Bar charts expressing the number of **(A)** $CD24^{+}CD49f^{+}$ mammary stem/progenitor cells **(B)** $CD24^{+}CD49f^{lo}$ luminal progenitor cells, and **(C)** $CD24^{+}CD49f^{hi}$ basal stem cells relative to the number of $CD24^{+}$ epithelial cells in the mammary gland for 13, 17, and 21-week socially-isolated and group-housed rats (mean ± sem), $*P < 0.05$.



reproductive hormone levels. We found no statistically significant correlation between

$CD24^+CD49f^+$ cell population and the number of days a rat was exposed to luteal progesterone (Figure 3.2A, R = 0.080, P = 0.99, n = 9), nor any statistically significant correlation with high estrogen days (Figure 3.2B, R = 0.029, P = 0.82, n = 9).

Figure 3.2: Correlation of CD24+CD49f+ progenitor cell population to progesterone, estrogen, and glucocorticoid exposure.
Regression of progenitor cell population size to **A,** luteal progesterone exposure (r = 0.081) **B,** high estrogen exposure (r = 0.293) **C,** corticosterone reactivity (r = 0.737); *P < 0.05, T-test, n = 9. **D**, Representative image of CD24+CD49f+ cells stained for **(A)** glucocorticoid receptor (brown). Arrows indicate positive receptor expression.



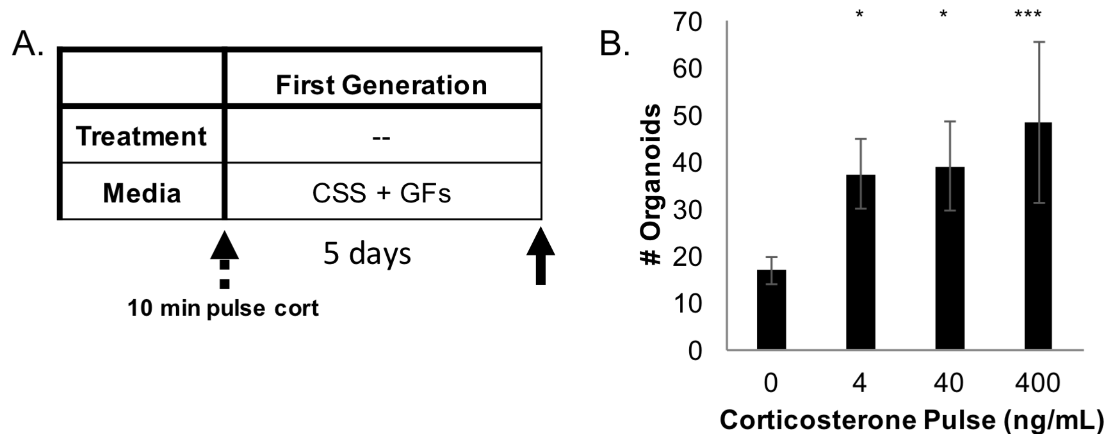
We have previously reported that psychosocial stress also increases glucocorticoid serum concentration during reactivity to and recovery from an acute stressor. We next determined if the $CD24^+CD49f^+$ population corresponded to the corticosterone exposure in response to an acute restraint test. Indeed, there was a moderate, positive correlation between $CD24^+CD49f^+$ cell population and corticosterone reactivity (Figure 3.2C, R = 0.727, P = 0.03, n = 9). This data suggests that psychosocial stress exposure may influence the $CD24^+CD49f^+$ population through

its effects on circulating corticosterone rather than indirect effects on progesterone and estrogen. Indeed, CD24^{+}CD49f^{+} cells include cells that express glucocorticoid receptor (Figure 3.3D.)

3.3.3: Corticosterone impairs differentiation of mammary progenitor cells

To determine if glucocorticoid receptor activation is sufficient for differentiation of mammary progenitor cells, we treated we treated a single-cell suspension of mammary cells from group-housed rats with a single ten-minute pulse of corticosterone (0, 4, 40 and 400 ng/mL). 400 ng/mL was chosen as the upper bound as that was the peak reactivity observed in isolated rats following a 30 minute restraint test. A ten-minute pulse mimics the response of a rat to a single acute stressor. The mammary cells were subsequently grown in 3D culture (Figure 3A).

Figure 3.3 : Short-term glucocorticoid receptor increases spheroid formation.
A. Experimental design: mammary cells were treated with a 10-minute pulse of corticosterone (0, 4, 40, 400 ng/mL), washed and plated in matrigel beads. The cells subsequently grown in charcoal stripped serum (CSS) and growth factors (GFs) for five days. B. the number of spheroids counted after 5 days (n = 4 technical replicates. *P < 0.05, and, ***P < 0.001, Students' T-test, four biological replicates were used.



Any treatment of corticosterone significantly increased the number of clonal colonies formed five days later (Figure 3.3B). Compared to vehicle treated cells, colony formation was doubled at the lower concentrations of corticosterone (4 and 40 ng/mL) and tripled at the highest concentration (400 ng/mL). These data indicate a permissive role for glucocorticoid receptor

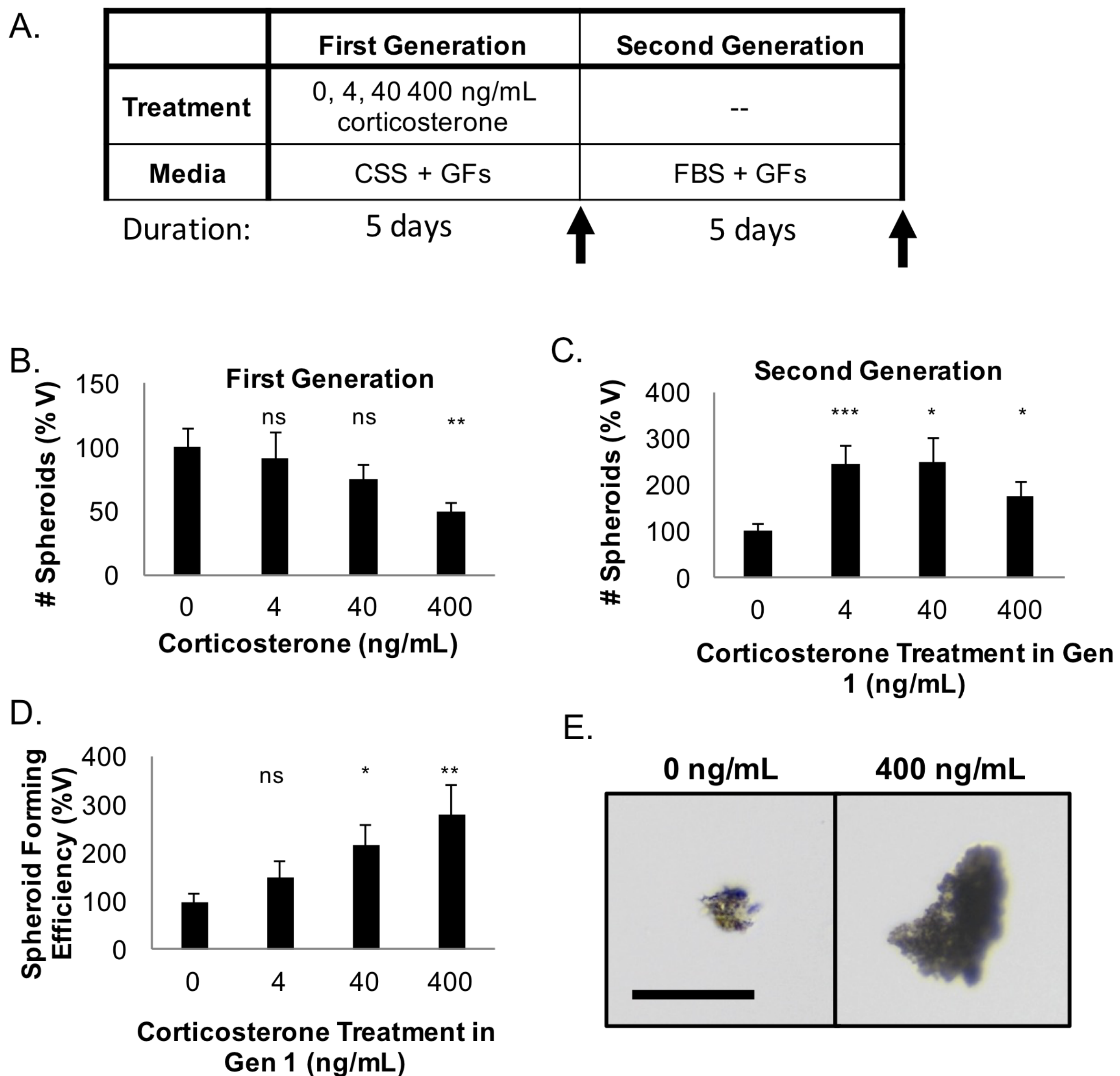
activation in mammary progenitor cell proliferation. There was a significant difference in the number of colonies formed between individual rats. Because of variance in the number of organoids formed between biological replicates, organoid number and forming efficiency was reported as a percent of vehicle in the subsequent experiments.

To mimic the effect of prolonged exposure to psychosocial stress on mammary progenitor cell differentiation, mammary cells were treated with corticosterone (0, 4, 40, or 400 ng/mL) for five days (Generation 1; Figure 4A). Contrary to the short-term pulse, long-term corticosterone treatment decreased the number of organoids observed at the highest concentration, indicating a decrease in proliferation of mammary progenitor cells (Figure 3.4B; 50.87 ± 6.79% of the number of organoids formed with vehicle, P = 0.009, Students' T-test). We next determined if the mammary cells treated with glucocorticoids maintained a population of stem and progenitor cells capable of forming organoids, despite undergoing less proliferation during glucocorticoid receptor activation.

The cells from Generation 1 were disaggregated, re-plated in Matrigel and grown in serum containing media. Cells previously treated with any concentration corticosterone grew at least 50% more organoids than cells treated with vehicle (Figure 3.4C, E). Indeed, the organoid forming efficiency increased as with increased concentration of corticosterone in the previous generation (Figure 3D). These data demonstrate that mammary progenitor and stem cells exposed to glucococortioid concentrations equivalent to concentrations in stressed rats undergo reduced proliveration, yet maintain their propagation potential once the elevated glucocorticoid concentration is relieved. Indeed, by inhibiting proligeration of these cells, glucocorticoid receptor activation prevents the production of more differentiated progeny.

3.3.4: Mammary adipose tissue secretome from isolated rats increases survival of mammary epithelial cells in suspension

Figure 3.4 : Glucocorticoid receptor activation decreases spheroid formation, and increases forming efficiency in subsequent generations.
A. Experimental overview: the first generation of spheroids were grown in 0, 4, 40, 400 ng/mL of corticosterone with charcoal stripped serum (CSS) and growth factors (GFs) for five days. The second generation was grown in fetal bovine serum (FBS) and GF with no additional corticosterone for five days. Sphereoids > 50 µm were counted and compared between treatments as indicated by bold arrows. The number of spheroids formed in the **(B)** first generation (n = 4 technical replicates) and **(C)** second generation (n = 3 technical replicates) expressed as a percent of vehicle. d, spheroid forming efficiency in the second generation. **B-D.** *P < 0.05, *P < 0.01, *P < 0.001, Students' T-test, four biological replicates were used**. E.** representative images of an spheroid produced from the second generation of growth after treatment with 400 ng/mL corticosterone in the first generation.



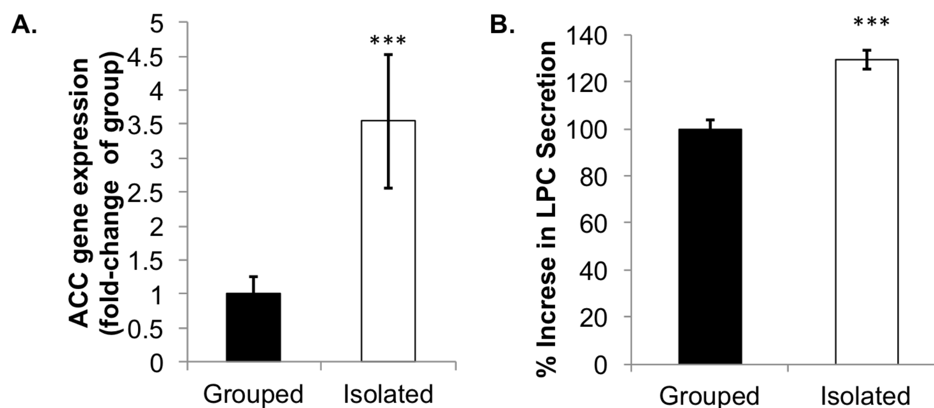
Mammary adipose tissue is an endocrine organ that secretes numerous factors that have been indicated in mammary stem cell biology (R. M. Esper et al. 2015). Moreover, our lab has demonstrated that exposure to psychosocial stress is associated with changes to the mammary adipose tissue secretome that increase the proliferation and invasion of pre-cancerous epithelial cells (Volden et al. 2016; Volden et al. 2013). This change in mammary adipose tissue was associated with an increase in lipid metabolism gene expression and an increase in secretion of fatty acids, including lysophosphatidic acid (LPA). Therefore, we hypothesized that psychosocial stress exposure would alter the secretion profile of mammary adipose tissue and increase the proliferation mammary stem cells.

We first looked for evidence that psychosocial stress affected rat mammary adipose tissue in a similar manner to that in the mouse model. At 17 weeks of age, we observed a significant increase in expression of lipid metabolism gene acetyl-CoA-carboxylase-alpha in mammary glands from rats exposed to psychosocial stress (3.54-fold increase in gene expression in isolated versus grouped rats, ***P< 0.003, T-Test; Supplemental Figure 3.S1A). Unlike, the SV-40 TAg mice exposed to psychosocial stress, there was no change in secreted LPA (data not shown). Yet, there was a significant increase in its metabolic precursor lysophosphatidylcholine (LPC; 30% increase in isolated compared to grouped 18:0, 18:2 and 20:4, *** P <0.001, Holm-Sidak Test; Supplementary Figure 3.1B).

After we confirmed alterations in lipid metabolism, mammary adipose tissue conditioned media from grouped or isolated rats was applied to mammary epithelial cells grown in suspension. Preliminary experiments using purified progenitor cells in 3D culture were inconclusive due to high levels of cell death induced by the flow sorting assay. Therefore, we used a the MCF10A cell line of normal mammary epithelial cells. These cells include epithelial cells throughout the stem cell hierarchy, but can be enriched for cells capable for forming clonal colonies through growth in suspension; anoikis-resistance is a hallmark of stem cells (Dontu and Wicha 2005).

Media derived from culturing mammary adipose tissue, regardless of animal housing, was sufficient to drive the formation of mammospheres. Media derived from culturing mammary adipose tissue from socially isolated rats resulted in significantly more MCF10A mammospheres formed than media from group-housed mammary adipose tissue (Figure 3.5A) (***P < 0.0001). The mammospheres derived from MCF10A cells treated with conditioned media from isolated rats maintained their number through 40 hours while no mammospheres were detectible in the cells treated with conditioned media from grouped rats. Moreover, there was significantly less cell death in MCF10A cells grown in conditioned media from isolated rats as compared to that derived from grouped rats (Figure 3.5B).

Supplemental Figure 3.S2: Acetyl CoA Carboxylase gene expression and LPC secretion from grouped and isolated rats mammary adipose tissue.
A. *Acc* gene expression of whole mammary gland and **(B)** secretion of lysophosphatidylcholine (LPC) from enriched mammary adipose tissue in grouped versus isolated rats at 17 weeks of age. *** P< 0.005, *T*-Test (**A**) and (**B**) Holm-Sidak Test.

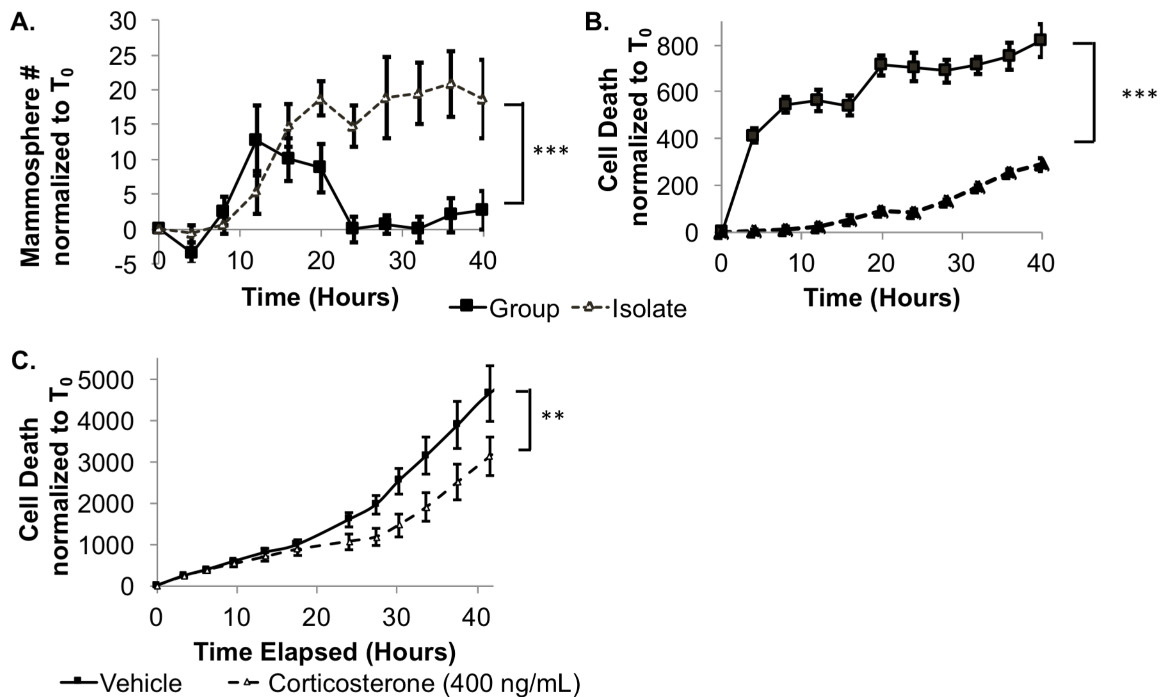


3.3.5: Corticosterone treatment increases secretion of pro-survival factors from mammary adipose tissue

Previous work has demonstrated that adipose tissue in the developing rat mammary gland expresses high levels of glucocorticoid receptor and is responsive to glucocorticoid receptor activation. Because isolated rats are exposed to higher levels of glucocorticoids in response to

an acute stressor, we hypothesized that glucocorticoid receptor activation of the mammary adipose tissue would increase secretion of pro-survival factors from mammary adipose tissue.

Figure 3.5. Mammary adipose tissue conditioned media from isolated rats promotes mammospheres formation and decreases cell death in MCF10A cells.
A-B. Mammary adipose tissue from grouped or isolated rats was cultured in serum free media for 8 hr to make CM, which was then applied to MCF10A cells grown in suspension. **C**, Mammary adipose tissue from grouped rats was treated with a ten-minute pulse of corticosterone or vehicle prior to culture. Cell proliferation (A) and death (B-C) was monitored using the Inucyte live content imaging system. Mammosphere number and cell death were calculated and the mammosphere number or cell death at t=0 was subtracted from all subsequent counts.



A 10-minute pulse of corticosterone was sufficient to increase secretion of pro-survival factors from mammary adipose tissue. When treated with the secretome of mammary adipose tissue treated with 400 ng/mL corticosterone, MCF10A cells underwent significantly less cell death (Figure 3.5C) despite having no change in mammosphere number (data not shown).

3.4 Discussion

We have previously demonstrated that socially isolated rats that have elevated glucocorticoid stress response have less differentiated morphology of their mammary ductal tree (Chapter 2) and a higher risk for developing invasive cancer (G. L. Hermes et al. 2009). Here we provide evidence that psychosocial stress and the resulting glucocorticoid stress response is associated with an increase in mammary progenitor populations.

Moreover, GR activation of mammary cells decreased proliferation of mammary stem and progenitor cells, and maintained their propagation potential. The mammary adipose tissue secretome promoted mammosphere formation of MCF10A cells grown in suspension. Moreover, the mammary adipose tissue secretome from rats exposed to psychosocial stress promoted the survival of these cells to a greater extent than that from grouped rats. Furthermore, treating mammary fat with pulse of corticosterone also increased the secretion of pro-survival factors. Together, these data demonstrate that aberrant glucocorticoid receptor activation can inhibit progenitor cell differentiation.

Our study also extends previous studies that examine the role reproductive steroids in the expansion of mammary stem/progenitor populations. Here, we tested the hypothesis that estrogen and progesterone mediate the effects of psychosocial stress. This is a plausible mechanism as isolation affects circulating estrogen and progesterone throughout the ovarian cycle (Chapter 2). Other work has demonstrated that these hormones increase the mammary stem and progenitor cell populations. However, our work did not support their role as a mediating mechanism for the effect of social isolation induced psychosocial stress. It is notable that neither previous exposure to progesterone nor to estrogen correlated to the size of the mammary progenitor population, supporting previous evidence that progesterone-induced expansion of mammary stem cells during diestrus is truly temporal (Joshi et al. 2010). Moreover, this is contradictory to the hypothesis that repeated estrous cycles alone could be responsible for the

increase mammary stem and progenitor cell populations observed in rats exposed to psychosocial stress.

Instead, there was a moderate correlation between glucocorticoid stress reactivity and mammary progenitor population. While exposure to single high level pulse of glucocorticoids increased stem cell differentiation (Figure 3.3), it is more likely that the corticosterone reactivity and recovery during an acute restraint test is representative of the rat's reactivity to daily stressors. Thereby, the mammary progenitor population is likely a result of repeated pulses of elevated glucocorticoids.

Glucocorticoid receptor activation of mammary cells decreased the differentiation of mammary progenitors and stem cell while preserving their ability to produce clonal colonies when elevated glucocorticoids were removed. This suggests that elevated glucocorticoid reactivity may be responsible for the decreased differentiation of the mammary ductal tree in isolated rats (Chapter 2). Moreover, the spheroid forming efficiency of cells in the second generation was significantly increased with previous treatment with glucocorticoids and increased in a dose response. This demonstrates that dose of previous glucocorticoid exposure correlates to the number of progenitors, which is supported by data that the size of a rat's progenitor cell populations correlate to their stress reactivity.

Because of the technical limitations in isolating viable $CD24^{+}CD49f^{+}$ cells, all assays used mammary cell suspensions that included epithelial stem and progenitor cells in addition to differentiated epithelial cells and non-adipocyte stromal cells (e.g. fibroblasts). Therefore, it remains unknown if GR activation of mammary stem and progenitor cells elicits the decrease in differentiation or if it is a result of paracrine activity of GR activated accessory cells. It is notable, however, that rats exposed to the psychosocial stressor of social isolation have significantly fewer differentiated epithelial cells and stromal cells in their mammary glands.

Future studies that clearly identify stem and progenitor cells in the adult mammary gland are needed to further elucidate the mechanism of action. It is also unclear whether the increase in clonal forming efficiency is a result of decreased proliferation and therefore a greater proportion of mammary progenitors to differentiated cells and if glucocorticoid receptor activation results in genetic or epigenetic change that manifest as increased mammary progenitor self-renewal. Because glucocorticoid receptor activation inhibits apoptosis of mammary epithelial cells, it is unlikely that cell death is responsible for the decrease in mammosphere formation. Because we observed a similar increase in forming efficiency after a 10-minute pulse of corticosterone, our data suggests a transcriptional or epigenetic component.

It has been proposed that the more undifferentiated the cell of origin, the more aggressive the resulting breast cancer (Rangel et al. 2016). Therefore, it is possible that elevated glucocorticoid reactivity induced by psychosocial stress increases mammary cancer risk by inhibiting the production of differentiated epithelium. Hypothetically, this would increase the likelihood of neoplastic transformation in an undifferentiated cell type and subsequent development of an aggressive cancer. This hypothesis needs to be tested, but it is corroborated with data that breast cancer aggressiveness, but not overall incidence of breast pathologies, is increased in populations exposed to high-levels of psychosocial stressors, such as low-SES (Akinyemiju et al. 2015).

Chapter 4: Conclusions and Future Directions

The research described throughout the previous chapters was prompted by the initial observation that social isolation of rats and mice induces larger mammary tumors and a shift toward malignancy as compared to their group-housed counterparts. Through our trans-disciplinary research program, we have continued this work to identify mechanisms by which the neuroendocrine response to unremittent stress exposure and resulting heightened glucocorticoid stress reactivity, disrupts normal mammary gland development to contribute to breast cancer progression. The data described in the previous chapters have utilized an integrative approach to understand the effect of psychosocial stressors and resulting increased glucocorticoid stress reactivity on mammary gland development and reproductive steroid exposure in the context of later mammary cancer risk.

Our findings underscore the role of glucocorticoids and glucocorticoid receptor activation in mammary gland development, likely independent of the effects of stress on circulating estrogen and progesterone. Exposure to elevated glucocorticoids during adolescence was associated with decreased alveolobular differentiation in early adulthood (17 to 25 weeks of age) which was preceded by an increase in the proportion of mammary progenitor cells to differentiated cells in the mammary gland (17 weeks of age). Notably, the population of mammary progenitors correlated to glucocorticoid reactivity, but not prior exposure to estrogen or progesterone, indicating a role for the glucocorticoid receptor in mammary progenitor cell differentiation that persists across ovarian cycles (Joshi et al. 2010). These data demonstrate a previously unappreciated role of glucocorticoid receptor activation on nulliparous mammary gland development and has implications across multiple scientific fields including public health, endocrinology, behavioral science, developmental biology, metabolism, and cancer biology.

The purpose of Chapter 4 is to provide perspective on some of the major questions raised by the data and to provide suggestions for research moving forward. Addressing the effects of social stress, reproductive endocrinology, and mammary stem cells using *in vitro* and *in vivo* methods will be discussed. This section will highlight some of the challenges moving forward in researching mammary gland development using available models and will also discuss some research tools that may aid in the process. The findings detailed in previous chapters raise many questions, and despite the progress that has been made, much work is still needed to fully understand how stress-mediated mechanisms are involved in the progression of breast cancer.

4.1: Pseudotemporal analysis of mammary epithelial cells and its implications

Epidemiological studies suggest a link between exposure to unrelenting stressors and breast cancer biology. This data is supported by research from our lab which described a shift toward malignancy in the Sprague-Dawley rat model of heterogeneous breast cancer and an increase in tumor burden in the TAg mouse model of human TNBC (G. L. Hermes et al. 2009; J. B. Williams et al. 2009). Research described in this dissertation demonstrated that this shift in tumor biology is associated with significant changes in mammary gland development (Chapter 2) and that purported targets for transformation, mammary stem cell and progenitor cells, can respond to elevated corticosterone (Chapter 3). These data suggest that the developing mammary gland is not only susceptible to elevated stress hormones, but also that changes in the mammary gland could mediate increased breast cancer risk. Moreover, these data suggest that a single stressor resulting in a pulse of corticosterone can change the differentiation potential of a progenitor's daughter cells. A logical next step would be to understand how an increase in the proportion of mammary stem and progenitor cells contribute to breast cancer risk.

There are several major challenges in mammary stem cell research as it pertains to breast cancer progression that would need to be addressed first: 1) Genetic signatures that enrich for

stem cell populations have been identified, but the genetic signature exclusive to stem cells has not, 2) the stem cell hypothesis has not been proven, and 3) how to track the purported cancer cell-of-origin from development to neoplastic transformation is technically difficult due to the relative rarity of mammary stem/progenitor cells.

A major obstacle in defining the mammary stem cell genetic signature has been the fact that mammary stem cells are fairly rare and have only been enriched for MRU at a rate of ~1/50 cells (Trejo et al. 2017). This has led to much confusion in the literature including, but not limited to the assertion that true bi-potent mammary stem cells may not exist in the adult mammary gland at all (Trejo et al. 2017; Chen, Liu, and Song 2017). Moreover, population-based gene expression profiling of this stem cell enriched population generates average measurements and masks the variation across individual cells, thus limiting insight into the genetic signature of rare populations such as the mammary stem cell.

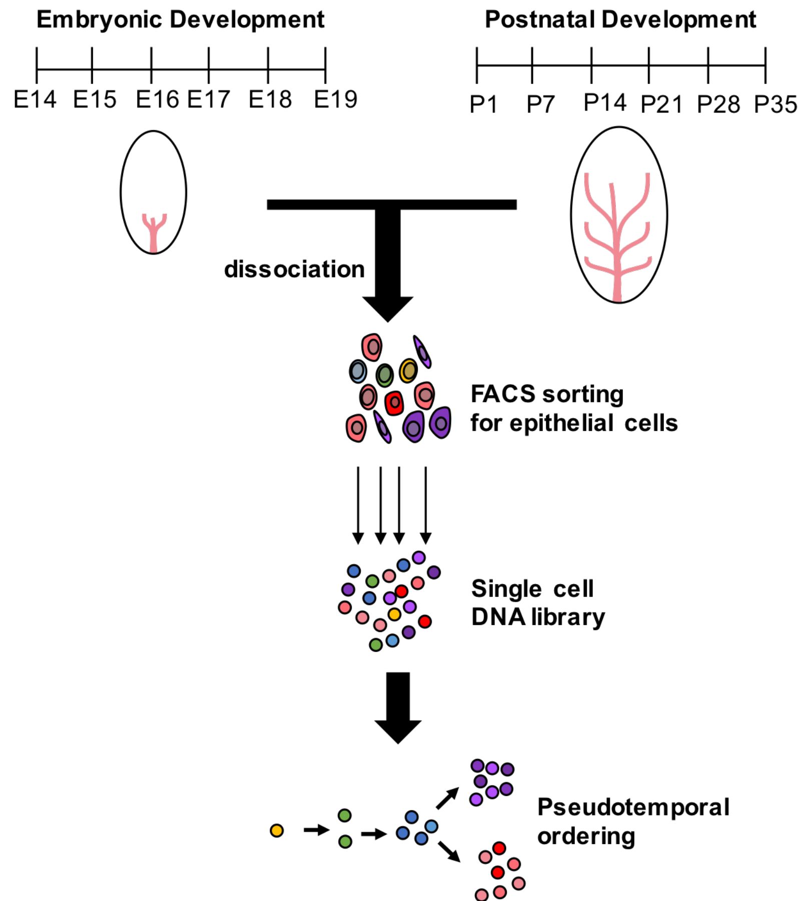
Single cell transcriptome analysis and subsequent psuedotemporal ordering of mammary cells would be a significant advancement toward identifying genetic signatures exclusive to mammary stem cells. Through providing gene expression profiles of individual cells, single-cell RNA sequencing (RNA-Seq) can overcome this problem. Subpopulations of cells can be identified based on transcriptional similarity. Moreover, if sampled throughout the developmental time course, single-cell expression profiles can be used to order cells along a “psudotemporal” developmental continuum. That is, one could sample mammary epithelial cells throughout mammary gland development beginning at fetal mammary gland development when mammary stem cells are relatively abundant and continuing through early adulthood when differentiated cells have populated the mammary ductal tree (Figure 4.1). After running single-cell sequencing at each of these time points, the cells can then be evaluated by transcriptional similarity and relative abundance as mammary gland development progresses. *In silico,* these cells can be computationally ordered according to the gradual transition of their transcriptomes, effectively

placing individual cells on a virtual time axis along which cells are presumed to travel as differentiation increases. This method can not only be used to determine the genetic signature of stem cells “early” in the pseudotemporal differentiation track, but it can also be used to determine what genes are involved in cell fate decision. Indeed, this process already been used to resolve cell fate decisions in B cells, beta-cells, and myoblasts (Bendall et al. 2014; Trapnell et al. 2014; C. Zeng et al. 2017).

After identifying stem cells signatures and regulators of stem and progenitor cell fate decision, the stem cell hypothesis can truly be interrogated. Using inducible, tissue-specific genetic engineering, researchers can then impair mammary ductal differentiation through limiting differentiation of certain cell types at different points in the lifespan to experimentally promote the accumulation of mammary stem or progenitor cells. Oncogenic mutations (e.g mutations in *p53* or *BRCA1)* can also be employed to aid in promoting transformation of these cells on a reasonable timeline. However, spontaneous cancer development with mammary stem and progenitor cell populations as the only variable would be the most informative for proving the stem cell hypothesis.

The experiment described above could also employ pseudotemporal sequencing of the stem and progenitor populations throughout cancer progression to track oncogenic changes that lead to invasive cancer progression. This has the potential to identify genetic mechanisms of progression from a benign mammary epithelial cell to a malignantly transformed cell. Moreover, this method can be used to formulate a stem cell hierarchy for future research.

Figure 4.1: Experimental overview of pseudotemporal ordering of mammary epithelial cell differentiation



4.2: Expanding research to include nulliparous mammary gland development.

Current models of mammary gland development have overlooked alveolobular development during young adulthood. To this point, the great majority of these models have focused on early puberty, when terminal end buds are most abundant, or during pregnancy, when final differentiation of the mammary ductal tree occurs. However, epidemiological studies have demonstrated that the breast is susceptible to environmental insults at least through a woman's early 20s and likely until first birth. Yet, current models of mammary gland development do not

include an emphasis on nulliparous alveolobular development or on mechanisms that impair this stage of development and could potentially lead to oncogenesis.

Broadening developmental models to include nulliparous mammary ductal differentiation is becoming increasingly important as women are delaying childbirth or choosing not to have children (Myrskylä et al. 2011). Women who have birth for the first time after 35 are approximately 40 percent more likely to get breast cancer than women who have their first child before age 20 and the risk is similar for women who remain nulliparous (Ewertz et al. 1990). Yet nulliparous mammary gland development has not been a focus to date. Indeed, xenograft and genetically engineered mouse models of breast cancer often induce oncogenesis as early as 6-8 weeks of age while the mammary gland is still developing. Moreover, studies on pregnancy-associated differentiation induce pregnancy at the onset of fertility, which is the equivalent of inducing pregnancy in a 13-year old.

Our model of social isolation underscores the importance of studying nulliparous alveolobular development in environmental exposure studies. Building on a rich history of work in rats, we have demonstrated that mammary gland development continues well past the presence of TEBs. Significant ductal differentiation continued through 25 weeks of age, which is equivalent to mid-twenties in a woman. Moreover, alveolobular development decreased in rats exposed to social isolation despite no difference earlier in development, including TEB number. Indeed, mice have a relatively rudimentary ductal tree compared to rats and humans, and as a result, expanding the age in which the nulliparous mammary gland is studied in mice may be fruitless. Therefore, current research studying environmental exposures and breast cancer risk would benefit by including rat models.

4.3: Individual variation in ovarian cycle hormones and breast cancer risk

One of the major findings described in this dissertation is that social isolation is associated with a decrease in mammary ductal differentiation despite an increase in exposure to luteal progesterone. These data underscore a gap in the rich research investigating the role of reproductive steroids in development and breast cancer. Firstly, progesterone and estrogen have been clearly defined as pro-proliferative and pro-differentiating factors (Andrechek et al. 2008; Young 2013; Paine and Lewis 2017; Joshi et al. 2010; Smith 1955; C Brisken et al. 1998; Beleut et al. 2010; Javed and Lteif 2013; Haslam and Shyamala 1980; Sharma et al. 2011; Shyamala 1999; Russell D Romeo, Lee, and McEwen 2004; Lee et al. 2013). It would follow that mammary gland differentiation would increase with an increasing exposure to these steroids. It has also been well characterized that a more differentiated mammary ductal tree is protected against breast cancer risk (Meier-Abt and Bentires-Alj 2014; Jose Russo et al. 2005; de Assis et al. 2010; J Russo, Wilgus, and Russo 1979). A logical hypothesis would be that more ovarian cycles would be associated with a more differentiated mammary ductal tree and therefore lower breast cancer risk. Instead the opposite is true; epidemiological studies have demonstrated that more ovarian cycles over the lifespan is associated with greater breast cancer risk (Colditz and Frazier 1995; Pike et al. 1983). This conflict in the literature needs to be investigated to more fully understand the associations between reproductive hormone exposure, mammary gland development, and breast cancer risk.

In Chapter 1, it was proposed that timing of menarche and ovarian maturity may deleteriously impact initial ductal elongation and differentiation of the mammary ductal tree and therefore be more important to increasing breast cancer risk than the number of estrous cycles, although these factors are themselves associated. Indeed, age of menarche increases a woman's risk for developing all subtypes of breast cancer (Ambrosone et al. 2015; Peeters et al. 1994; Turkoz et al. 2013). However, studies on how mammary gland development mediates the

effect of reproductive life history on cancer risk remains unclear. The studies described in Chapter 2 have hinted at experimental methods by which researchers can induce alteration in ovarian maturity and reproductive steroid exposure in rodents (i.e. by the imposition of psychosocial stress). These methods could potentially be expanded to a lifespan approach in rodent models of breast cancer to measure the development of mammary cancer (i.e. tumor volume and grade) and determine exactly which reproductive factors (i.e. ovarian cycle onset, period of anovulatory cycles, length of ovarian cycles, exposure to estrogen and progesterone, etc.) are significantly associated with increased cancer progression. Once those reproductive factors are identified, mammary gland development in relation to reproductive life history can be assessed. The proposed study has the potential to identify the underlying endocrine and developmental mechanisms by which pubertal timing and ovarian hormone exposure alters mammary gland development and cancer progression; moreover, these data will provide a developmental window for targeted interventions in women.

4.4: Psychosocial interventions: rodents and beyond

This dissertation expands on epidemiological studies in women that psychosocial stress exposure increases breast cancer progression (Taylor et al. 2007; Vona-Davis and Rose 2009; Liao et al. 2008). Indeed, data in Chapter 2 and 3 suggests that adolescence is a key time in which psychosocial stress, specifically glucocorticoid receptor activation, can impair mammary gland development to contribute to the increased breast cancer risk in these populations. Rescue interventions are necessary to determine what stage of mammary gland development (i.e. growth or differentiation) is associated with later breast cancer risk.

As determined in Chapter 2, mammary growth and differentiation continues through late puberty and early adulthood. The growth phase is characterized by the presence of terminal end buds, which cavitate by 13 weeks of age, and is bookended by the completion of ductal extension. The differentiation phase, while it overlaps with the growth phase, occurs largely after ductal

extension, between 17 and 25 weeks of age. A social intervention at the intersection of these phases (17 weeks of age), would inform whether one or both of these phases are necessary for conferring breast cancer risk. This study could determine 1) if heighted GC reactivity and slowed mammary gland development in social isolated females can be counteracted by a return to group living and 2) if mammary gland developmental changes are associated with the number and size of spontaneous mammary tumors.

Implementation of social programs in the public also provide an opportunity for intervention. There has been a recent increase in mindfulness and meditation programs in schools, particularly in low-income areas and in neighborhoods with high levels of violence (O'Connel 2017). The purpose of these programs is to decrease incidence of violence, but their implementation also has an effect on the stress response of the participants. Indeed, a 2014 meta-analysis of nineteen controlled studies has demonstrated a significant decrease in reported stress and an increase in stress resilience ($P<0.05$) in adolescents taking part in mindfulness programs (Zenner, Herrnleben-Kurz, and Walach 2014); this benefit has endured despite variation in implementation and exercises. Indeed, the David Lynch Foundation has worked to implement mindfulness programs within schools in low-income areas and neighborhoods with high levels of violence, such as South Side Chicago.

Because these programs are already in place, it would be plausible to follow the adolescents that take part in these programs in a prospective study of health outcomes. We would hypothesize that adolescent girls that have training in meditation and mindfulness would have lower stress reactivity during mammary gland development and therefore are at a lower risk for developing breast cancer.

Many of the current approaches on addressing health disparities in breast cancer include increasing access to healthcare and providing patient navigation. While important, these may

methods may not address the differences in incidence between racial/ethnic minorities. However, the data described in this dissertation and future work that builds off this data has the potential to provide a comprehensive understanding of the effect of psychosocial stress on mammary gland development as it relates to breast cancer risk. This would be a significant step toward understanding how biological mechanisms mediate health disparities in the onset of breast cancer.

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