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THE EFFECT OF CHRONIC PSYCHOLOGICAL STRESS DURING PUBERTY AND EARLY ADULTHOOD ON MAMMARY GLAND DEVELOPMENT

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BRIANA BANKS

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Dedication

With profound gratitude and a heart overflowing with appreciation, I dedicate this dissertation to my unwavering support system—my family. To each member who has stood by my side through every twist and turn of this academic journey, I owe an immeasurable debt of gratitude. Yet, amidst this collective love and encouragement, there is one figure who shines brightly—my mommy. Your boundless love, patience, and belief in me have been the guiding light illuminating my path.

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Chapter 1 : Introduction

1.1: Breast Cancer

Breast cancer is the most common cancer amongst women in the US, and the second leading cause of cancer death [1]. About 1 in 8 women will be diagnosed with breast cancer within their lifetime [2]. Neoplasms occur when cells within the breast start to grow uncontrollably leading to the formation of tumors. Tumors can be characterized as malignant or benign. A benign tumor cannot metastasize to other parts of the body. Malignant tumors are classified as "cancer" as they are characterized by their ability to invade nearby tissues and metastasize [3]. Breast cancer can initiate at the lobules, ducts, or connective tissue [4]. Invasive ductal carcinoma accounts for 80% of all diagnosed invasive breast cancer cases [5]. Less frequent occurrences include invasive lobular carcinoma, tubular carcinoma, papillary carcinoma, metaplastic carcinoma, and squamous cell carcinomas [6].

The development of DCIS with invasion can be characterized in sectors (Ductal Hyperplasia, Ductal Hyperplasia with Atypia, DCIS, DCIS with invasion) and begins in the normal breast characterized by a lumen surrounded by a layer of differentiated epithelial cells, supported by a basement extracellular matrix [7]. In a normal breast duct the lumen remains a barren space, however, during ductal hyperplasia proliferating cells accumulate and begin to form a hyperplastic lesion in the lumen. As the proliferative and presumptive malignant cells fill the lumen, atypia ductal hyperplasia occurs [8]. In DCIS, neoplastic cells composing the hyperplastic lesion remain within the lumen. As the hyperplastic and mass of proliferating cells begin to disrupt the myoepithelial cell layer and evade basement membrane, the lesion is classified as invasive ductal carcinoma [9] (**Figure 1.1**). DCIS with invasion can metastasize through the richly vascularized structure of the breast through the lymph nodes or bloodstream.

In breast cancer biology, two primary hypotheses explain the origin of breast cancer cells: the sporadic clonal evolution model and the cancer stem cell model [5]. According to the sporadic clonal evolution model, any breast epithelial cell can accumulate deleterious mutations over time, leading to epigenetic and genetic changes that drive tumor progression. As tumors progress, cells acquire additional mutations, resulting in increased heterogeneity and the emergence of subpopulations with enhanced characteristics such as heightened proliferation and resistance to apoptosis [10] . This heterogeneity ultimately fosters the development of more invasive tumors that are resistant to therapies [11]. Evidence of this theory is supported by studies that have shown breast cancers with high mutational heterogeneity [12], indicating that these tumors do not arise from a single cancerous cell or mutation. For example, comparative genomic hybridization analysis from the same in situ breast cancer has shown that various breast cancer cells within the tumor are comprised of significant genetically and spatially separated cell populations with distinct allelic losses [13].

The cancer stem cell model supports the theory that stem and progenitor cells initiate cancer through the accumulation and acquisition of deleterious alterations during their division. The accumulation of deleterious stem cells results in the generation of cancer stem cells, which contribute to subsequent tumor formation and progression [14] . Similar to normal stem cells, cancer stem cells possess self -renewal properties and the ability to differentiate indefinitely. It is postulated that these properties give rise to various cell types, thus contributing to tumor heterogeneity [15]. While tumors remain largely heterogeneous, with cells having non-stem cell progeny, under this theory, cancer stem cells are hypothesized as the “drivers” of tumor progression and metastasis [16]. A substantial amount of literature has supported the existence of

mammary stem cells in the mammary gland, as the mammary gland undergoes significant morphologic changes during puberty and before and after pregnancy [17-19]. The mammary gland is mainly comprised of luminal and myoepithelial cells, hierarchal data postulates that mammary stem cells give rise to committed progenitor cells for the luminal and myoepithelial lineages [18]. This has been supported by evidence in mouse models that has shown a functional mammary gland can arise from a single mammary stem cell [20, 21]. Further evidence supports that early first full-term pregnancy is associated with reduced breast cancer risk. It is hypothesized that lactation triggers terminal differentiation of milk producing-luminal epithelial cells [22]. This differentiation is associated with fewer mammary stem cells as this apoptotic clearing removes these cells from the potential pool of precursor mammary stem cells [23]. Finally, it has been exhibited that women with the BRCA1 mutation, who are at higher risk of developing breast cancer and more aggressive subtypes, have less differentiated mammary morphology [24]. It is postulated that loss of BRCA1 function leads to inhibition of epithelial differentiation, causing an expansion of the undifferentiated stem/progenitor cell population [25, 26]. Therefore, the mammary stem cell theory is substantial, however, lineage tracing and more evidence is required to full support this theory.

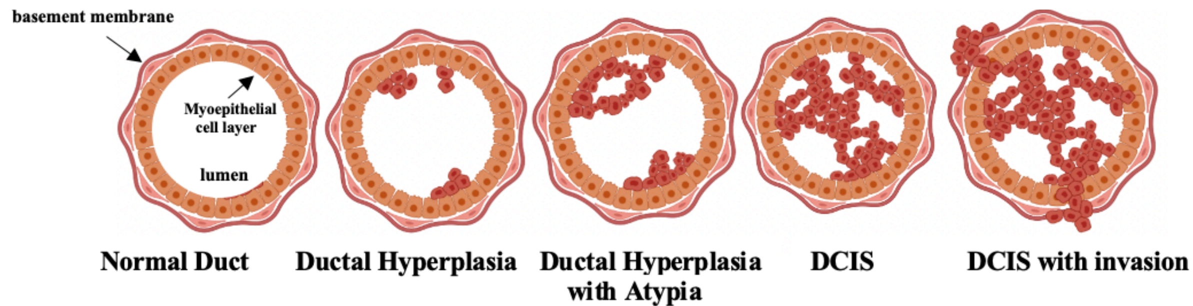


Figure 1.1. Schematic of Advancement of DCIS

The typical duct comprises a distinct layer of cells myoepithelial and luminal cells, which create a barrier between an empty lumen and the stroma. During the development of ductal carcinoma in situ , epithelial cells start to occupy the duct's lumen. Invasive breast cancer occurs when these cells breach the basement membrane (modified from [9]).

The treatment of breast cancer hinges significantly on the assessment of its stage, grade, and subtype. This assessment is conducted through various techniques, including advanced imaging tools such as breast ultrasound, diagnostic mammogram, and MRI, along with targeted breast biopsy procedures. Staging utilizes both clinical and pathological approaches. At the forefront of this staging methodology is the internationally recognized TNM system. The TNM pathologic prognostic system categorizes breast cancer based on tumor size (T), lymph node spread (N), and the presence of metastasis (M) [27]. In this context, Stage 0 denotes Ductal Carcinoma In Situ [9], confined to breast ducts. Stage I represents the initial invasive phase, spreading to nearby tissue while remaining relatively small (<2 cm). Subcategories IA and IB are dependent on the tumor's hormonal receptor status. Stage II indicates a larger tumor (>2 cm) and may further classify into IIA or IIB if lymph nodes are affected. Stage III can be recognized as “inflammatory breast cancer”, and involves advanced lymph node spread and larger tumors. Finally, Stage IV designates metastatic breast cancer, as the disease has spread to distant organs or lymph nodes [27].

Staging serves to gauge the tumor's size and extent of spread, complemented by the essential concept of grading which is a part of prognostic stage. This further categorizes tumors according to cellular characteristics compared to normal breast cells. Tumor grades are stratified into three levels: Grade 1, Grade 2, and Grade 3 [28]. In Grade 1, tumor cells closely resemble normal tissue and exhibit a lower level of proliferation. Grade 2 introduces a moderate level of proliferation and a less differentiated cell profile. In Grade 3, tumor cells are highly proliferative and display a poorly-differentiated state. This systematic grading framework refines the understanding of the tumor's cellular makeup [29].

Invasive breast cancer is broadly classified into five subtypes: Luminal A, Luminal B, HER2, basal, and normal [30, 31]. These categorizations hinge on the immunohistochemical expression of the hormone receptors: progesterone receptor (PR), estrogen receptor [32], and human epidermal growth factor receptor 2 (HER2). Complementing this classification, Ki67 serves as a crucial cellular marker for proliferation [33], providing a comprehensive framework for understanding the diverse characteristics of these subtypes. **Luminal A breast cancer**, encompassing 50-60% of all breast cancer cases [34], stands as the predominant subtype. It is distinguished by being estrogen and progesterone receptor positive [32] while lacking HER2 expression (HER2-). Additionally, Luminal A is marked by lower levels of Ki-67, further defining its better prognosis and high survival rate. **Luminal B breast cancer**, constituting 15-20% of all breast cancer cases, exhibits a more aggressive phenotype. Emerging research indicates the involvement of growth factor pathways, including fibroblast growth factor receptor 1, HER1, and PI3K, in contributing to the heightened proliferation and aggressiveness observed in this subtype [35]. Luminal B cancers are characterized by being estrogen receptor-positive [32] and negative for progesterone and HER2 (PR-/HER2-). Notably, Luminal B is distinguished

by elevated levels of Ki-67. **HER2-enriched breast cancer** also accounts for 15-20% of breast cancer subtypes. It can be estrogen and progesterone receptor positive and/or negative [32] and HER-2 positive (HER2+). HER2-enriched breast cancers undergo HER2 amplification, activating pro-oncogenic pathways. This amplification leads to increased proliferation through the RAS-MAPK pathway and enhanced survival via the PI3K-AKT pathway [36]. Despite a poorer prognosis, HER2 positive tumors exhibit heightened sensitivity to specific cytotoxic agents. **The basal-like breast cancer subtype**, constituting 8-15% of all cases, is recognized as the most clinically aggressive subtype with a notable metastatic propensity. This subtype is characterized by elevated levels of basal myoepithelial markers: CK5, CK14, CK17, and laminin [34]. Lacking ER, PR, and HER2, basal-like cancers are commonly termed Triple Negative Breast Cancer, with BRCA1 mutations accounting for 75% of cases [37]. While the terms "triple negative" and "basal-like" are often used interchangeably, basal-like tumors demonstrate greater immunohistochemical homogeneity. **Normal breast-like cancers**, comprising approximately 10-15% of all breast cancer cases, remain the least characterized subtype. Similar to basal-like cancers, they lack ER, PR, and HER2 expression but differ by being negative for CK5 and EGFR [38]. In certain contexts, normal breast-like cancers present an intermediate prognosis between luminal and basal-like cancer subtypes.

Upon completion of the comprehensive assessment of breast cancer encompassing stage, grade, and subtype considerations, treatment strategies exhibit variability contingent upon menopausal status. In the context of non-invasive breast cancer, exemplified by DCIS, standard treatment options involve lumpectomy coupled with radiation or, alternatively, mastectomy [39]. In cases where tumors progress to stage I or stage III, yet exhibit positivity for estrogen receptor [32], progesterone receptor (PR), or ERBB2, hormone therapy emerges as a viable intervention

[40]. Aromatase inhibitors, notably anastrazole, letrozole, and exemestane, play a pivotal role in suppressing estrogen production, particularly tailored for postmenopausal women. Complementing this approach, selective estrogen receptor modulators [41] like tamoxifen serve as competitive inhibitors to impede estrogen activity, with tamoxifen FDA-approved for hormonal treatment in both pre and postmenopausal women [41]. For tumors lacking hormone receptors, such as triple-negative breast cancer, therapeutic considerations encompass preoperative chemotherapy or immunotherapy. Surgically, excisable tumors may undergo lumpectomy with adjuvant radiation. Metastatic breast cancer, while challenging to cure, remains amenable to treatment with modalities including immunotherapy, chemotherapy, or hormone therapy, all geared towards enhancing the quality of life [42].

1.2: Breast Cancer Disparities

Over the past decade, treatment for breast cancer has seen remarkable evolution, resulting in more targeted therapies and an overall decrease in mortality rates. Despite these advancements, a troubling trend persists; while women of European descent are more frequently diagnosed with breast cancer, women of African-descent face higher mortality rates. Shockingly, black women are 41% more likely to succumb to breast cancer compared to their non-Hispanic white counterparts, and they are also twice as likely to develop aggressive subtypes like triple-negative breast cancer [2].This disparity in breast cancer outcomes is complex and influenced by a myriad of factors, encompassing genetics and socioeconomic variables. It becomes imperative to delve deeper into these underlying factors to address the existing gaps in knowledge and treatment.

Uncovering the genetic underpinnings of cancer mutations across diverse ancestry populations holds paramount importance. Currently, much of the knowledge regarding genetic predisposition to breast cancer predominantly stems from European ancestry. The extensively studied BRCA1 and BRCA2 mutations, often denoted as "founder mutations," have been extensively explored in populations such as Ashkenazi Jews, Icelanders, and French-Canadians [43-45]. However, there has been a notable absence of data on African ancestry and BRCA1/BRCA2 mutations. Pioneers in the field, including Dr. Funmi Olopade, have played a pivotal role in bridging this gap, advancing the understanding of genetic variants within this population. Their work has notably unveiled the existence of BRCA1/BRCA2 mutations that are more prevalent in African and African American populations compared to individuals of European ancestry [46].

In a significant stride towards precision medicine, researchers from Weill Cornell employed case-control, case-series, and race-nested approaches, identifying an ancestry-specific allele associated with triple-negative breast cancer outcomes. Specifically, the "Duffy null allele" in women of West African descent, impacting the polymorphic gene variant ANKLE1, has been linked to an increased risk of triple-negative breast cancer, as elucidated by the groundbreaking work of Dr. Melissa Davis [47]. These findings not only shed light on the intricate genetic landscape but also pave the way for the development of more accurate risk assessment models for breast cancer in racially-ethnic populations. The collective efforts of these researchers contribute significantly to the ongoing endeavors in understanding and addressing the complexities of cancer genetics across diverse communities.

With genetic factors accounting for a modest 5-10% of breast cancer cases [48], it is important to note the impact of socioeconomic factors in relation to breast cancer disparities. In

the US, a larger proportion of black women live in poverty compared to whites [49]. Low-income women have limited access to early detection via breast cancer screening, resulting in advanced and later-stage diagnoses. Living in areas with limited infrastructure involves the lack of regular healthcare visits, encompassing not only later-stage diagnosis, but also a myriad of undiagnosed health ailments that can further contribute to the disease. This juxtaposition will be discussed in more detail (below). Lack of health insurance is another socioeconomic factor between black and white women. Black women are twice as likely to be uninsured [50]. Healthcare systems in low-income communities do not always have the up-to-date equipment for proper screening or diagnosis. For example, in the Death Gap, Dr. Ansell noted that screening centers in low infrastructure areas where black women would receive their mammograms were 33% less likely to have their mammogram read by a specialist and 50% less likely to receive screening with the latest technology [51]. This is to note the impact of structural racism on healthcare access and its inequities.

Living in low-income areas with poorer infrastructure can be overlapped with a "food desert". Food deserts can be defined as geographic areas where residents have limited access to affordable healthy food options [52]. In low-income areas, liquor stores and convenient stores are typically widely available. This leads to the overconsumption of processed foods that are high in sugar, fat, and salt. This, in turn, leads to the higher rates of obesity experienced by black women [53]. As obesity is a risk factor for poorer breast cancer prognosis, it's important to note its contribution to the disparity [54-56].

While breast cancer disparities stem from multifactorial origins, it's intriguing to observe how one factor can cascade into others, particularly the physiological responses triggered by socioeconomic influences. Chronic stress, a consequence of racism, discrimination, and sexism

among black women, not only amplifies inflammatory responses but also heightens the risk of cardiovascular diseases [57]. Moreover, studies indicate that women reporting racial discrimination face an increased risk of breast cancer [2, 58]

In the daily lives of black women, elevated levels of loneliness, hypervigilance, and stress contribute to this complex interplay. For instance, research reveals that African American female adolescents exhibit higher evening cortisol levels compared to their Caucasian counterparts [59]. Extensive epidemiological studies have delved into the effect of chronic stress on breast cancer development and progression, consistently demonstrating that women exposed to stress face a heightened risk of developing breast cancer [60]. Notably, chronic psychological stress induced by everyday stressors emerges as the most potent stressor associated with increased breast cancer risk [61].

Timing of stress exposure, particularly during adolescence, emerges as a significant factor impacting breast cancer development. It has been shown that significant stress experienced from losses or social deficits during adolescence correlates with breast cancer incidence in adulthood [61]. The Baltimore Epidemiologic Catchment Area Study found a significant association between the death of a mother during a girl's early adolescence and breast cancer incidence in adulthood [62]. Furthermore, a prospective study linked stress exposure to the Holocaust among women under 18 years old with the highest risk and mortality of breast cancer [63]. Recognizing puberty and early adolescence as critical developmental windows, the probability of chronic stress exerting a substantial impact on breast physiology is high. Therefore, it becomes imperative to comprehend the link between exposure to chronic stress during puberty and early adulthood and its consequential influence on breast cancer risk and development later in life.

1.3: The Mammary Gland across the Lifespan

While puberty is a critical period of mammary development, rendering it sensitive to environmental insults it is important to note the state of the mammary gland during the stages: embryogenesis, puberty, pregnancy, and post-menopause. The mammary gland is a complex organ composed of adipocytes, epithelial, endothelial, fibroblasts, and immune cells [64] . Epithelial cells are organized in the order: mammary stem cells, mammary progenitor cells, and committed mammary epithelial cells: luminal and myoepithelial [65]. The formation of the mammary gland begins during embryogenesis [66]. At this time, development begins with the delineation of the ectoderm as the mammary line. The mammary line serves as initial site from which mammary glands originate. Wnt signaling initiates specification of the mammary lines. As development of the embryo progresses, signals from the mesenchyme regulate the formation of placodes. These signals also include Wnt signaling and are marked by the expression of transcription factors such as lymphoid enhancer-binding factor 1 (LEF1) and T-box transcription factor 3 (Tbx3) [67]. Under the influence of parathyroid hormone-related protein (PTHLH) signals, mammary placodes invaginate the mesenchyme to form mammary buds. These are the rudimentary structures of the mammary gland. Embryonic development concludes with formation of the ductal lumen and nipple structure. After birth before the onset of ovarian cycles, development of the mammary gland is aligned with body growth [68, 69].

In the field of developmental biology, puberty is regarded as the “window of susceptibility”[70]. It is the time where the breast is undergoing rapid development under the response of various hormones and growth factors. During puberty, the mammary gland transforms from a rudimentary tree into a complex branched epithelial network of ducts [68]. Puberty typically starts between the ages of 8 and 14 years of age in humans and between 4 and

7 weeks in rodents [71, 72]. At the onset of ovarian cycles, differential growth is induced by hormones and growth factors [22, 73, 74]. During puberty mammary development can be placed into the two categories: growth phase and differentiation phase.

During the growth phase, increased production of estrogen and progesterone, in conjunction with pituitary growth hormones promote the formation of terminal end buds (TEBs) [75, 76]. Terminal end buds are bulb-like structures comprised of an outer layer of cap cells with an inner layer of luminal epithelial cells located at the leading edge of the duct [77]. The elongation of the ducts, involve the invasion of TEBs through the mammary fat pad forming a network of ductal structures as TEBs are composed of highly proliferative cells. Upon reaching the edge of the mammary fat pad, TEBs regress and cavitate to form terminal ducts [78, 79].

During the differentiation phase, these ductal structures become more ornate with each ovarian cycle as primary ducts segment into secondary ducts forming more extensive branching [80, 81]. These secondary branches occupy up to 60% of the fatty stroma [82]. Further differentiation occurs when alveolar buds begin to form. In humans, lateral branches lead to terminal ducts that give rise to acini structures [83]. During pregnancy hormonal fluctuations cause the expansion of alveolar cells that mature to acini structures. Acini structures produce milk during lactation [84-86].

Throughout adulthood, the mature breast typically remains a state of quiescence. However, the onset of pregnancy prompts significant anatomical and physiological transformations within the breast, priming it for the process of lactation [87, 88]. During pregnancy, elevated levels of estrogen instigate the stimulation of the pituitary gland, resulting in heightened production of prolactin [89]. Prolactin and progesterone promote the differentiation of alveoli, which are the structures that synthesize and secrete milk throughout lactation. This is

activated through the JAK2/STAT5 signaling pathway which help control crosstalk between prolactin and progesterone [90]. The initiation of involution is triggered by a decrease in prolactin and oxytocin, as well as alterations in estrogen and progesterone levels. Involution involves extensive tissue remodeling including restructuring of the extracellular matrix and glandular architecture as apoptotic cells are cleared [91]. This leads to a noticeable reduction in the size of the mammary gland. Immune cells help to facilitate remodeling and clearing of apoptotic cells. This involution process restores the breast to a quiescent state, resembling its pre-pregnancy condition [92].

Menopause, typically occurring in women aged between 45 and 55, heralds a significant hormonal shift characterized by declining levels of estrogen and progesterone [93]. This hormonal decline precipitates a cascade of changes within the mammary gland. Foremost among these changes is the atrophy of mammary gland tissue, leading to a decrease in breast size and firmness [94]. Additionally, the mammary gland undergoes structural alterations, becoming progressively more fibrous and less elastic. Aging of the mammary gland is further characterized by a shift in cellular composition, with the proportion of epithelial cells increasing from 45% to 82%, while stromal and hormone-sensing cells decline from 55% to 18% and 53% to 9%, respectively [95]. These intricate cellular transformations collectively contribute to the physiological adaptations observed in the mammary gland during the menopausal transition.

1.4: Molecular Mechanisms of Mammary Development during Puberty: Influence of Hormones and Growth Factors

As puberty marks the beginning of the ovarian cycle, understanding the mechanisms through which reproductive hormones kick start mammary gland development becomes crucial.

It has been shown that mammary gland development is affected directly and indirectly by estrogen, progesterone, glucocorticoids, and growth hormone [77, 96, 97]. The mammary gland is highly heterogeneous; comprised of adipocytes, fibroblasts, and an array of cell types including immune, lymphatic, and vascular cells. Evidence has shown adipocytes' role in regulating epithelial growth and crosstalk with other cell types within the gland [98]. Fibroblasts are integral to the extracellular matrix as they synthesize collagens, fibronectin, and enzymes that release growth factors [99, 100]. Immune cells play a critical role in differentiation and branching morphogenesis as they arbitrate extension in the mammary fat pad [100].

In parallel with human mammary gland, the rat mammary gland undergoes a phase of embryonic development where it remains in the form of rudimentary epithelial buds [101]. The initiation of pubertal development in rats is characterized by the establishment of normal gonadal function and the onset of the ovarian cycle, typically commencing between 4 and 7 weeks [102, 103]. Subsequent weeks witness the development of the rat mammary gland, marked by ductal extension within the mammary fat pad and the transformation of epithelium into terminal end buds (TEBs).

The primary hormones driving mammary gland development are estrogen and progesterone, which are produced in the ovaries in response to hypothalamic-pituitary-gonadal- (HPG) axis activation. Developmental modifications are correlated with ovarian cycles; estrus cycle in rodents (4-5 days) and menstrual cycle in humans (25-30 days)[9, 104] . The cycle consists of two phases: follicular (proestrus and estrus in rodents) and luteal (metestrus and diestrus in rodents). Follicular phase starts at the first day of the cycle, where progesterone levels decrease, and estrogen levels rise. In ovulation (estrus in rodents), high levels of estrogen stimulate the high production of luteinizing hormone from the pituitary gland, contributing to the

onset of the luteal phase [65]. The mid-luteal phase is considered the most proliferative phase of the cycle as both estrogen and progesterone levels are high. High levels of progesterone are associated with formation of epithelial alveolar bud formation and tertiary branching [80]. As progesterone levels decrease, the onset of late luteal phase is marked by apoptosis of newly formed alveolar buds and tissue remodeling.

As ovarian cycles persist, the ductal tree undergoes continuous branching, evolving into a more intricate structure. Upon activation of the estrogen receptor α, it acts as a transcription factor, transcribing genes associated with growth and elongation. Additionally, estrogen receptor α can also act via paracrine signaling to stimulate the release of amphiregulin (AREG) [105]. AREG serves as a ligand for the epidermal growth factor receptor in stromal cells. Upon binding to its receptor, AREG induces the expression of fibroblast growth factors (FGFs). These FGFs, in turn, induce luminal cell proliferation [82]. Growth hormone is also essential to mammary gland development, with signaling through the growth hormone receptor in stromal fibroblasts inducing the expression of insulin-like growth factor 1 (IGF1). IGF1 works synergistically with estrogen to promote luminal cell proliferation. Furthermore, the transcription factor forkhead box A protein 1 (FOXA1) serves as a mediator of estrogen signaling by facilitating chromatin accessibility [32]. This helps promote interaction between the estrogen receptor and its gene targets. FOXA1 is regulated by the GATA3 network and plays a pivotal role in branching morphogenesis. Studies have demonstrated that loss of GATA3 disrupts terminal end bud formation and impairs the development of estrogen receptor-positive mammary epithelial cells, thereby perturbing pathways regulated by progesterone [75, 106, 107].

Progesterone acts via hormone and paracrine signaling to promote side-branching morphogenesis. The release of receptor activator of nuclear factor kappa-B ligand [108] interacts

with receptor activator of nuclear factor kappa-B [108] and stimulates proliferation by upregulating expression of Cyclin Delta 1 (CCND1)[41, 109]. CCND1 forms complexes with cyclin-dependent kinases (CDKs), facilitating the activation E2F transcription factors [110]. E2F target genes promote progression through the G1 phase of the cell cycle thus facilitating in cell proliferation. Depletion of CCND1 shows similar phenotypes to PR-null mice, further displaying their synergistic roles [111, 112].

Other growth factors and hormones, such as glucocorticoids, have been implicated in mammary gland development. For instance, studies employing Cre-LoxP models with deletion of the GR gene demonstrated the essential role of GR in cell proliferation during lobuloalveolar development [113]. Additionally, glucocorticoids can influence the hypothalamic-pituitary-gonadal (HPG) axis. Acute or chronic administration of glucocorticoids has been shown to suppress HPG activity, with glucocorticoids specifically slowing luteinizing hormone pulse frequency [114]. Since luteinizing hormone contributes to the onset of the luteal phase, excess glucocorticoids may contribute to stress-associated menstrual disturbances by altering its release. The proposition is that disturbances in the ovarian cycle induced by excess glucocorticoids could subsequently affect mammary gland development. However, the effects of excess glucocorticoid exposure on mammary gland development remain an area for further exploration.

Similarly, puberty represents a rapid and critical phase of development in both rodents and humans, making the breast particularly vulnerable to environmental insults—both physical and social. Exposures during puberty may significantly influence mammary cancer risk in adulthood, underscoring the importance of comprehending the complex interplay between developmental stages and subsequent susceptibility to breast cancer.

1.5: Effect of Physical Environmental Insults during Puberty on Mammary Gland Development and Breast Cancer Risk

During the developmental window of puberty, the breast undergoes crucial physiological changes, making it particularly vulnerable to environmental insults [115-117]. Epidemiological data highlight a correlation between exposure to chemical toxins during this period and an elevated incidence of breast cancer later in life. For instance, a study identified a link between exposure to Dichlorodiphenyltrichloroethane (DDT), a synthetic insecticide widely used in the mid-1900s (before its US ban in the 1970s), and premenopausal breast cancer[117]. Specifically, women exposed to DDT before the age of 14 exhibited an increased risk of premenopausal breast cancer. Women who were not exposed prior to 14 years of age showed no association [118].

Furthermore, an epidemiological investigation delved into the ramifications of radiation exposure in childhood Hodgkin's lymphoma treatment on subsequent breast cancer incidence. Shockingly, the findings highlighted that exposure during puberty and early adolescence (ages 10-16) was linked to a staggering 74% incidence of breast cancer cases, surpassing those treated before the age of 10 [119]. Another study investigating the impact of ionizing radiation exposure timing from the atomic bombings of Hiroshima and Nagasaki, Japan, on breast cancer incidence in adulthood echoed these findings. By scrutinizing epidemiological data of breast cancer cases and matched controls among atomic bomb survivors, it was elucidated that those exposed between the ages of 0-19 exhibited a higher percentage of radiation-related cancers in late adulthood (40-60 years) compared to those exposed between 20-39 years [120]. These compelling studies underscore the heightened susceptibility of the breast during the critical

period of puberty, emphasizing the imperative of comprehending and mitigating environmental risks during this developmental phase.

Animal models have also shown the correlation between carcinogen exposure and increased mammary tumor burden. Using the Sprague Dawley Rat model of spontaneous mammary tumor development, the impact of carcinogen exposure on mammary ductal development and differentiation was measured[121] [122]. Exposure to carcinogens during early puberty between 5-8 weeks had a correlative effect with tumor induction. Exposure to carcinogens at 8 weeks had the greatest tumor burden as the TEBs are rapidly proliferating. Russo et al. hypothesized that during early puberty (6-8 weeks) is when the mammary gland is most susceptible, as this time period is the mammary growth period and there are greater number of TEBs [122]. In late puberty, TEBs reduce as the mammary gland begins to differentiate. However, it was hypothesized that TEBs primed to differentiate to ABs are most susceptible to neoplastic transformation. It was postulated that changes in DNA synthesis during this time is what causes the increased tumor incidence [123].

1.6: Effect of Social Environmental Insults during Puberty on Mammary Gland Development and Breast Cancer Risk

While exploring the influence of one's physical environment during puberty on breast cancer risk and development yields intriguing insights, it is crucial to emphasize the significant role of insults originating from one's social environment and the resultant psychological stress. Social insults encompass a wide array of factors including, but not limited to, low socioeconomic status, experiences of racial discrimination, feelings of loneliness, and limited social support

networks [124-126]. These insults can profoundly impact an individual's psychological well-being, potentially exacerbating stress levels and contributing to long-term implications.

Stress is defined as an alteration in the body's "hormonal and neuronal secretions in response to a perceived threat" [127]. Stress response can be perceived as acute or chronic. The acute stress response is synonymous to the "fight or flight" response or activation of the sympathetic-adreno-medullar axis. The fast-acting acute response induces section of norepinephrine and epinephrine from the adrenal medulla and norepinephrine from the sympathetic nerves [128, 129]. This catecholamine release of epinephrine and norepinephrine binds the α-adrenergic and β-adrenergic receptors present in the central nervous system and cell membranes of key surfaces and organs throughout the body i.e. heart, liver, muscles, sweat glands. Once bound, downstream cAMP signaling leads to the activation of these adrenergic receptors resulting in increased blood pressure, heart rate, muscle contraction, increased sodium retention, etc. [130, 131].

The chronic stress response includes activation of the hypothalamic-pituitary-adrenal axis of hormonal signaling. The perceived stressor induces the hypothalamic released of corticotropin-releasing hormone (CRH) which binds to corticotropin release factor (CRF) receptors on the anterior pituitary gland [132-135]. Once CRH is bound, it stimulates the release of adrenocorticotropin. In turn, adrenocorticotropin signals the adrenal cortex to produce cortisol (cortisol in humans and corticosterone in rodents). Cortisone, cortisol's inactive form, is catalyzed by 11 beta-hydroxysteroid dehydrogenases to become cortisol in its active state [136]. Glucocorticoid hormones such as cortisol or corticosterone generate a physical response to the stress signal by binding to its cytoplasmic receptor (glucocorticoid receptor).

Upon bound glucocorticoid receptor (GR) entering the cytoplasm, the receptor homodimerizes and translocates to the nucleus acting as a nuclear transcription factor [137] . Glucocorticoid receptor binds to DNA sequences that contain glucocorticoid-response elements (GREs) in the promoter of target genes which comprise up to 10-20% of the human genome and can promote transactivation or transrepression [138]. In addition, GR can regulate the transcription of target genes by interacting with other transcription factors. For example, GR interaction with AP-1 and NF-κB suppresses their activity [139].

As dexamethasone has been used widely as an antiemetic to prevent chemotherapy-induced nausea and vomiting, recent studies have studied glucocorticoid receptor activation in the context of breast cancer progression and metastasis [140, 141]. Reports have shown an association between high GR expression and increased mortality and chemoresistance in TNBC [142-144] . Moreover, a human breast cancer xenograft model demonstrated the use of a GR antagonist results in chemotherapy efficacy [145]. Glucocorticoid receptor target genes include SGK-1, MKP-1, and AP-1 have also been proven to promote breast cancer progression through the inhibition of apoptosis [145, 146].

It has also been postulated that systemic glucocorticoid can indirectly induce tumor progression through the activation of insulin resistance in adipocytes. Insulin resistance leads to the secretion of pro-inflammatory cytokines and growth factors that have been associated with tumor advancement [147]. Adipocytes exhibit elevated expression of 11β-HSD1 (enzyme responsible for converting inactive glucocorticoids into active forms). 11β-HSD1 in breast tissue has been implicated in breast cancer development and progression by promoting tumor growth, invasion, and metastasis [148]. 11β-HSD1 within adipose tissue has been implicated to contribute to the tumor microenvironment's pro-inflammatory and pro-tumorigenic

characteristics [149]. Thus, GR can affect tumor signaling through the upregulation of local glucocorticoid levels [150].

Furthermore, in mouse models, the activation of GR at distant metastatic sites was observed to enhance the colonization, heterogeneity, and metastasis of breast cancer cells [140] (**Figure 1.2**). Although glucocorticoid receptor activation has been proven to lead to breast cancer progression, scant information is present regarding its activation during mammary gland developmental periods (puberty-early adulthood), and subsequent breast cancer initiation later in life [141].

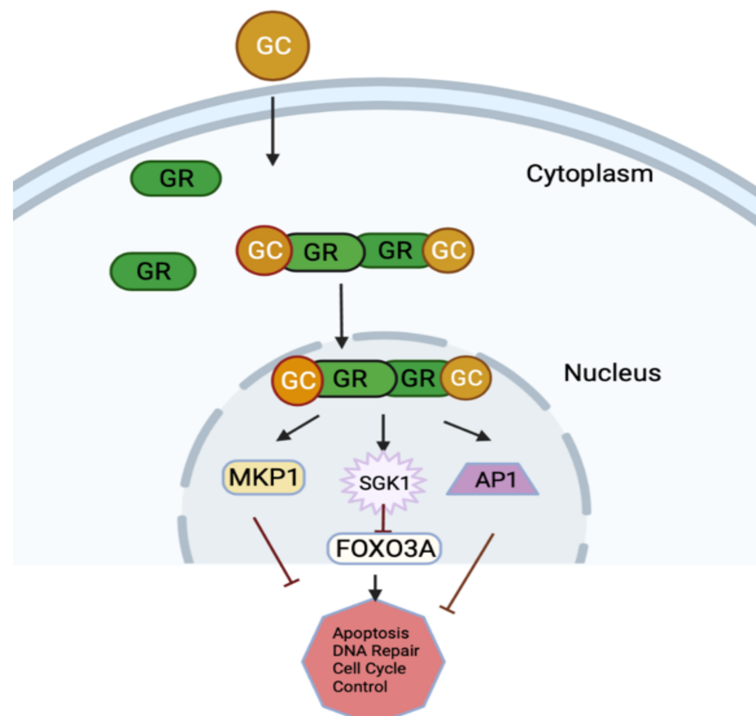


Figure 1.2. Glucocorticoid Receptor Pathway.
Activation of the glucocorticoid receptor results in the transcription of GR target genes involved in breast cancer progression. (Modified from [60]).

To study the effects of chronic psychological stress on mammary gland development and subsequent mammary cancer risk, McClintock et al. established a model of social isolation in the Sprague Dawley Rat model [151]. Sprague-Dawley rats are genetically-predisposed to spontaneously develop diverse types of mammary cancer [e.g. ductal carcinoma (both invasive and in situ), carcinosarcoma, fibrosarcoma and lobular carcinoma in situ], they also develop, as do humans, several types of non-malignant mammary tumors (lactating adenomas, tubular adenomas and papillary cystadenomas) [152].

Like humans, rats are a very social species. They co-habituate together, rear their young together, and use their sense of smell to recognize others [153, 154]. The lab has found by socially-isolating the animals to where they can still see, hear, and smell their group-housed counterparts they lack the network to dissipate the effects of external stressors. These external stressors include the everyday husbandries of lab. i.e. cage cleanings, feedings, etc. [155, 156](**Figure 1.3)**. In response to an acute restraint test, socially-isolated animals showed elevated levels of corticosterone [151]. Even after the stressor ends, glucocorticoids continue to rise in isolates and stress recovery is delayed [157]. Using this model of breast cancer and stress reactivity, they discovered that social isolation from weaning increases levels and duration of glucocorticoid release in response to everyday stressors, causes earlier onset of more aggressive and malignant mammary gland cancers in adulthood, with the most prevalent malignant tumors being DCIS [9] and DCIS with invasion [151].

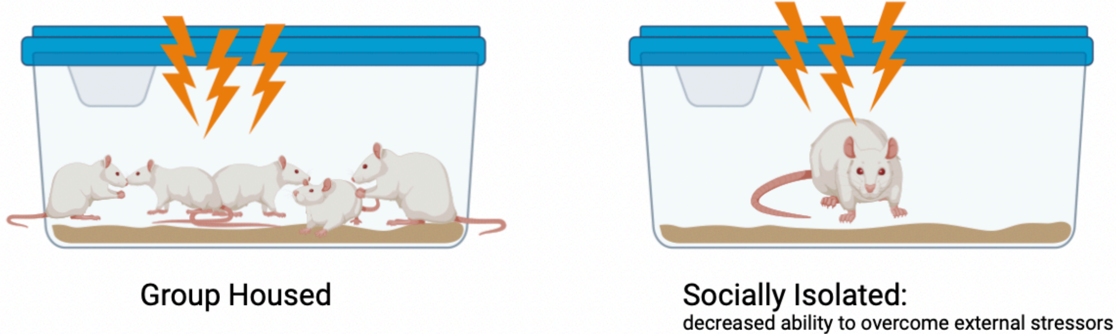


Figure 1.3. **Schematic of the Sprague Dawley Rat Model of Social Isolation**.
Socially-isolated animals are housed where they can still see, hear, and smell their group-housed counterparts. It has been shown they lack the social network to dissipate the effect of external stressors. (Original content)

As the time period of social isolation was from weaning at 5 weeks until death, a critical timeline into which these alterations were occurring was not established. It was hypothesized that these alterations were occurring during puberty. As aforementioned, puberty is a "critical window of susceptibility"[70]. This rapid period of development renders it sensitive to insults from not only the physical environment (previously mentioned on regards to carcinogen exposure and TEB number), as well as the social environment [121, 151]. To test this hypothesis, animals were socially isolated from early puberty through early adulthood [158]. It was shown that social isolation and exposure to chronic psychological stress from early puberty through early adulthood had no effect on estrogen or progesterone receptor activity. This study revealed two novel findings: nulliparous mammary development continues through early adulthood and social isolation through early puberty to early adulthood (5-22 weeks) impairs maximal ductal extension growth and alveolobular differentiation, which are key processes in mammary gland development.

It was shown exposure to chronic psychological stress had a scant effect on TEB number [158]. Suggesting that impaired development later in life is impactful when mediating mammary cancer risk. It was also shown that maximal ductal development is reached by late puberty (17 weeks of age). At this crux, the primary focus shifts from mammary ductal extension to mammary ductal differentiation into ABs and lobules which continues into early adulthood at 25 weeks of age. Decreased alveolobular differentiation in socially-isolated animals was associated with an increase mammary adipose tissue [158]. The hypothesis poses that this increase in adipose tissue can increase the proliferation and survival of mammary cancer cells [159]. As it was shown that adipose tissue in socially-isolated animals contain greater expression of genes involved in energy production and lipid metabolism [159]. To further support that hypothesis, a study following the effect of chronic social isolation on C3(1)/SV40 large T-antigen (Tag) transgenic mice found that social isolation was linked to heightened glucocorticoid reactivity to acute stressors and an accelerated tumor growth rate. Furthermore, chronic social isolation also led to a significant of upregulation of genes associated with lipid synthesis and glycolytic pathways in the mammary gland even before the onset of invasive cancer formation [160]. Although these findings provide intriguing evidence on the effect of increased exposure to chronic psychological stress on mammary ductal development and differentiation, what remains unknown are the molecular mechanisms linking this in delayed pubertal mammary ductal development and differentiation with increased mammary cancer risk later in life.

1.7: The Integrated Stress Response

The integrated stress response is an adaptive intracellular signaling network that has been evolutionary conserved amongst organisms. This network plays an integral role in helping cells,

tissues, and organisms restore and maintain homeostasis in response to stress which includes various environmental, physiological, and pathological conditions [161-163] These conditions can include amino acid deprivation, viral infection, iron deficiency, and misfolded proteins. In response, stress-activated eukaryotic translation initiation factor-α (eIF2α) kinases general control nonderepressible 2 (GCN2), Protein Kinase RNA-dependent (PKR), Heme Regulated Inhibitor Kinase (HRI), and PKR-like ER kinase (PERK) phosphorylate eIF2α which not only halts global protein synthesis but stimulates the preferential translation of specific mRNAs resulting in the reprogramming of gene expression [162].

GCN2 is activated in response to amino acid deprivation. Amino acid deprivation can occur in various physiological contexts, such as during prolonged periods of fasting, nutrient scarcity, or certain disease conditions [164]. Upon these conditions, uncharged (deacetylated) transfer RNAs (tRNAs) accumulate and bind to GCN2 facilitating its activation. It has been postulated that ribosomes assist in the recognition and binding of deacetylated tRNAs [165, 166].

PKR is activated in response to viral infections. During viral infection double stranded RNA (dsRNA) promotes the dimerization of PKR and its subsequent activation [167]. It has been postulated in addition to viral infections; oxidative stress, bacterial infection, and riboxotic stress have been shown to stimulate PKR [168-170]. However, through this function, PKR is activated independently of dsRNA.

HRI activated in response to iron deficiency [171, 172]. HRI is a well-defined heme sensor in erythroid progenitor cells [173]. It is regulated by homeostatic heme levels, heme inhibits HRI kinase activity by preventing dimerization via disulfide bond formation [174]. When heme is deficient, HRI becomes an active dimer and its subsequent activation results in the phosphorylation of eIF2α.

PERK is activated in response to ER stress i.e. unfolded protein response. Activation of PERK has two models of activation. The well-defined model of PERK activation involves the response to the accumulation of unfolded proteins located in the lumen of the ER, the luminal domain protein 78-kDa glucose-regulated protein (GRP78) which plays an integral role in ER quality control will release from PERK [175]. This disassociation promotes the dimerization of PERK and its subsequent autophosphorylation and activation. The second model of PERK activation involves inositol-requiring enzyme type 1 (IRE1) which also plays an integral role in ER quality control [176]. In response to ATP depletion, PERK becomes activated through in the inhibition of sarcoplasmic/ER Ca^{2+} - ATPase pump. PERK's subsequent activation leads to the phosphorylation of eIF2α [162].

As aforementioned, the highlighted four kinases are well-defined to facilitate in the phosphorylation of eIF2α that halts global protein synthesis. The mechanism by which this happens will be outlined below. eIF is a heterotrimeric GTPase consisting of the 3 subunits: α,β, and γ, with γ as the GTPase and eIF2α and eIF2β as accessary functions [177, 178]. As protein synthesis in eukaryotes is initiated by methionyl transfer RNA (Met-tRNAi), eIF2 along with other translation initiation factors within the preinitiation complex (PIC) play the crucial role of delivering Met-tRNAi to the 40S subunit of the ribosome [179-183]. In addition to the initiation of protein synthesis, eIF2 also plays a role in the recognition of start codons. Met-tRNAi has a high affinity to eIF2-GTP. eIF GTP hydrolysis is dependent upon guanine nucleotides exchange factors and its GTPase activating protein (GAP) eIF5 [184]. Upon codon recognition, eIF5 releases Pi and an eIF2-GDP complex is formed and disassociated from the ribosome. eIF5 helps to regulate GDP release. Due to its low affinity for Met-tRNAi, eIF2-GDP undergoes reactivation by exchanging bound GDP for GTP. This transformation results in the formation of

eIF2-GTP, which now possesses an affinity for Met-tRNAi, consequently initiating protein synthesis. This process is tightly regulated by eIF2β. eIF2β initially removes eIF5 and acts as guanine nucleotide exchange factor which subsequently activates eIF2 facilitating Met-tRNAi interaction and eIF5 rebinding. eIF2β's role as a guanine nucleotide exchange factor is crucial for maintaining the pool of active eIF2-GTP, facilitating proper codon recognition and the initiation of protein synthesis [185, 186].

In the integrated stress response, the kinases aforementioned (GCN2, HRI, PRK, and PERK) attenuate global protein synthesis by phosphorylating Ser51within the eIF2α subunit [187, 188]. This phosphorylation hinders the guanine nucleotide exchange factor activity of eIF2β, and forms a tight eIF2α/eIF2β inhibitory complex. By impeding the recycling of inactive eIF2-GDP to its active from eIF2-GTP, protein synthesis is attenuated. However, in response, a range of stress-responsive mRNAs are induced to restore homoeostasis. This includes the selective transcription of ATF4 and GCN4 which facilitate in the activation of pro-survival and pro-apoptotic pathways [189, 190](**Figure 1.4**).

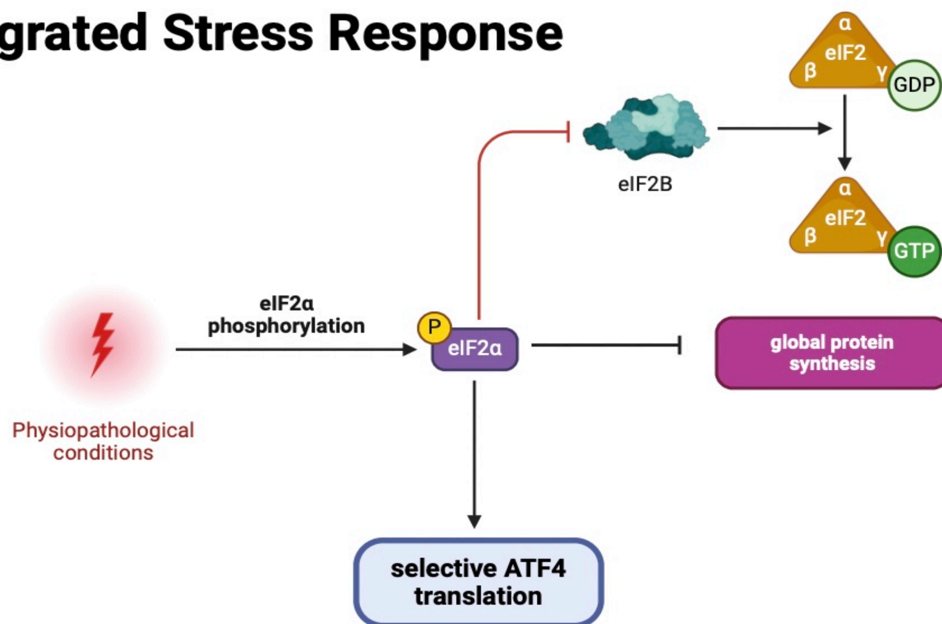


Figure 1.4. Schematic of the Mechanisms involved in the Activation of the Integrated Stress Response.
Upon phosphorylation of the eIF2α subunit, global protein synthesis is attenuated. Selective translation of ATF4 is induced. (adapted from "Stress Response Mechanism in Cells" by BioRender.com (2024))

The connection between cellular stress and chronic psychological stress has been briefly explored. Recent studies show a correlation between ER stress and/or unfolded protein response and neuropsychiatric disorders [191-195]. The postulated mechanism includes the activation of the HPA in response to chronic psychological stress. This activation increases circulation of cytokines that may be transported to the brain or released from the microglia. As pro-inflammatory cytokines increase reactive oxygen species, oxidative and ER stress is induced [131]. Mitochondrial dysfunction also induces reactive oxygen species generation. ER stress which is characterized by the accumulation of misfolded proteins can be sensed by PERK [163]. Which as aforementioned plays a critical role in ER quality control. In response, PERK can

phosphorylate eIF2α resulting in the attenuation of global protein synthesis to suppress accumulation of misfolded proteins.

The integrated stress response has also been implicated as a pivotal player in cancer progression, offering cancer cells the ability to evade apoptosis and bolstering their survival, proliferation, and metastatic potential. A hallmark of neoplastic transformation is deregulation of cell division, culminating in uncontrolled proliferation and aberrant global protein synthesis. As the tumor progress, rapid oncogenic growth increases genomic instability, metabolic rate, and depletes oxygen availability [196]. As this creates arid conditions, cancer cells can hijack machinery to promote oncogenic survival. By activating the integrated stress response, which attenuates protein synthesis, cancer cells strategically alleviate the demand on cellular resources. This orchestrated modulation not only enables cells to cope with the heightened metabolic burden but also plays a pivotal role in fostering pro-oncogenic survival [197].

Activation of the integrated stress response (ISR) extends beyond its role in cancer progression, demonstrating a significant impact on treatment resistance. In TNBC, cells exhibiting heightened resistance to radiotherapy exhibit the most robust activation of the integrated stress response [198]. Notably, the phosphorylation of eIF2α and subsequent ATF4 activation within this context drives the transcription of genes facilitating glutathione biosynthesis. This, in turn, enhances susceptibility to reactive oxygen species. Given that elevated levels of reactive oxygen species are recognized as a hallmark of cancer, their activation in TNBC cells results in the development of a radioresistant phenotype [198].

While the link between ISR activation and cancer progression, as well as therapy resistance, has been elucidated, there remains a scant data on how chronic activation of this pathway during mammary development may influence breast cancer risk later in life. Exploring

this aspect could provide valuable insights into the nuanced interplay of the integrated stress response in mammary development and its potential implications for future breast cancer development.

1.8: Conclusions and Project Overview

As aforementioned, the mammary gland is a highly intricate and multifaceted organ, composed of a myriad of cell types and tissues. An in-depth comprehension of mammary gland development is imperative, considering its susceptibility to external influences such as chronic psychological stress, which can alter its developmental trajectory and subsequently heighten the risk of mammary cancer. This is particularly relevant during pivotal developmental stages like puberty and early adulthood.

Moreover, investigating the repercussions of stressors during puberty represents a novel avenue for discerning risk factors contributing to the disparities observed in human breast cancer incidence. Within this context exploring the direct link between social environment and mammary gland biology. As one's social community and context is a factor in the social determinants of health, this project is helping to elucidate how factor such as social integration, discrimination, and chronic psychological stress during developmental periods such as puberty and early adulthood influences breast cancer risk in later adulthood. We hypothesized that social isolation and prolonged exposure to chronic psychological stress during puberty and early adulthood exert a substantial influence on mammary gland development, inducing delayed maturation and escalating the likelihood of mammary cancer onset later in life. This hypothesis underscores the importance of understanding the interplay between psychosocial factors and mammary gland biology in shaping cancer susceptibility.

This study has a dual focus: first, to solidify puberty's pivotal role in the onset of stress-induced effects, pinpointing it as a crucial window for interventions. We'll delve into the complex molecular pathways that underlie the stalling of mammary development in response to social isolation during this critical phase. Secondly, we'll explore whether these impacts are reversible and assess the effectiveness of environmental and pharmacological interventions in counteracting the negative effects experienced during puberty. By taking this multifaceted approach, we aim to glean insights into actionable strategies for mitigating the adverse consequences of social isolation during this developmental period. Ultimately, we aspire to utilize our findings to advocate for targeted interventions that can address health disparities and improve clinical outcomes, particularly among vulnerable populations.

Chapter 2 : Reducing Psychosocial Stress during Puberty and Early Adulthood Restores Mammary Gland Development

2.1: Abstract

In the context of developmental biology, puberty emerges as a critical "window of susceptibility," marking a pivotal phase in which the mammary gland undergoes rapid development and becomes highly responsive to physiological and environmental influences, potentially escalating the risk of breast cancer in adulthood. Utilizing the Sprague-Dawley rat model of social isolation, our laboratory has unveiled the previously unexplored phenomenon of mammary development extending into early adulthood, demonstrating that exposure to chronic psychological stress during the crucial period of early puberty through early adulthood (5-21 weeks) impedes maximal ductal extension growth and alveolobular differentiation. Our investigation delves into the lingering question of how heightened exposure to chronic psychological stress correlates with decreased mammary development and differentiation. In our study, animals were socially isolated throughout this developmental window, resulting in a manifestation of hypervigilant temperament, reduced mammary development and differentiation, and an elevated preservation of mammary stem/progenitor cells compared to the control group (group-housed animals). Seeking a comprehensive understanding, we conducted a bulk RNA-seq analysis, pinpointing the eIF2α signaling pathway as the most modulated in socially-isolated animals. The critical observation of phosphorylated eIF2α exclusively in socially-isolated subjects suggests that chronic psychological stress during early puberty through early adulthood induces decreased protein translation, potentially contributing to the observed decrease in mammary gland development. Understanding the continuous trajectory of mammary

development from early puberty to early adulthood, we investigated the potential reversal of detrimental effects through an environmental intervention initiated at the onset of late puberty through early adulthood (13-22 weeks). During this phase, animals underwent social isolation, exposing them to chronic psychological stress in early puberty, but were subsequently returned to group housing at the onset of late puberty through early adulthood. The environmental intervention of rehousing yielded notable outcomes, including the restoration of mammary gland development and differentiation, a reduction in hypervigilant temperament to baseline/control measures, and a decrease in the proportion of mammary stem/progenitor cells. This study not only reveals a molecular target for the impact of chronic psychological stress during early puberty through early adulthood but also emphasizes the temporal importance of mammary developmental windows. Such a comprehensive understanding contributes significantly to the discourse on preventive strategies for breast cancer, highlighting the necessity for targeted interventions during these critical developmental periods.

2.2: Introduction

In the realm of developmental biology, puberty is commonly referred to as the "window of susceptibility" [199] signifying a pivotal phase wherein the mammary gland undergoes rapid development and becomes highly susceptible to environmental influences. Traditionally, research has primarily focused on physical environmental insults, such as exposure to chemical carcinogens or toxins, during early puberty and their subsequent impact on altered mammary gland development, ultimately contributing to an increased risk of breast cancer in adulthood [122] .

However, a previously overlooked dimension pertains to the influence of social environmental factors from early puberty through early adulthood, specifically chronic psychological stress. The understanding of how exposures to stress during this critical period might affect mammary gland development and elevate the risk of breast cancer in adulthood has been a significant knowledge gap. This knowledge is particularly crucial, considering that women are most vulnerable to environmental exposures before their first pregnancy, during puberty, and in young adulthood [200]. Moreover, the global trend of delaying childbearing has extended this sensitive period [201].

Given the physiological similarities between rats and humans, especially in mammary gland structure, rats offer an appealing model for studying the effects of chronic psychological stress on mammary gland development. The Sprague Dawley Rat model of social isolation, a well-established model for mild unremitting chronic psychological stress, proves to be particularly valuable. Socially isolated animals lack the network to dissipate the effects of secondary stressors [151]. Previous research conducted in our laboratory using this model demonstrated that mammary development and differentiation span from early puberty to early adulthood [60]. Notably, social isolation during this critical period (5-22 weeks) was found to impair maximal ductal extension growth and alveolobular differentiation, which are key processes in mammary gland development [158] . These findings underscore the significance of considering both physical and social environmental factors in the complex interplay influencing mammary gland development and subsequent breast cancer risk.

The lingering inquiry revolved around how heightened exposure to chronic psychological stress translates to decreased mammary development and differentiation. Seeking comprehensive understanding, this current study embarked on an environmental intervention commencing at the

onset of late puberty through early adulthood (13-22 weeks). During this period, animals experienced social isolation (exposure to chronic psychological stress) in early puberty but were subsequently returned to group housing at the onset of late puberty through early adulthood.

The selected timeline aligns with key observations indicating decreased terminal end buds (TEBs) and optimal ductal growth, extension, and differentiation in the late puberty to early adulthood phase. Our hypothesis postulates that animals subjected to social isolation during early puberty but reintegrated into group housing at the onset of late puberty would manifest a protective and restorative effect against the detrimental impacts of social isolation. Notably, the concept of rehousing socially-isolated animals has predominantly been explored to gauge acute behavioral responses in male rats [202-204].

Distinguishing our approach, this novel study extends the exploration to resocializing socially-isolated female rats, aiming to elucidate the specific impact on mammary development during critical developmental windows. By delving into the intricacies of how social reintegration may mitigate the effects of early stress on mammary gland development, this research contributes to a more nuanced comprehension of the interplay between chronic psychological stress and mammary physiology.

Upon the environmental intervention of rehousing, we observed a restoration of mammary gland development and differentiation, accompanied by a reduction in hypervigilant temperament (apart from two outliers) to baseline/control measures. These novel results emphasize the temporal importance of mammary developmental windows, highlighting the critical impact of social environmental exposures during late puberty through early adulthood. The reduction in exposure to chronic psychological stress was associated with the restoration of

mammary development, providing mechanistic insights into how decreased mammary development is affected by increased exposure to chronic psychological stress.

2.3: Material and Methods:

2.3.1: Sprague-Dawley Rats

Female Sprague-Dawley Rats were bred at Charles River Laboratories (Kingston, NY). The animals were transitioned into all-female groups at three weeks of age and subsequently transported to our laboratory by the time they reached four weeks of age. Upon their arrival, they were initially housed in groups for 1 week to rectify the stress of transportation. When they reached 5 weeks of age, they were transferred to either individual housing (n=10) or group housing (n=5), after being carefully balanced for vigilant temperament as assessed during an open field test at 4.5 weeks of age (outlined below;[155]).

All animals were accommodated in solid stainless-steel cages suspended on shared racks in the same colony room maintained at 21 ± 1 °C. The cages featured wire coverings on the front and floor, situated above shared bedding pans (Harlan Teklad Aspen Sani-Chips, 7090A). The animals could see, hear, and smell their counterparts, however, social interaction was limited to group-housed animals. Group-housed animals were placed in a 46 × 61 × 36 cm cage, and socially-isolated animals in individual 20 × 24 × 18 cm cages. The animals followed a reversed light-dark cycle (14:10, with dark onset at 08:00 h), and all experimental procedures occurred during the rats' behavioral day. Food (Harlan Teklad Rodent Diet, 8604) and water were provided *ad libitum*. Routine handling during cage changes and health inspections adhered to the National Institutes of Health and University of Chicago Animal Care Guidelines.

2.3.2: Experimental Design

To determine the effect of exposure to chronic psychological stress and heightened stress reactivity on MG development, the experimental design included three conditions (**Figure 2.1**): animals that were group-housed at 5 weeks and sacrificed at 22 weeks , socially-isolated at 5 weeks and sacrificed at 22 weeks, and socially-isolated from 5 weeks to 13 weeks; returned to group-housing at 13 weeks and sacrificed at 22 weeks (n= 5 group-housed, n= 5 socially-isolated, and n=5 socially-isolated then returned to group housing).The time of sacrifice is during early adulthood (22 weeks of age). Ovarian cyclicity was quantified the week prior to sacrificed, and animals were sacrificed in proestrus/estrus to minimize ovarian cycle variability in mammary morphology [155, 205]. This experiment was repeated twice independently.

Reproductive Lifespan Events: Mammary, Adrenal, and Ovarian		Weaning Prepubertal Temperament	Early and Mid-Puberty - Terminal End Buds (TEBs) differentiate to Terminal Ducts and Alveolar Buds - Hypothalamic-pituitary-ovarian axis is matured	Late Puberty and Early Adulthood - Ducts differentiate - Duct tree lengthens and expands - Mammary stem/progenitor cell population drops
Age (weeks)		3-4	5-12	13-22
A	G ➡ G			
B	SI ➡ SI			
C	SI ➡ G			
Social Environment:		Grouped (grey)	Socially-Isolated (white)	

Figure 2.1. Schematic of experimental design and timeline. 15 animals were placed into three social conditions to determine the effect of social isolation during puberty and early adulthood on mammary development. An ***environmental intervention*** was employed to investigate the potential reversibility of the impacts resulting from social isolation during this time period. Animal conditions were as followed: n = 5 group housed (group housed animals from 5-22 weeks of age) n = 5 socially-isolated (socially-isolated animals from 5-22 weeks of age) n = 10 rehoused (socially isolated animals from 5-13 weeks of age, returned to group housing from 14-22 weeks of age) Sacrifice of animals occurred during early adulthood (22 weeks). Experiment was performed two independent times (30 animals total, 10 per each experimental condition).

2.3.3: Behavioral Response to an Open Field Test

An open field test measures an animal's novelty-seeking behavior and willingness to explore (Padilla et al. 2010). Exploration in an unfamiliar environment is a stress model to test vigilance and temperament in rodents. The exploration area consisted of Plexiglas walls, a home base enclosure in one corner infused with familiar scents, and a small overturned ceramic bowl in the center. The animal was carefully transferred from its home cage in the neighboring colony room and placed into the home base. Vigilance was assessed by measuring the time it took for the rat to emerge from the home base within a five-minute period. Consistency was maintained by using the same handler throughout, and the open field base was cleaned with Cavi-wipes between animals to eliminate stress-related odors. We measured vigilance and temperament twice during the lifespan of the animal, with testing at 4.5 weeks of age and 20 weeks of age.

2.3.4: MG Whole Mount Preparation and Analyses

Upon sacrifice the right inguinal mammary gland of the animal is excised, applied onto a glass slide and fixed with Carnoy's fixative. Once the mammary gland is fixed, xylene is applied to remove the lipid layer and carmine alum is used for staining. Once the staining is dried, an image of the whole mount slide is acquired through a 3600 dpi flatbed scanner [158]. 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 mammary gland ductal tree. Distal MG ductal area was the area between a horizontal line transecting the LN and the traced perimeter of the mammary gland ductal tree. A minimum of 10 animals in each experimental condition were used for statistical comparison of mammary gland ductal extension and ductal area.

2.3.5: Quantifying Mammary Progenitor/Stem Cells

Mammary Cell Isolation: Mammary glands in the left inguinal quadrant were excised using nipple 12 as an anatomical marker. Upon excision, mammary gland was minced into a paste and placed in serum-free media supplemented with 1% penicillin/streptomycin (P/S), 1% bovine serum albumin (BSA), and 1% of Type 1 collagenase for 15 hours at 37 degrees [159]. The collagenase digestion separates individual mammary cells (mammary stem/progenitor, epithelial, endothelial, hematopoietic, and immune cell) from the adipocyte fraction. Upon overnight digestion, the cell pellet was formed through centrifugation and removal of the adipocyte fraction. The pellet of cells was treated with NH4Cl to lyse blood cells and DNase/dispase to prevent cell clumping prior mammary progenitor/stem cell quantification.

Mammary Progenitor/Stem Cell Quantification: The mammary cell suspension was blocked with unconjugated Fc antibody (BD Biosciences 550271) and dyed with Live/Dead solution (catalog number) to distinguish cell viability. Subsequently, the mammary cell suspension was stained with anti-CD31 ((Abcam 33858), anti-CD45 (Abcam 33916), anti-CD24 (BD Biosciences 56210), and anti-CD29 (BioLegend 02225). The mammary cell suspension was then analyzed and quantified through flow cytometry (LSR Fortessa 4-15) . The CD31+CD45+ hematopoietic and endothelial cells were excluded through gating. Thus, we were able to detect CD24+CD29+ amongst the Lin- (CD31-CD45-) population of cells, which are highly enriched for undifferentiated normal mammary stem/progenitor cells (Velten et al. 2020). The quantification of mammary progenitor/stem cells was reported as proportion of CD24+CD29+/CD31-CD45- (Lin-) cells. The undifferentiated normal mammary stem/progenitor cells (CD24+CD29+/Lin-) were further sorted based on CD24+CD29+ levels into basal and luminal progenitor stem/cells

(basal= CD24+cD29hi and luminal= CD24+CD29lo). A minimum of 10 animals in each experimental condition were used for statistical comparison of proportion of CD24+CD29+/CD31-CD45- (Lin-) cells.

2.3.6: RNA Sequence Analysis

Total RNA was extracted from the right pectoral whole mammary gland of the animal using E.Z.N.A Total RNA Kit II (Omega bio-tek), and messenger RNAs were subsequently eluted. A Nanodrop (Thermo Scientific NanoDrop 2000C) was used to determine RNA concentration and threshold of 260/280 ratio ranged from 2.0-2.05. RNA samples with a volume of 13 ul and concentration of 100 ng/ul from all experimental conditions (n=15) were submitted to the genomics core at the University of Chicago for RNA-seq analysis. A library was created using NovaSeq with 30M clusters/sample and 60M PE reads/sample. Samples were run twice on a flow cell in the HiSeq 4000 system. Downstream analysis was performed on the web based BaseSpace-Sequence Hub (Illumina) platform. While read alignment/mapping was done with the RNA-seq Alignment app (Illumina).

Gene expression data was expressed in fold change between experimental conditions, (socially-isolated vs group housed, socially-isolated vs rehoused, rehoused vs group-housed). Differential expression analysis was performed using a two-tailed t test. Unequal variances were accounted for each expressed gene between the three groups resulting in ordered ranked p-values. Genes that had a lower p value than 0.05 were used for further analysis.

2.3.7: Ingenuity Pathway Analysis

Gene lists of significantly differentially expressed genes between the three experimental conditions socially-isolated vs group housed, socially-isolated vs rehoused, rehoused vs group-housed were entered into the Ingenuity Pathway Analysis software by Qiagen. Using this software, gene lists were narrowed to determine differential canonical pathways, diseases and functions, signal pathway interactions, and genes and networks of upstream regulators.

2.3.8: Quantitative Real-Time Polymerase Chain Reaction

To confirm the gene expression findings of the RNA-seq and IPA analysis, qRT PCR was performed. Upon acquiring concentrated and relatively pure RNA (acquisition steps mentioned above), a complementary DNA (cDNA) synthesis kit (Quantbio 95048-100) was used to synthesize cDNA. cDNA was used for the quantitative real time polymerase chain reaction. Primers listed in supplementary materials. Gene expression quantification was performed wth SYBR green using a Bio-Rad CFX Connect Real-Time PCR Detection System. The housekeeping gene HRPT1 was used as an endogenous control to normalize mRNA levels in each sample. Using the C(T) method, gene expression levels were quantified.

2.3.9: Immunoblotting

Protein was extracted from the right pectoral whole mammary gland of the animal for all experimental conditions (n=15, socially-isolated, socially-isolated to group housed (rehoused), and group-housed. Tissue was cut, weighed at 100 mg, and immediately placed in ice cold 1x RIPA buffer (EMD Millipore; 20-188) containing 1X phosphatase and protease inhibitor cocktail

tablets (Roche). Samples were homogenized and placed on ice for 30 minutes. Cooled samples were then sonicated (Sonics Vibra-cell) and spun at 1000xg for 10 minutes at 4°C. Supernatant was then collected and spun at 12000xg for 10 minutes at 4°C. The supernatant was stored at -80°C.

Using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific; 23227), concentration of protein was quantified. Samples were then diluted in 2X Laemilli Sample Buffer (Bio-Rad 1610737) and ran on 4-15% mini–Protean TGX gels (Bio-Rad 4561021). Upon overnight transfer onto Immobilon-P-PVDF membranes, membranes were then blocked with 3% non-fat dry milk in TBST for an hour. Membranes were blocked overnight with primary antibodies (1:10000 B-actin (Thermo Fisher Scientific: 15G5A11/E2) and 1:1000 p-eIF2α (ab32157), 1:1000 eIF2α (Cell Signaling 9721S), or 1:1000 ATF4 (Cell Signaling 11815). Membranes were then incubated with IRDye secondary antibodies (LI-COR) for an hour. Detection was performed using Odyssey CLx System, (LI-COR). Using Image Studio software, blots were analyzed. (protocol modified from [206]).

2.3.10: Statistical Analysis

Statistical analyses were conducted GraphPad Prism Software 10 (GraphPad Software, Inc., La Jolla, CA ($P > 0.05$, two-tailed tests), all means ± SEM)

2.4: Results

To determine the effect of exposure to chronic psychological stress and heightened stress reactivity on vigilance and temperament, MG development and differentiation, and subsequent gene expression, the experimental design included three conditions. Animals were group-housed

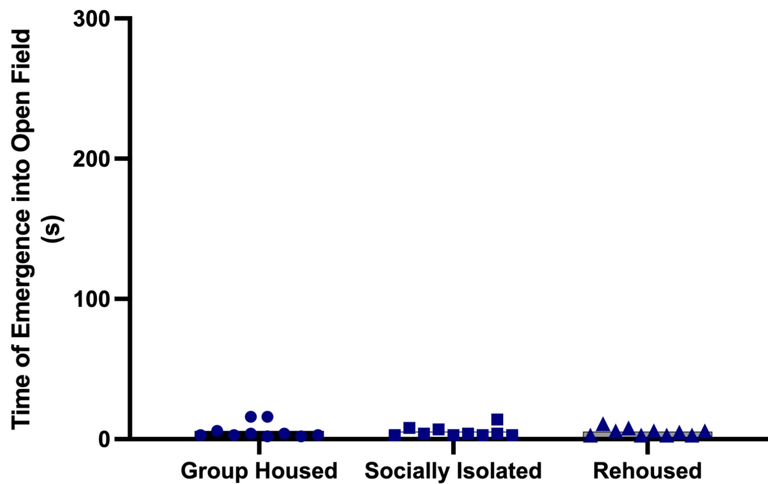
with 5 rats/cage at 5 weeks and sacrificed at 22 weeks (group housed), socially-isolated at 5 weeks and sacrificed at 22 weeks (socially-isolated), and socially-isolated from 5 weeks to 13 weeks; returned to group-housing at 13 weeks and sacrificed at 22 weeks (rehoused); (n= 5 group-housed, n= 5 socially-isolated, and n=5 socially-isolated then returned to group housing). The time of sacrifice is during early adulthood.

2.4.1: Increased Vigilance in Socially-Isolated Animals, Rehousing Reduces Vigilant Temperament and Behavior

The Open Field Test serves as a valuable metric for assessing an animal's inclination towards novelty-seeking behavior and willingness to explore [207]. In the context of our study, exploration in an unfamiliar environment serves as a stress model, providing insights into vigilance and temperament in rodents. Specifically, we sought to investigate the impact of social isolation during late puberty and early adulthood on temperament. Additionally, we explored the potential mitigating effects of an environmental intervention, i.e. rehousing into group-housed condition. The primary focus was on measuring the time of emergence from home base within the 0-300 seconds timeframe into the open field. Temperament measurement was conducted on all animals at 4.5 weeks prior to their placement in experimental conditions, ensuring control for temperament differences. Animals in all conditions displayed similar emergence times; animals placed in group-housing displayed an averaged emergence time of 5.9 seconds, animals placed into social isolation displayed an average emergence time of 5.3 seconds, and animals placed in rehousing displayed an average emergence time of 5.4 seconds (**Figure 2.2A**). Temperament was re-measured 2 weeks prior to sacrifice after animals had been in experimental conditions for 6.5 weeks.

Vigilance and Temperament
Prior to Placement in Experimental Conditions (4.5 weeks)

2.2A.



Vigilance and Temperament Prior to Sacrifice (20 weeks)

2.2B.

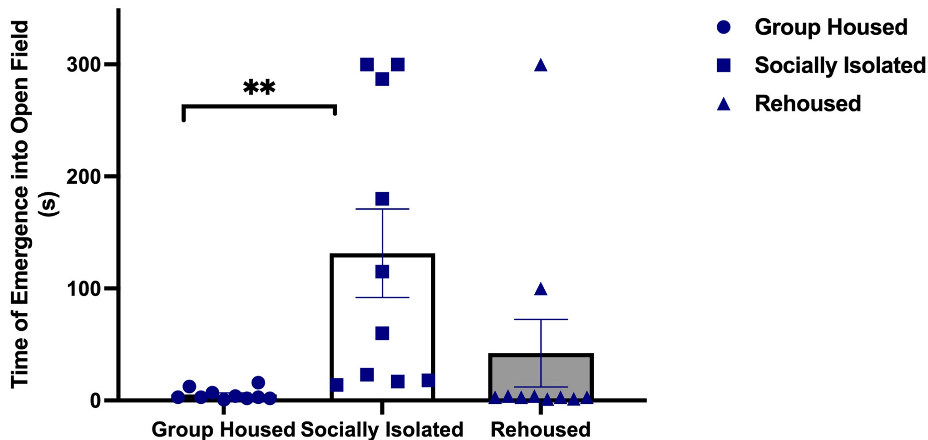


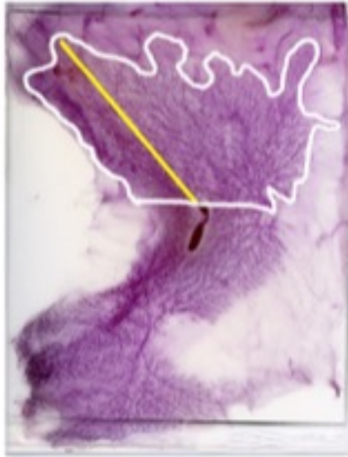
Figure 2.2. Effect of social environment on vigilance and temperament. A) Time of animal emergence (seconds) into an open field was quantified for all animals at 4.5 weeks prior to placement in experimental condition at 5 weeks **B)** Time of animal emergence (seconds) into an open field was quantified prior to sacrifice (20 weeks) for experimental conditions: group housed (animals were group housed from 5-22 weeks of age), socially-isolated (animals were socially-isolated from 5-22 weeks of age), rehoused (animals were socially-isolated from 5-13 weeks of age, then returned to group housing from 14-22 weeks of age).** $p<0.01$ (n=10 for all experimental conditions; each animal shown as an individual datum point).

Group-housed animals demonstrated rapid emergence times, with an average time of 5.3 seconds, indicative of a more exploratory and less hesitant temperament. In contrast, socially-isolated animals exhibited prolonged periods at the home base, taking the longest time to emerge (**Figure 2.2B**) with an average time of 131 seconds indicative of significantly heightened vigilance ($p<0.01$). Interestingly, rehoused animals exhibited an average time of emergence of 42.3 seconds, which was further reduced to 2.9 seconds when two outliers were excluded. This reduced emergence time in rehoused animals indicates an alleviation of the hypervigilant behavior exhibited by socially-isolated counterparts, particularly in 8 of the 10 rehoused animals. The findings from this Open Field Test provide valuable insights into the impact of social isolation and the potential ameliorative effects of rehousing on rodent temperament during late puberty and early adulthood.

2.4.2: Social Isolation Slows Mammary Ductal Development, Rehousing is Associated with Restorative Effect in Mammary Ductal Development

In the field of developmental biology, mammary development during late puberty and early adulthood is a readout of intricate hormonal signaling pathways and genetic regulation that orchestrate the maturation of mammary glands [82]. Using the right inguinal mammary gland, maximal ductal extension and distal ductal area was measured at the time of sacrifice at 22 weeks. **Ductal Extension.** Maximal 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 mammary gland ductal tree at 22 weeks of age (**Figure 2.3A-C,** yellow line; extension quantitated in **Figure 2.3D**). Group housed animals displayed physiological ductal extension of approximately 45 mm. As previously reported, social isolation led to significantly reduced mammary ductal

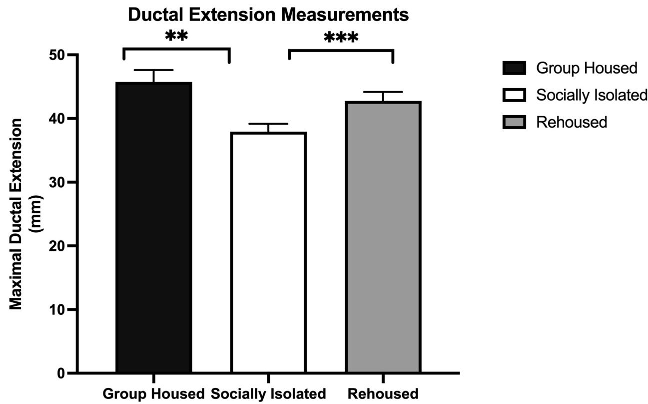
2.3A.



2.3B.

2.3C.

2.3D.



2.3E.

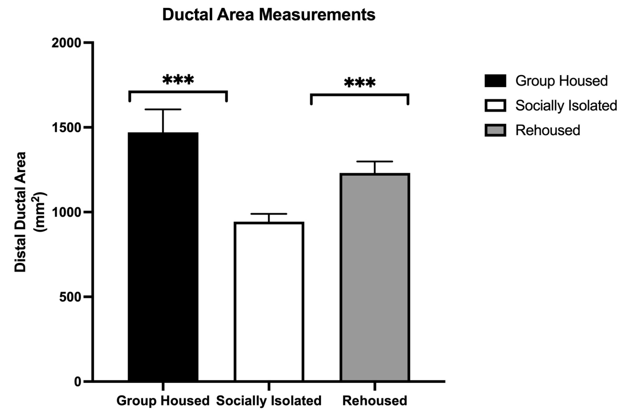


Figure 2.3. Effect of social environment on mammary development measured by maximal ductal extension and distal ductal area A-C) Representative images of carmine alum staining of the whole mount derived from right inguinal mammary gland. Ductal extension was determined as distance from lymph node (LN) to furthest terminal end bud (yellow line). Ductal area was determined by the area between the horizontal line transecting the LN and the traced perimeter of the mammary gland ductal tree. A) Group housed animals from 5-22 weeks of age: B) Socially isolated animals from 5-22 weeks of age; C) Socially isolated animals from 5-13 weeks of age, returned to group housing from 14-22 weeks of age. D) Quantification of the effect of social condition on mammary ductal extension (mm) E) Quantification of the effect of social condition on mammary ductal area (mm^2) (All measures were performed blinded by two independent observers). **p<0.01, ***p<0.001. (n= 10 group housed, n=10 socially-isolated, n=10 rehoused animals)

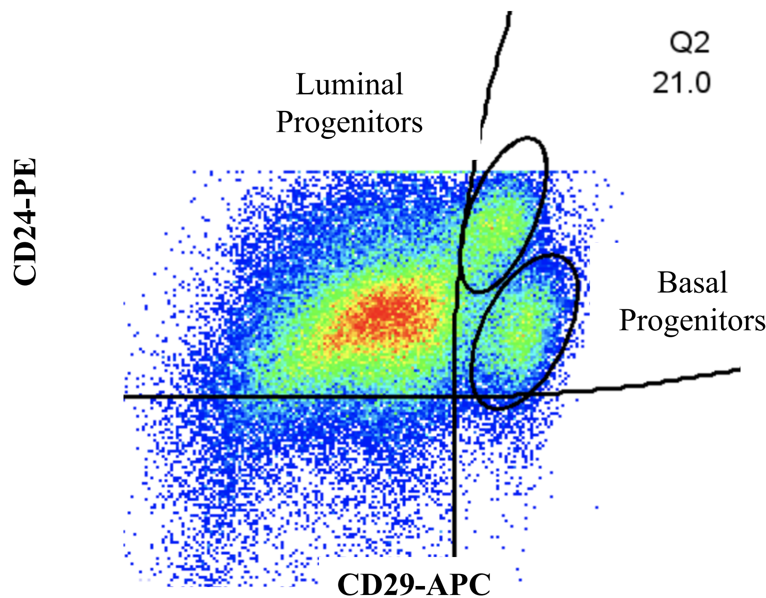
extension averaging 37 mm ($p < 0.001$) [158]. Interestingly, rehousing resulted in significant restoration of mammary ductal extension to an average of 42 mm ($p < 0.001$).

Ductal Area. Distal ductal area was measured as the area between a horizontal line transecting the LN and the traced perimeter of the mammary gland ductal tree (**Figure 2.3A-C**, white outline; quantitated in **Figure 2.3E.)** Group housed animals displayed physiologic mammary development with a distal ductal area of 1411 mm^2. Socially-isolated animals had significantly reduced distal ductal area at 929 mm^2 ($p<0.001$). The environmental intervention of rehousing, significantly restored distal ductal area with rehoused animals having similar ductal area to the group housed animals at 1230 mm^2 ($p<0.001$).

2.4.3: Social isolation Increases Mammary Progenitor populations, Rehousing Reduces Mammary Progenitor/Stem population

In the rat mammary gland, the process of mammary ductal differentiation and a decline in the mammary stem/progenitor cell population are well-documented phenomena during early adulthood [208]. Consequently, the proportion of mammary stem/progenitor cells serves as a valuable indicator of mammary development. Given that socially-isolated animals exhibit less developed mammary glands, we formulated the hypothesis that these animals would display an elevated proportion of mammary stem/progenitor cells compared to group housed or rehoused animals. This hypothesis aligns with the notion that mammary stem/progenitor cells in socially-isolated subjects may not be undergoing the typical differentiation process during the critical period from early puberty through early adulthood.

2.4A



2.4B

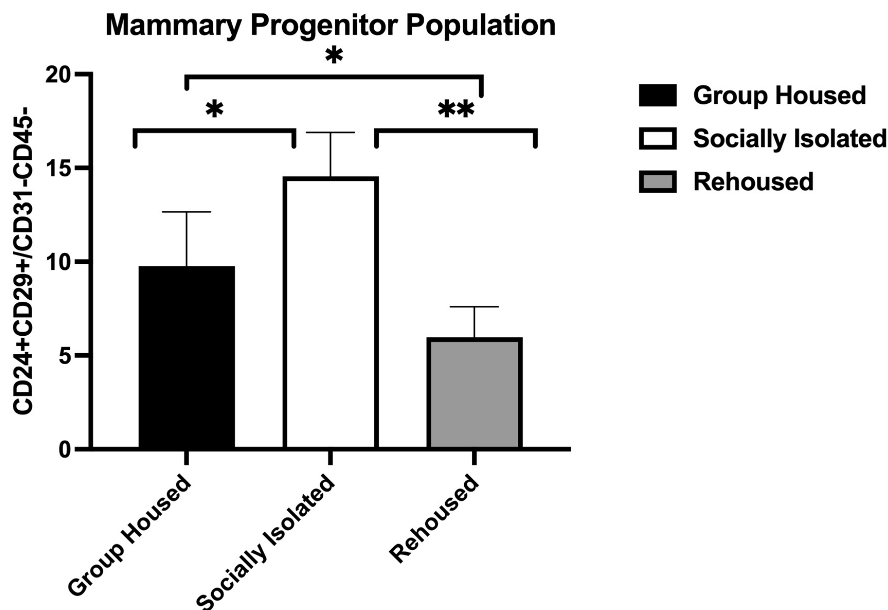


Figure 2.4. Effect of social environment on mammary stem/progenitor proportion A) Representative flow cytometry of mammary progenitor/stem cells ($CD24^{+}CD29^{+}$) further separated as $CD24^{+}CD429^{lo}$ (luminal progenitor) and $CD24^{+}CD29^{hi}$ (basal progenitor) cells in the $CD45^{-}CD31^{-}$ mammary-gland cell population B) The proportion of total CD24+CD29+ progenitor cells relative to $CD45^{-}CD31^{-}$(Lin-) cells from grouped, socially-isolated, and rehoused animals). **$p<0.01$ *$p<0.05$ (n = 10 group housed, n = 10 socially-isolated, n = 10 rehoused).

Using flow cytometry, we were able to clearly identify luminal progenitor ($CD24^{+}CD429^{lo}$) and basal progenitor ($CD24^{+}CD29^{hi}$) cell populations (**Figure 2.4A**). Showing this clear distinction to substantiate the identification of the mammary stem/progenitor population, the population of stem/progenitor cells was measured as a proportion $CD24^{+}CD29^{+}$ undifferentiated normal mammary stem/progenitor cells divided by CD31+CD45+ hematopoietic and endothelial cells (Lin-)(**Figure 2.4B**). Group housed animals had a reduced proportion of mammary stem/progenitor cells, suggesting greater ductal differentiation and mammary development. As hypothesized, socially-isolated animals had a significantly greater proportion of mammary stem/progenitor cells ($p<0.001$). This finding corroborated with less mammary ductal differentiation and development. The environmental intervention of rehousing displayed the lowest proportion of mammary stem/progenitor cells ($p<0.05$). The hypothesis posits that there was a delay in differentiation from early to late puberty; however, once the subjects were regrouped, the process of differentiation was significantly enhanced.

2.4.4: Social Isolation Decreases Pathways involved in Mammary Development; Rehousing, shows a restorative effect, displaying a similar genetic phenotype to group-housed (control)

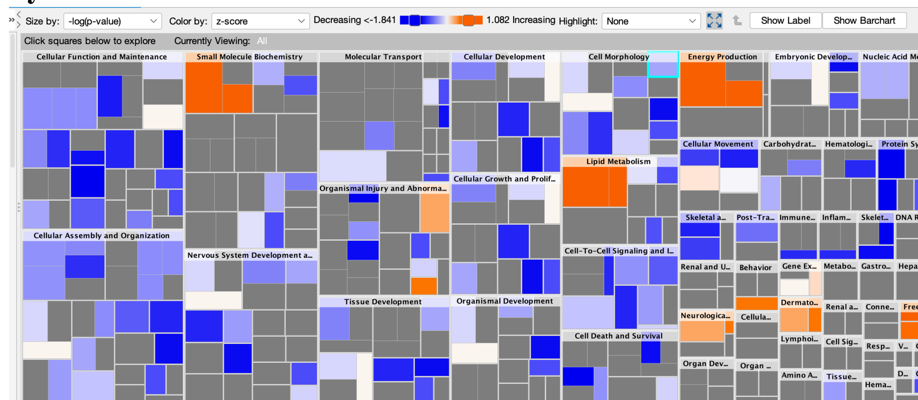
To determine the mechanistic basis of the reduced mammary development in socially-isolated animals, we performed a comprehensive RNA-sequence analysis using RNA eluted from the right pectoral mammary gland in all experimental conditions (group housed, socially-isolated, rehoused). The RNA-sequence analysis generated significant ($p<0.05$) differentially expressed genes in all three conditions socially-isolated vs group housed, socially-isolated vs rehoused, and rehoused vs group housed. These differentially expressed gene sets were input into an Ingenuity Pathway Analysis (IPA) to determine differential canonical pathways, diseases and functions, signal pathway interactions, and genes and networks of upstream regulators [209].

The IPA software showed of slew of modulations changing in response to each social condition. When comparing socially isolated conditions to group housed, there was a significant reduction in cellular growth and proliferation, cellular development, and tissue development. We hypothesized these diseases and functions were being modulated in the epithelial cell fraction (**Figure 2.5A**). Upregulation of lipid metabolism and energy production was hypothesized to be upregulated in the adipocyte fraction of the whole mammary gland. Similar trends were observed when comparing socially-isolated conditions to rehoused, there was a significant reduction in cellular growth and proliferation, cellular development, and tissue development, and an upregulation of lipid metabolism and energy production (**Figure 2.5B)**. When comparing rehoused vs group housed due to low number of significant differential genes (<150), IPA was unable to produce z-scores indicating upregulation or downregulation. However, p-values indicated significant differences in lipid metabolism and nervous system development (**Figure 2.5C).** To hone in on pathways of interest, IPA generated bubble charts that showed genes involved in cellular growth, proliferation, and development, second messenger signaling, and translation were highly significantly downregulated in socially-isolated vs group housed and socially-isolated vs rehoused (**Figure 2.6A. and 2.6B**). Rehoused vs group housed pathways were also compared, however, the pathways that had significant modulations showed no clear directionality and had the least amount of overlapping genes in a given pathway as indicated by the p-value in the bubble chart (**Figure 2.6C**).

Based upon these initial findings, we took it a step further to define what specific canonical pathways were modulated. In socially-isolated vs group housed, the canonical pathways of

2.5A. **Socially-Isolated vs Group Housed**

2.5B. **Socially-Isolated vs Rehoused**



2.5C. **Rehoused vs Group Housed**

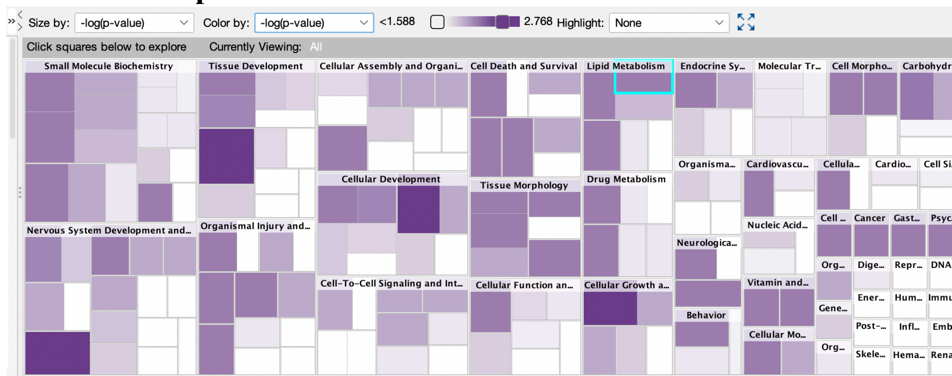
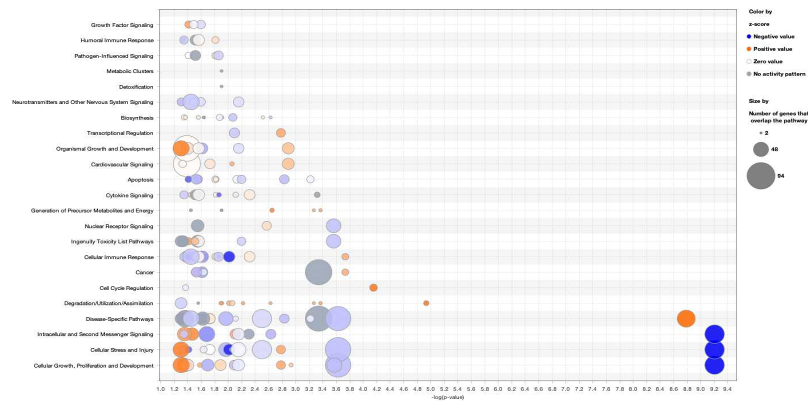
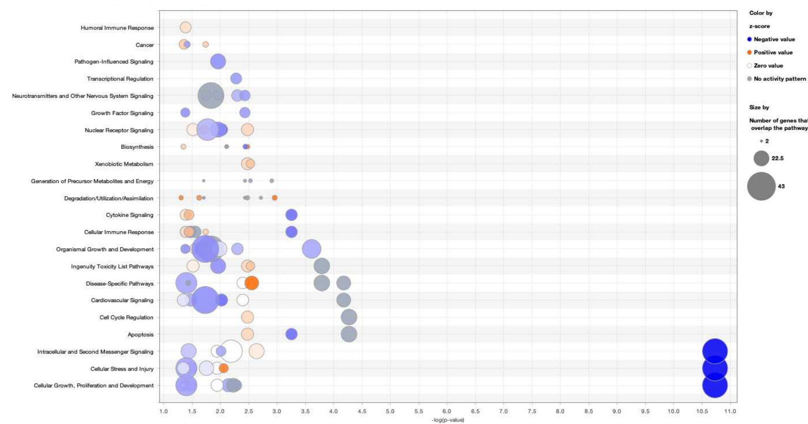


Figure 2.5. Diseases and Functions from Ingenuity Pathway Analysis configured from bulk RNA-Seq A) Diseases and Functions inhibited or activated in socially-isolated vs group housed animals B) Diseases and Functions inhibited or activated in socially-isolated vs rehoused C) Diseases and Functions inhibited or activated in rehoused vs group housed animals (n=5 animals per experimental condition).

2.6A. **Socially-Isolated vs Group Housed**



2.6B. **Socially-Isolated vs Rehoused**



2.6C. **Rehoused vs Group Housed**

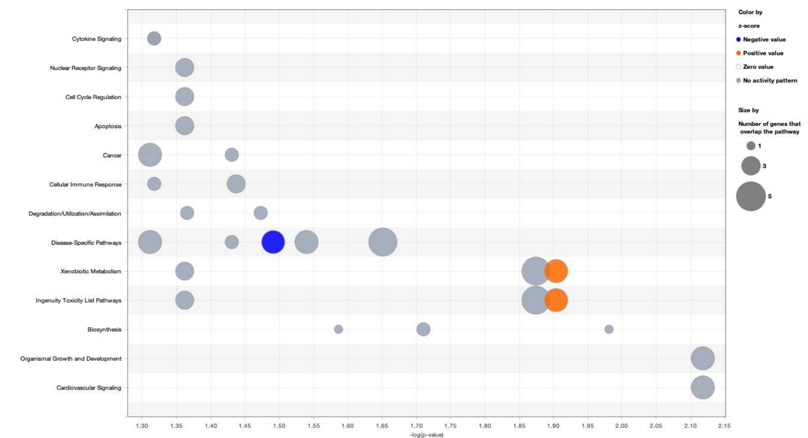
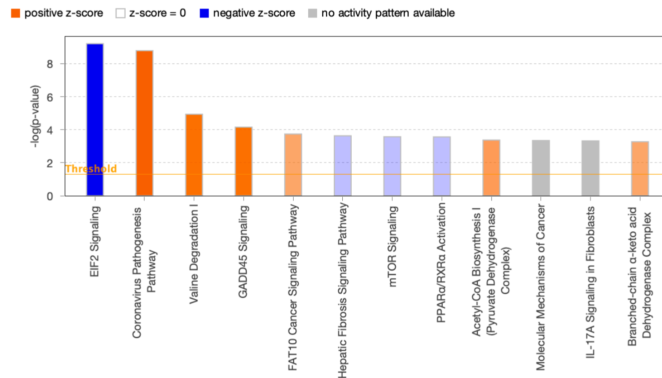
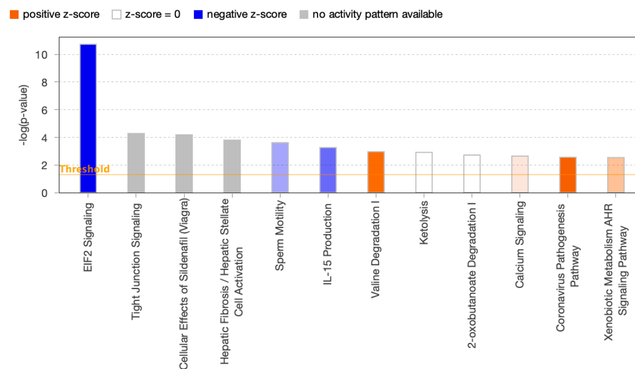


Figure 2.6. Bubble Chart from Ingenuity Pathway Analysis configured from bulk RNA-Seq A) Bubble chart of pathways inhibited or activated in socially-isolated vs group housed animals B) Bubble chart of pathways inhibited or activated in socially-isolated vs rehoused C) Bubble chart of pathways inhibited or activated in rehoused vs group housed animals (n=5 animals per experimental condition).

2.7A. **Socially-Isolated vs Group Housed**



2.7B. **Socially-Isolated vs Rehoused**



2.7C. **Rehoused vs Group Housed**

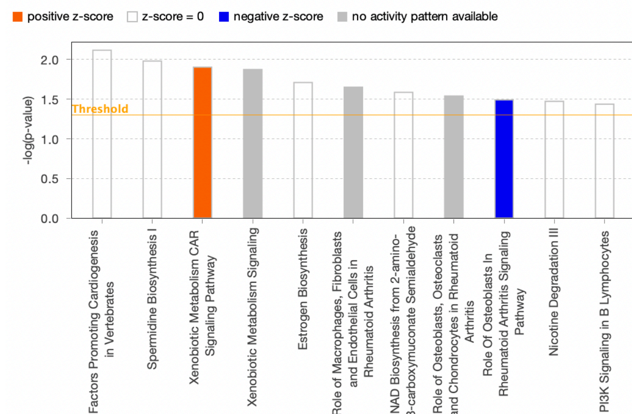


Figure 2.7. Canonical Pathways configured in Ingenuity Pathway Analysis from bulk RNA-Seq

A) Canonical pathways inhibited or activated in socially- isolated vs group housed animals B) Canonical pathways inhibited or activated in socially- isolated vs rehoused C) Canonical pathways inhibited or activated in rehoused vs group housed (n=5 animals per experimental condition)

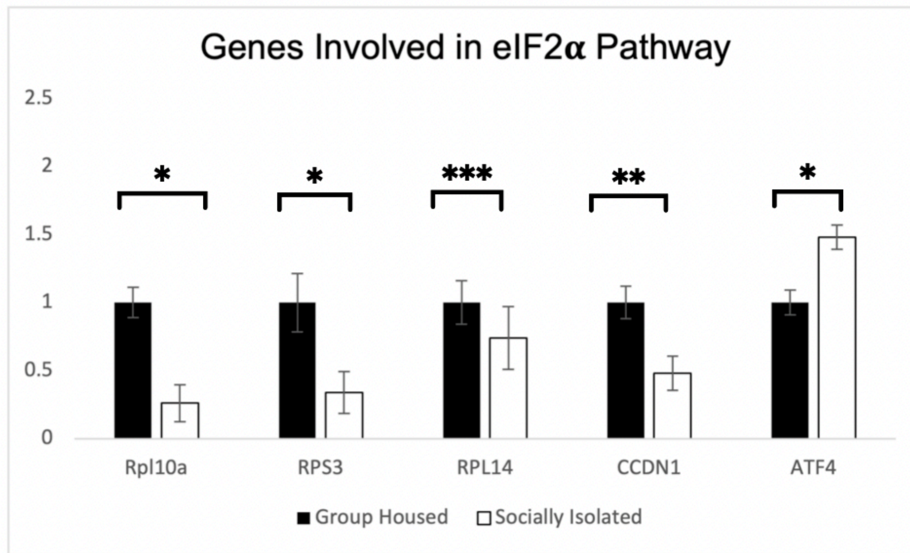
eIF2α , Coronavirus pathogenesis, Valine Degradation, and GADD45 signaling were the most significantly modulated (**Figure 2.7A**). In socially-isolated vs rehoused, the eIF2α, IL-15 production, and Tight junction pathways were the most significantly modulated (**Figure 2.7B**). As the eIF2α signaling pathway was the most significantly modulated pathway in the socially-isolated vs group housed and socially-isolated vs rehoused. The focal point of attention was directed towards the eukaryotic initiation factor 2 alpha (eIF2α) signaling pathway.

2.4.5: eIF2α Signaling Pathway

IPA determines "downregulation of a signaling pathway "as a readout of downregulation of the main genes involved in that canonical pathway. Majority of downregulated genes in the eIF2α signaling pathway between socially-isolated vs group housed and socially-isolated vs rehoused were ribosomal. To confirm findings from the RNA-seq and IPA qRT PCR was performed using primers of ribosomal RNA (Rp10a, RPS3, RPL14) and eIF2α targets (CCDN1, ATF4). As shown in IPA, ribosomal RNA (Rp10a, RPS3, RPL14) and eIF2α target (CCDN1) was significantly downregulated in socially-isolated vs group housed (**Figure 2.8A**) and socially-isolated vs rehoused (**Figure 2.8B**). eIF2α target ATF4 was significantly upregulated in socially-isolated vs group housed and socially-isolated vs rehoused. ($p<0.0005$, $p<0.005$, $p<0.05$)

While the IPA was able to provide a pathway to interrogate genes that were downregulated, it did not shed light on phosphorylation or post-transcriptional status. When eIF2α is phosphorylated, it inhibits the initiation of translation, leading to a general reduction in protein synthesis [182]. We were able to get a readout of downregulation of ribosomal genes (**Table 2.1**) however, we performed immunoblotting to determine phosphorylative state of eIF2α.

2.8A.



2.8B.

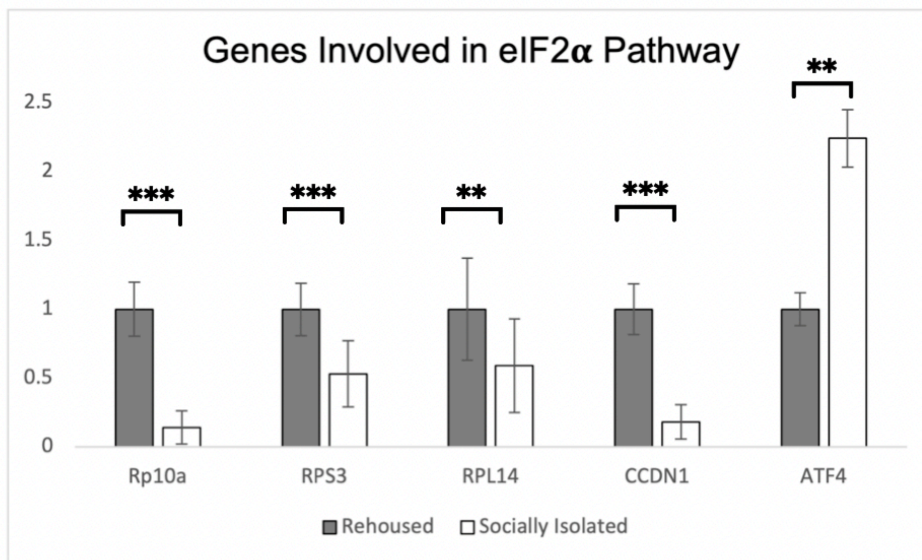


Figure 2.8. qRT PCR measurement of genes involved in the eIF2α signaling pathway in Socially-Isolated vs Group Housed and Socially-Isolated vs Rehoused RNA eluted from rat mammary gland A) Expression of RPl10a, RPS3, RPL14, CCND1, ATF4 normalized to housekeeping gene HRPT1 in socially –isolated vs group housed B) Expression of RPl10a, RPS3, RPL14, CCND1, ATF4 normalized to housekeeping gene HRPT1 in socially –isolated vs rehoused C) List of overlapping genes in the eIF2α signaling pathway in socially-isolated vs group housed and socially-isolated vs rehoused (***p<0.001, **p<0.01, *p<0.05) (n=10 animals per experimental condition)

Genes In EIF2α Signaling pathway	Gene Expression in Socially-Isolated vs Group Housed	Gene Expression in Socially-Isolated vs Rehoused
ACTA2	down	down
ACTG2	down	down
CCND1	down	down
CCND2	up	up
CCND3	down	down
RPL10A	down	down
RPL11	down	down
RPL12	down	down
RPL13	down	down
RPL14	down	down
RPL15	down	down
RPL17	down	down
RPL18	down	down
RPL21	down	down
RPL22	down	down
RPL23	down	down
RPL24	down	down
RPL26	down	down
RPL31	down	down
RPL35	down	down
RPL39	down	down
RPL4	down	down
RPL5	down	down
RPL6	down	down
RPL9	down	down
RPS10	down	down
RPS11	down	down
RPS12	down	down
RPS16	down	down
RPS19	down	down
RPS21	down	down
RPS23	down	down
RPS3	down	down
RPS4Y1	down	down
RPS6	down	down
RPS8	down	down
Rpl36a	down	down
MAPK1	up	up

Table 2.1. List of overlapping genes in the eIF2α signaling pathway. Compared in socially-isolated vs group housed and socially-isolated vs rehoused (***p<0.001, **p<0.01, *p<0.05) (n= 5 animals per experimental condition)

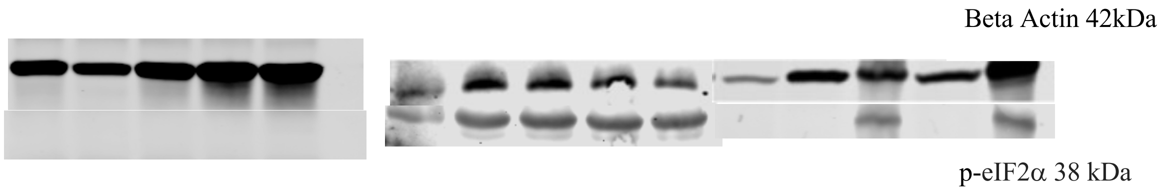
It was shown that eIF2α was phosphorylated in socially-isolated animals, but not in group housed or rehoused animals. Interestingly, there were two outliers on the rehoused immunoblot, going back to records these same bands correlate with the behavioral outliers confirmed from the hypervigilant testing (**Figure 2.2B**) (**Fig. 2.9A-C**). We took it a step further by immunoblotting for eIF2α, and found that general protein levels were similar between socially-isolated, group housed, and rehoused (**Figure 2.9D-F**). When looking at the proportion of protein levels for p-eIF2α/ eIF2α, it was significantly higher in socially-isolated compared to group housed or rehoused animals (**2.9G**).

2.5: Discussion

This study not only underlines mechanistic insights into how increased exposure to chronic psychological stress during puberty through early adulthood leads to delayed mammary gland development and differentiation by pinpointing the phosphorylation of the eIF2α pathway but shows the restorative effect of an environmental intervention at onset of late puberty to early adulthood. Upon the environmental intervention of rehousing, we observed a restoration of mammary gland development and differentiation, accompanied by a reduction in hypervigilant temperament (apart from two outliers) to baseline/control measures. These novel results emphasize the temporal importance of mammary developmental windows, highlighting the critical impact of social environmental exposures during late puberty through early adulthood.

Our laboratory has previously investigated the impact of increased exposure to chronic psychological stress from early puberty through early adulthood on mammary gland development and differentiation [158] .

2.9A. **Group Housed** 2.9B. **Socially-Isolated** 2.9C. **Rehoused**



2.9D. **Group Housed** 2.9E. **Socially-Isolated** 2.9F. **Rehoused**

2.9G.

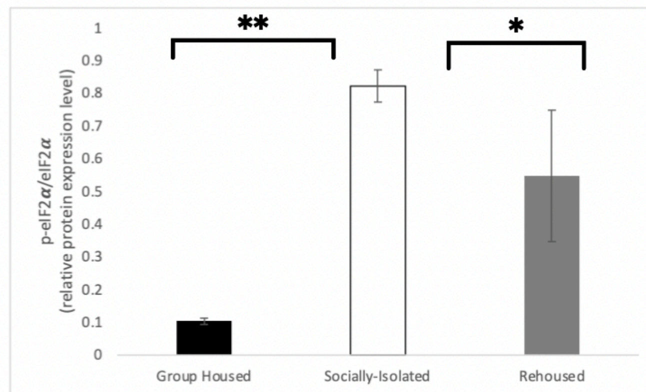


Figure 2.9. Immunoblot of homeostatic and phosphorylative levels of eIF2*α*. Protein derived from right pectoral mammary gland in all animals. A-C) Representative immunoblot showing group housed, socially-isolated, and rehoused lysates probed with antibodies targeting phospho-eIF2*α* and Beta Actin proteins on the same blot. D-F) Representative immunoblot showing group housed, socially-isolated, and rehoused lysates probed with antibodies targeting eIF2*α* and Beta Actin proteins on the same blot G) Proportion of relative p- eIF2*α* / eIF2*α* protein expression in group housed, socially-isolated, and rehoused animals **$p<0.01$ *$p<0.05$

These findings sparked interest, especially since earlier studies mainly focused on environmental insults during early puberty and their effects on terminal end buds (TEBs) [123]. Surprisingly, our research revealed that social isolation had no discernible effect on TEBs but significantly influenced mammary ductal extension and differentiation into adulthood [158].

Expanding on these observations, our current study underscores the significance of extending this developmental period through early adulthood. By reducing exposure to chronic psychological stress from late puberty at 13 weeks to early adulthood at 22 weeks, we observed restored mammary development and ductal differentiation.

To determine which pathways were altered an agnostic bulk RNA-sequence analysis was performed on rat mammary glands harvested at 22 weeks of age from animals housed in the three experimental conditions: group housed, socially-isolated, and rehoused. The analysis revealed the eIF2α signaling pathway as the most significantly modulated in both socially isolated vs group housed and socially isolated vs rehoused conditions. The modulation of this pathway served as a readout for the downstream regulation of major ribosomal genes and the upregulation of MAPK1. To validate and confirm these findings, the downregulation of specific genes (Rpl10a, RPS3, RPlL14, CCND1) was further corroborated through qRT-PCR. While RNA-seq provided valuable insight into a mechanism to target, it fell short of offering information about the active state of the mechanism, prompting us to take a more in-depth investigative approach.

The eIF2α signaling pathway holds significance due to its crucial role in cellular responses to various stress conditions. eIF2α, a component of the translation initiation machinery in eukaryotic cells, undergoes phosphorylation in response to cellular stress, thereby inhibiting translation initiation and reducing overall protein translation rates save for select transcript that

are translated to overcome the cellular stressor [210]. Although the RNA-seq results indicated reduced translation through the downregulation of ribosomal genes, we employed phospho-specific immunoblotting for p-eIF2α to determine potential covalent modifications that cannot be detected by mRNA analysis. Despite similar levels of eIF2α between socially-isolated, group-housed, and rehoused conditions, the increased phosphorylation of eIF2α upon social isolation strongly suggests that exposure to chronic psychological stress during early puberty through early adulthood leads to decreased protein translation, potentially contributing to the reduction in mammary gland development observed. Between the socially-isolated vs group housed and socially-isolated vs group housed, there was a significant decrease in Cyclin D1 (CCND1). In the developing mammary gland, CCND1 plays a key role through promoting mammary epithelial cell proliferation and ductal elongation and branching [211]. While the exact mechanisms linking p-eIF2α and CCND1 have not been clearly elucidated, literature has shown the link between ER stress and inhibited translation of CCDN1 [212]. As eIF2α is phosphorylated in response to cellular stresses like the unfolded protein response and ER stress, it is postulated that this activation leads to the subsequent decrease in CCDN1 expression. However, the exact mechanisms and upstream signaling pathways remain to be elucidated.

It's intriguing to highlight that eIF2α is typically viewed as an immediate response to acute stress, yet in our study, it serves as an adaptive response to a chronic psychological stressor persisting for eight weeks. This novel perspective adds depth to the understanding of the mechanism, particularly in its reversal by the environmental intervention of rehousing. Furthermore, the observation that the two behavioral outliers in the rehousing condition displayed phosphorylated eIF2α creates a direct link from hypervigilant temperament to a covalent modification. This underscores the intricate connections between the environment,

behavioral and psychological states, changes in gene expression, molecular pathways, and mammary development, illustrating the direct impact of one's social environment on mammary development.

Our findings carry significant implications for potential therapeutic interventions targeting the effects of chronic psychological stress during the pivotal developmental stages from early puberty through early adulthood. This research not only elucidates the molecular mechanisms that underlie stress-induced impacts on mammary development but also provides a data-driven foundation for addressing the needs of vulnerable human populations. Established evidence indicates that social isolation and exposure to chronic psychological stress can exert profound effects on mammary development, potentially heightening the risk of aggressive subtypes of mammary cancer later in life.

However, our study introduces a promising perspective by demonstrating that adverse outcomes associated with stress may be varied and potentially mitigated with appropriate interventions. This suggests that targeted interventions, whether environmental or eventually pharmacologic, during these critical developmental periods hold the potential to alter the trajectory of mammary cancer outcomes later in life. This presents a compelling avenue for future research and therapeutic exploration, offering hope for developing strategies that can positively influence mammary health and reduce the impact of chronic stress on cancer risk.

Chapter 3 : The Effect of GR Antagonist CORT125134 during Puberty and Early Adulthood on Mammary Gland Development

3.1: Abstract

Chronic psychological stress has emerged as a significant risk factor for breast cancer, prompting extensive investigation into its effects on disease development and progression. Epidemiological studies consistently demonstrate an elevated risk of breast cancer among individuals exposed to persistent stress. Utilizing the Sprague-Dawley rat model of social isolation, our lab has previously unveiled a compelling association between chronic psychological stress and the development of more aggressive spontaneous mammary tumors at an earlier stage compared to group-housed counterparts. This study focuses on elucidating the role of glucocorticoid receptor (GR) activation in mediating the effects of chronic stress on mammary development, particularly during puberty and early adulthood—a critical period of vulnerability. While GR activation has been implicated in breast cancer progression and metastasis, its influence on mammary development during puberty has remained underexplored. The central hypothesis of our investigation posits that heightened glucocorticoid reactivity and prolonged exposure to glucocorticoids impede mammary ductal development and differentiation. To test this hypothesis, socially-isolated rats were administered a competitive GR inhibitor, CORT125134, during puberty through early adulthood. This study sheds light on the intricate interplay between chronic stress, GR activation, and mammary development.

3.2: Introduction

Exposure to chronic psychological stress has been linked to an increased risk of breast cancer. Extensive epidemiological research has explored the impact of persistent stress on the development and progression of breast cancer, consistently revealing that women exposed to stress face an elevated risk of developing the disease [60]. Notably, everyday perceived stressors emerge as particularly potent stressors [61] . These everyday stressors encompass heightened levels of loneliness, hypervigilance, and environmental stressors related to socioeconomic factors and racial distinctions, including both micro and macroaggressions.

For Black women as a demographic group, these stressors are integral parts of their daily lives. Studies indicate that Black women exhibit the highest levels of evening cortisol compared to other racial and ethnic groups, coupled with a propensity to develop the most aggressive subtypes of breast cancer [213]. Consequently, the intricate interplay between exposure to chronic stress and the development of breast cancer has become a focal point of keen interest in research.

To simulate the impact of chronic psychological stress, our laboratory established the Sprague Dawley rat model of social isolation. Given the highly social nature of rats, akin to humans, where they engage in cohabitation and communal rearing, subjecting them to social isolation - even when they could still perceive their group-housed counterparts through sight, hearing, and smell - led to a diminished ability to cope with external stressors [151, 156]. This resulted in an elevated glucocorticoid stress response. Despite Sprague Dawley rats being genetically predisposed to spontaneous mammary tumors, those subjected to social isolation developed more malignant and aggressive mammary tumors at an earlier stage compared to their group-housed counterparts [151]. At the time, these findings were not only novel but also

striking, shedding light on the intricate interplay between social environment and mammary development using an animal model.

The hypothesis was formulated that these detrimental effects could be attributed to an increased glucocorticoid stress reactivity during the susceptible period of mammary development during puberty. In response to chronic stressors, the activation of the HPA axis leads to an elevated release of corticosterone (cortisol in humans), subsequently activating the glucocorticoid receptor pathway [138]. The glucocorticoid receptor (GR) serves as a nuclear transcription factor and plays a pivotal role in either transactivating or transrepressing key target genes. GR activation has been implicated in breast cancer progression and metastasis, with identified target genes like SGK1, MKP-1, and AP1 proven to promote breast cancer progression by inhibiting apoptosis [139, 145, 146]. While the promotion of breast cancer progression due to GR activation has been established, its impact on mammary development during puberty remained to be explored.

Crucially, the hypothesis asserts that GR activation exerts its greatest impact on mammary development during puberty and early adulthood, consequently increasing the risk of breast cancer. In developmental biology, puberty represents a recognized "window of susceptibility," marking a phase when the breast undergoes rapid development and becomes particularly vulnerable to environmental influences [70]. Employing the model of social isolation, our laboratory demonstrated that animals subjected to social isolation during puberty through early adulthood (5-22 weeks) exhibited diminished mammary development and differentiation [158]. Notably, elevated glucocorticoid reactivity became apparent at the onset of late puberty (13 weeks) and persisted through early adulthood. The delayed progress in mammary development and differentiation during this critical period becomes a potential risk

factor for mammary cancer in adulthood, with studies indicating that women at high risk for breast cancer often exhibit less differentiated ductal trees [24].

The focal point of the current study revolves around testing the hypothesis that heightened glucocorticoid reactivity and increased glucocorticoid exposure result in slowed mammary ductal development and differentiation through activation of GR. To investigate this, a competitive GR inhibitor (CORT125134) was administered to socially-isolated animals during puberty through early adulthood. Unlike mifepristone, CORT125134 does not cross-react with other nuclear receptors. This intervention aims to block glucocorticoid stress reactivity, with the anticipated outcome of restored mammary gland development and a subsequent decrease in mammary cancer risk later in life.

3.3: Material and Methods

3.3.1: Sprague-Dawley Rats

Female Sprague-Dawley Rats were bred at Charles River Laboratories (Kingston, NY). The animals were transitioned into all-female groups at three weeks of age and subsequently transported to our laboratory by the time they reached four weeks of age. Upon their arrival, they were initially housed in groups for 1 week to rectify the stress of transportation. When they reached 5 weeks of age, they were transferred to either individual housing (n=10) or group housing (n=5), after being carefully balanced for vigilant temperament as assessed during an open field test at 4.5 weeks of age (outlined below; ([155]).

All animals were accommodated in solid stainless-steel cages suspended on shared racks in the same colony room maintained at 21 ± 1 °C. The cages featured wire coverings on the front and

floor, situated above shared bedding pans (Harlan Teklad Aspen Sani-Chips, 7090A). The animals could see, hear, and smell their counterparts; however, social interaction was limited to group-housed animals. Group-housed animals were placed in a 46 × 61 × 36 cm cage, and socially-isolated animals in individual 20 × 24 × 18 cm cages. The animals followed a reversed light-dark cycle (14:10, with dark onset at 08:00 h), and all experimental procedures occurred during the rats' behavioral day. Food (Harlan Teklad Rodent Diet, 8604) and water were provided *ad libitum*. Routine handling during cage changes and health inspections adhered to the National Institutes of Health and University of Chicago Animal Care Guidelines.

3.3.2: Experimental Design

15 animals were placed into three experimental conditions to determine the effect of exposure to chronic psychological stress during puberty and early adulthood on mammary development. A ***pharmacologic intervention (CORT125134***) was employed to investigate the potential reversibility of these impacts resulting from social isolation during this time period. CORT125134 was shipped from Corcept Therapeutics, Menlo Park, CA, USA at a stock concentration of 250 mg. Prior to injections, compound was stored at room temperature with no exposure to sunlight.

Animal conditions were as followed: n = 5 group housed + vehicle (group housed animals from 5-22 weeks of age, administered vehicle (ethanol) from 14-22 weeks of age), n = 5 socially-isolated + vehicle (socially-isolated animals from 5-22 weeks of age, administered vehicle (ethanol) from 14-22 weeks of age), n = 5 socially-isolated + CORT125134 (socially isolated animals from 5-22 weeks of age, administered CORT125134 from 14-22 weeks of age). Sacrifice of animals occurred during early adulthood (22 weeks)(**Figure 3.1**).

3.1.

Reproductive Lifespan Events: **Mammary, Adrenal, and Ovarian**		**<u>Weaning</u>** Prepubertal Temperament	**<u>Early and Mid-Puberty</u>** - Terminal End Buds (TEBs) differentiate to Terminal Ducts and Alveolar Buds - Hypothalamic-pituitary-ovarian axis is matured	**<u>Late Puberty and Early Adulthood</u>** - Ducts differentiate - Duct tree lengthens and expands - Mammary stem/progenitor cell population drops
Age (weeks)		**3-4**	**5-12**	**13-22**
A	**G➡ G**			
B	**SI➡ SI**			
C	**SI➡~~GR~~**			~~GR~~
Social Environment:		**Grouped (grey)**	**Socially-Isolated (white)**	

Figure 3.1. Schematic of experimental design and timeline. 15 animals were placed into three social conditions to determine the effect of social isolation during puberty and early adulthood on mammary development. A ***pharmacologic intervention (CORT125134)*** was employed to investigate the potential reversibility of the impacts resulting from social isolation during this time period. Animal conditions were as followed: A) n = 5 group housed (group housed animals from 5-22 weeks of age, administered vehicle (ethanol) from 14-22 weeks of age), B) n = 5 socially-isolated (socially-isolated animals from 5-22 weeks of age, administered vehicle (ethanol) from 14-22 weeks of age), C) n = 5 socially-isolated + GR Modulator (socially isolated animals from 5-22 weeks of age, administered CORT 125134 from 14-22 weeks of age). Sacrifice of animals occurred during early adulthood (22 weeks). Experiment was performed two independent times (30 animals total, 10 per each experimental condition).

Stock concentration of CORT125134 was 200mg/ml in ethanol stored at -20°C. Administration of room temperature vehicle (ethanol) or CORT125134 was through subcutaneous injections performed three times a week during the rats' behavioral day (Monday, Wednesday, and Friday). Dosing concentration of CORT125134 and vehicle (ethanol) was 30 mg/kg dissolved in sesame oil, and weight was measured weekly. Experiment was performed two independent times (30 animals total, 10 per each experimental condition).

3.3.3: MG Whole Mount Preparation and Analyses

Upon sacrifice the right inguinal mammary gland of the animal is excised, applied onto a glass slide and fixed with Carnoy's fixative. Once the mammary gland is fixed, xylene is applied to remove the lipid layer and carmine alum is used for staining. Once the staining is dried, an image of the whole mount slide is acquired through a 3600 dpi flatbed scanner [158]. 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 mammary gland ductal tree. Distal MG ductal area was the area between a horizontal line transecting the LN and the traced perimeter of the mammary gland ductal tree. A minimum of 10 animals in each experimental condition were used for statistical comparison of mammary gland ductal extension and ductal area.

3.3.4: Quantifying Mammary Progenitor/Stem Cells

Mammary Cell Isolation: Mammary glands in the left inguinal quadrant were excised using nipple 12 as an anatomical marker. Upon excision, mammary gland was minced into a paste and placed in serum-free media supplemented with 1% penicillin/streptomycin (P/S), 1% bovine serum albumin (BSA), and 1% of Type 1 collagenase for 15 hours at 37 degrees [159]. The

collagenase digestion separates individual mammary cells (mammary stem/progenitor, epithelial, endothelial, hematopoietic, and immune cell) from the adipocyte fraction. Upon overnight digestion, the cell pellet was formed through centrifugation and removal of the adipocyte fraction. The pellet of cells was treated with NH4Cl to lyse blood cells and DNase/dispase to prevent cell clumping prior mammary progenitor/stem cell quantification.

Mammary Progenitor/Stem Cell Quantification: The mammary cell suspension was blocked with unconjugated Fc antibody (BD Biosciences 550271) and dyed with Live/Dead solution (catalog number) to distinguish cell viability. Subsequently, the mammary cell suspension was stained with anti-CD31 ((Abcam 33858), anti-CD45 (Abcam 33916), anti-CD24 (BD Biosciences 56210), and anti-CD29 (BioLegend 02225). The mammary cell suspension was then analyzed and quantified through flow cytometry (LSR Fortessa 4-15) . The CD31+CD45+ hematopoietic and endothelial cells were excluded through gating. Thus, we were able to detect CD24+CD29+ amongst the population of cells which are highly enriched for undifferentiated normal mammary stem/progenitor cells (Velten et al. 2020). The quantification of mammary progenitor/stem cells was reported as proportion of CD24+CD29+/CD31-CD45- (Lin-) cells. The undifferentiated normal mammary stem/progenitor cells (CD24+CD29+/Lin-) were further sorted based on CD24+CD29+ levels into basal and luminal progenitor stem/cells (basal= CD24+CD29hi and luminal= CD24+CD29lo). A minimum of 10 animals in each experimental condition were used for statistical comparison of proportion of CD24+CD29+/CD31-CD45- (Lin-) cells.

3.3.5: RNA Sequence Analysis

Total RNA was extracted from the right pectoral whole mammary gland of the animal using E.Z.N.A Total RNA Kit II (Omega bio-tek), and messenger RNAs were subsequently eluted. A nanodrop (Thermo Scientific NanoDrop 2000C) was used to determine RNA concentration and threshold of 260/280 ratio ranged from 2.0-2.05. RNA samples with a volume of 13 ul and concentration of 100 ng/ul from all experimental conditions (n=15) were submitted to the genomics core at the University of Chicago for RNA-seq analysis. A library was created using NovaSeq with 30M clusters/sample and 60M PE reads/sample. Samples were run twice on a flow cell in the HiSeq 4000 system. Downstream analysis was performed on the web based BaseSpace-Sequence Hub (Illumina) platform. While read alignment/mapping was done with the RNA-seq Alignment app (Illumina).

Gene expression data was expressed in fold change between experimental conditions, (socially-isolated vs group housed, socially-isolated vs rehoused, rehoused vs group-housed). Differential expression analysis was performed using a two-tailed t test. Unequal variances were accounted for each expressed gene between the three groups resulting in ordered ranked p-values. Genes that had a lower p value than 0.05 were used for further analysis.

3.3.6: Ingenuity Pathway Analysis

Gene lists of significantly differentially expressed genes between the experimental conditions socially-isolated + CORT125134 vs socially-isolated + vehicle, socially-isolated+CORT125134 vs socially-isolated (without vehicle i.e. from previous cohort). Using this software, gene lists

were narrowed to determine differential canonical pathways, diseases and functions, signal pathway interactions, and genes and networks of upstream regulators.

3.3.7: Statistical Analysis

Statistical analyses were conducted GraphPad Prism Software 10 (GraphPad Software, Inc., La Jolla, CA ($P > 0.05$, two-tailed tests), all means ± SEM)

3.4: Results

3.4.1: CORT 125134 Administration Results in a Steady Weight decline, Compared to Group Housed or Socially-Isolated Counterparts

Animal conditions were as followed: n = 5 group housed + vehicle (group housed animals from 5-22 weeks of age, administered vehicle (ethanol/sesame oil) from 14-22 weeks of age), n = 5 socially-isolated + vehicle (socially-isolated animals from 5-22 weeks of age, administered vehicle from 14-22 weeks of age), n = 5 socially-isolated + CORT125134 (socially isolated animals from 5-22 weeks of age, administered CORT 125134 from 14-22 weeks of age). Sacrifice of animals occurred during early adulthood (22 weeks). CORT125134 and vehicle were administered through subcutaneous injections three times a week, spanning from 14 to 22 weeks of age. The concentration of CORT125134 and vehicle was maintained at 30mg/kg prior to the initiation of injections, at the 13-week timepoint, animals across group-housed, socially-isolated, and socially isolated (pre-CORT125134) conditions exhibited comparable weights. The average weight for group-housed and socially isolated (pre-CORT125134) animals was 302 grams, while socially-isolated animals weighed slightly less at 297 grams. Over the course of 8 weeks, group-housed and socially-isolated animals receiving the vehicle (ethanol) displayed a

consistent weight gain reaching an average of 335 and 339 grams, respectively at the time of sacrifice (22 weeks).

In contrast, socially isolated animals treated with CORT125134 exhibited a distinct weight trajectory. Upon the first week of CORT125134 injections, these animals experienced a slight drop in weight, reaching 295 grams by end of week 14. Over the subsequent 8 weeks, there was no significant weight gain observed, with these animals averaging 296 grams at the time of sacrifice (22 weeks), significantly less than the other two experimental arms (**Figure 3.2**).

3.4.2: CORT125134 Further Slows Mammary Ductal Development

In the field of developmental biology, mammary development during late puberty and early adulthood is a readout of intricate hormonal signaling pathways and genetic regulation that orchestrate the maturation of mammary glands [82]. Using the right inguinal mammary gland, maximal ductal extension and distal ductal area was measured at the time of sacrifice at 22 weeks.

Ductal Extension. Maximal 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 mammary gland ductal tree at 22 weeks of age (**Figure 3.3A-C**, yellow line; extension quantitated in **Figure 3.3D**.) Interestingly, group housed and socially-isolated animals who received vehicle injection (14-22 weeks) displayed similar ductal extension measurements at 39 mm and 38 mm, respectively (p=n.s.) Interestingly, socially isolated animals receiving CORT125134 from (14-

3.2.

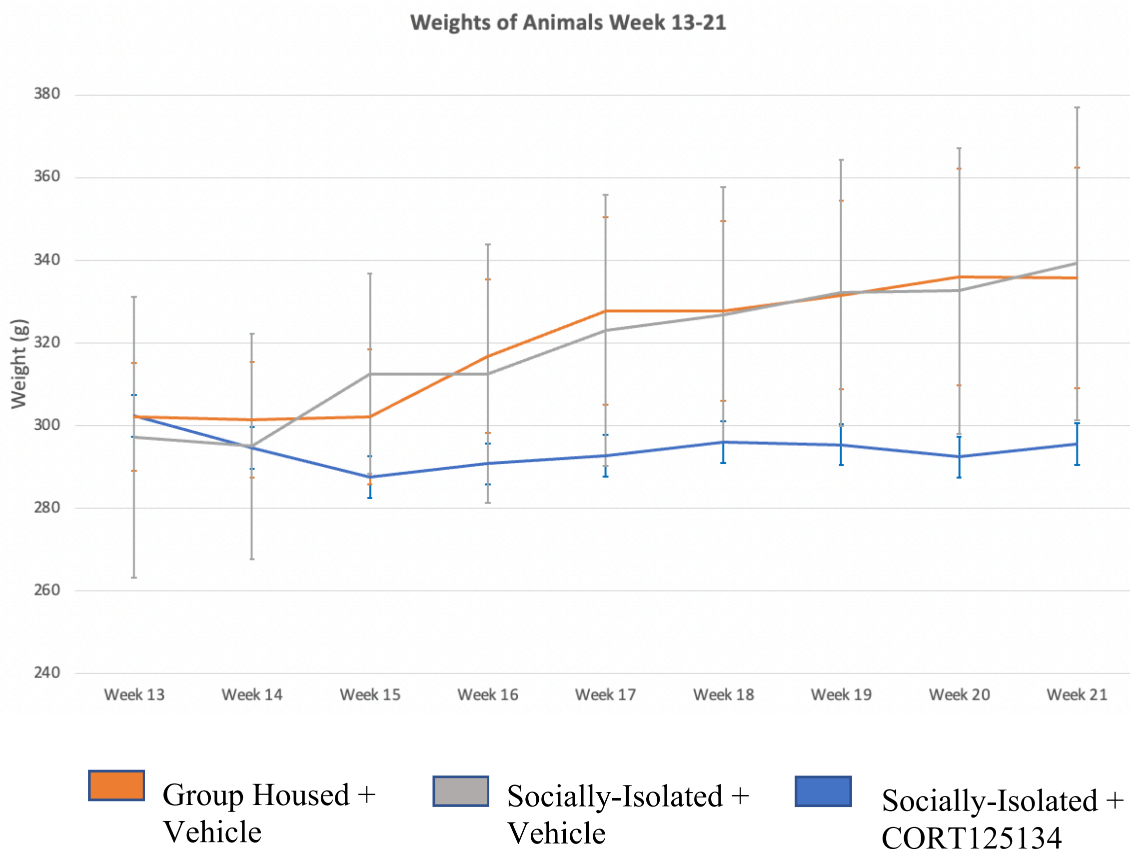
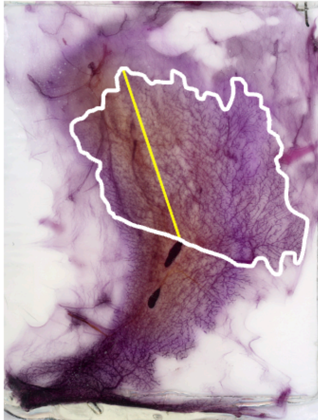
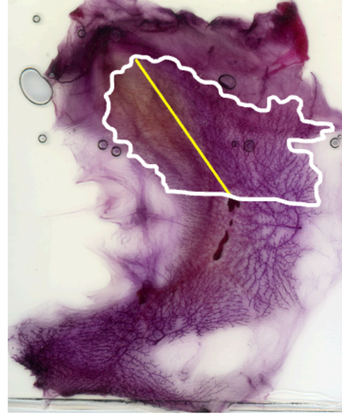


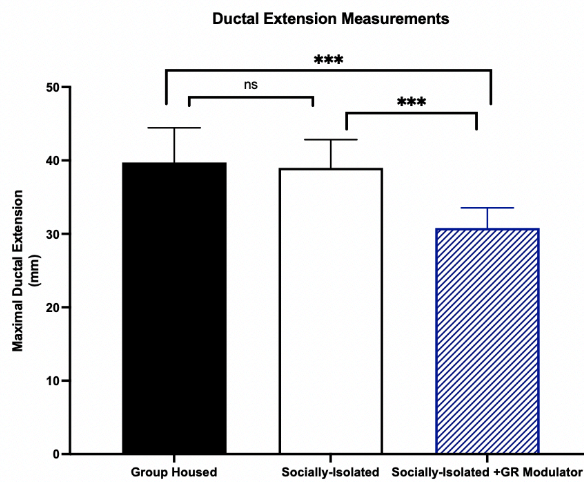
Figure 3.2. Chart of weight distribution of animals from all experimental conditions. Weights were measured at the start of each injection week. Shown at 13 weeks, are the weights of animals from all experimental conditions a week prior to subcutaneous injections. Weeks 14-21 are the weights of animals after vehicle or CORT125134 has been administered. n=10 animals per experimental condition

3.3A. 3.3B. 3.3C.





3.3D. 3.3E.



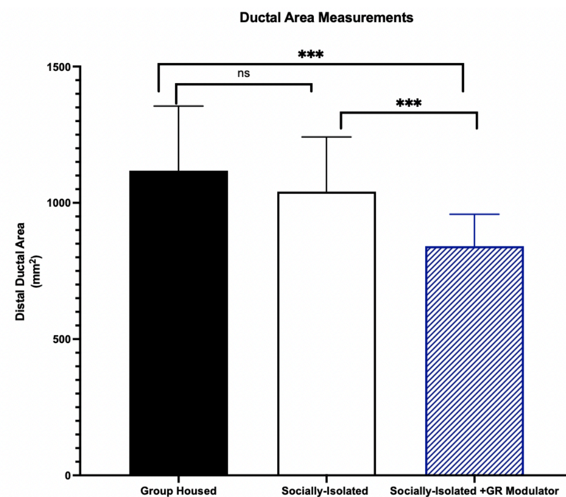


Figure 3.3. Effect of experimental condition on mammary development measured by maximal ductal extension and distal ductal area A-C) Representative images of carmine alum staining of the whole mount derived from right inguinal mammary gland. Ductal extension was determined as distance from lymph node (LN) to furthest terminal end bud (yellow line). Ductal area was determined by the area between the horizontal line transecting the LN and the traced perimeter of the mammary gland ductal tree. A) Group housed (group housed animals from 5-22 weeks of age, administered vehicle (ethanol) from 14-22 weeks of age) B) Socially-isolated (socially-isolated animals from 5-22 weeks of age, administered vehicle (ethanol) from 14-22 weeks of age) C) Socially-isolated + GR Modulator (socially isolated animals from 5-22 weeks of age, administered CORT 125134 from 14-22 weeks of age) D) Quantification of the effect of experimental condition on mammary ductal extension (mm) E) Quantification of the effect of experimental condition on mammary ductal area (mm^2) (All measures were performed blinded by two independent observers). n.s. $p>0.05$ ***$p<0.001$. (n=10 animals per experimental condition)

22 weeks) displayed significantly reduced mammary ductal extension averaging 30 mm ($p < 0.001$, $p<0.001$).

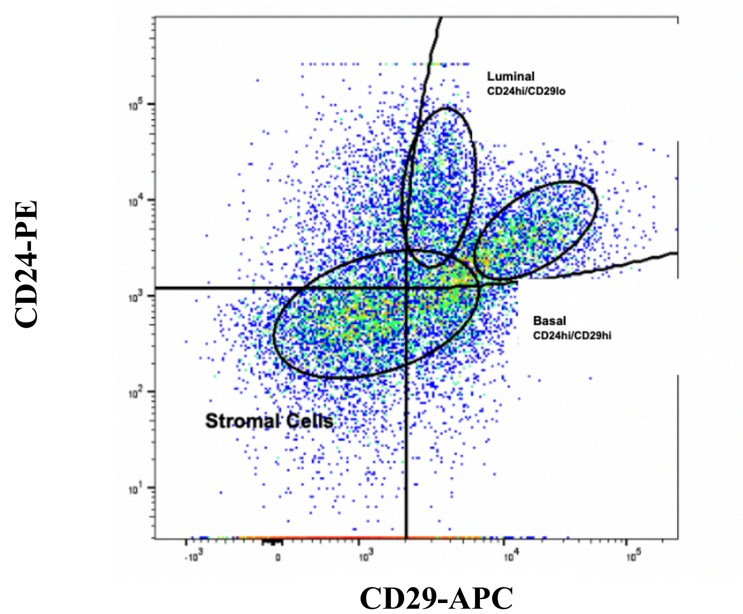
Ductal Area. Distal ductal area was measured as the area between a horizontal line transecting the LN and the traced perimeter of the mammary gland ductal tree (**Figure 3.3A-C,** white outline quantitated in **Figure 3.3E**.) Group housed and socially-isolated animals who received vehicle injection (14-22 weeks) displayed similar distal ductal areas of 1118 mm^2 and 1041 mm^2 , respectively (p=n.s.) Interestingly, socially isolated animals receiving CORT125134 from (14-22 weeks) displayed significantly reduced distal ductal area at 841 mm^2 ($p<0.001$, $p<0.001$).

3.4.3: CORT125134 Administration Results in a Slight Increase in Proportion of Mammary Progenitor/Stem Cells

In the rat mammary gland, the process of mammary ductal differentiation and a decline in the mammary stem/progenitor cell population are well-documented phenomena during early adulthood [208]. Consequently, the proportion of mammary stem/progenitor cells serves as a valuable indicator of mammary differentiation. The hypothesis is the lesser the proportion of mammary stem/progenitor cells during the critical period from early puberty through early adulthood the more developed the mammary gland will be.

Using flow cytometry, luminal progenitor ($CD24^{+}CD429^{lo}$) and basal progenitor ($CD24^{+}CD29^{hi}$) cell populations (**Figure 3.4A**) were identified. Showing this clear distinction to substantiate the identification of the mammary stem/progenitor population, the population of stem/progenitor cells was measured as a proportion $CD24^{+}CD29^{+}$ undifferentiated normal mammary stem/progenitor cells divided by CD31-CD45- hematopoietic and endothelial cells

3.4A.



3.4B.

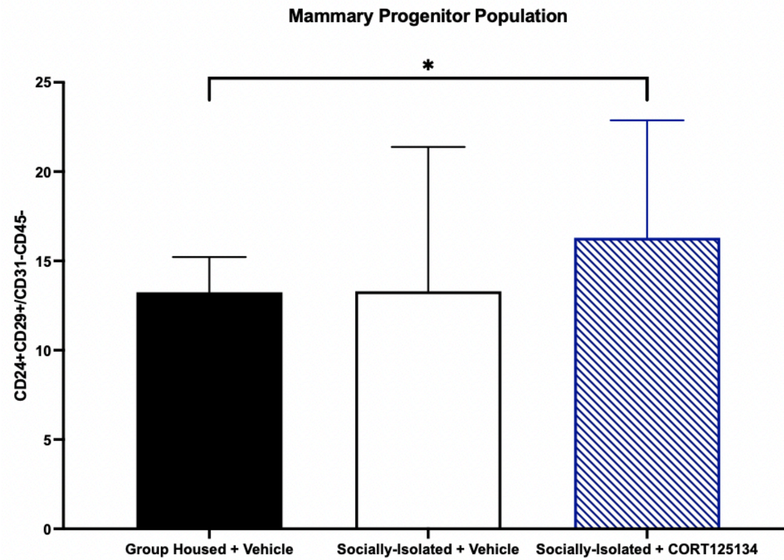


Figure 3.4. Effect of experimental condition on mammary stem/progenitor proportion A) Representative flow cytometry of mammary progenitor ($CD24^{+}CD29^{+}$) further separated as $CD24^{+}CD429^{lo}$ (luminal progenitor) and $CD24^{+}CD29^{hi}$ (basal progenitor) cells in the $CD45^{-}CD31^{-}$ mammary-gland cell population B) The proportion of total CD24+CD29+ progenitor cells relative to $CD45^{-}CD31^{-}$ cells. (n=10 group housed + vehicle, n=10 socially-isolated + vehicle, n=10 socially-isolated + CORT125134)

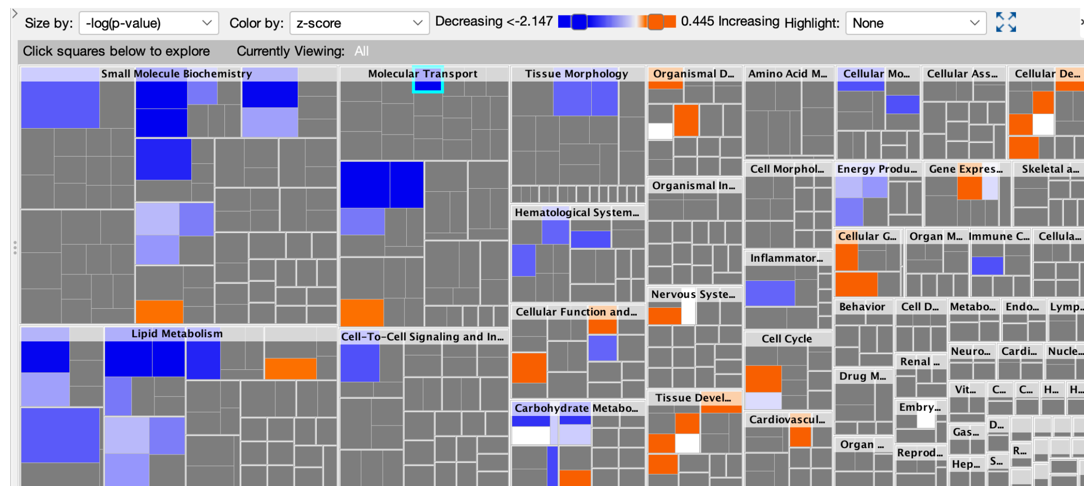
(Lin-)(**Figure 3.4B**). Interestingly, group housed + vehicle animals and socially-isolated + vehicle had similar proportion of mammary stem/progenitor cells. This aligns with the similarity observed in ductal extension and distal ductal area measurements. Socially-isolated + CORT125134 animals exhibited a significantly greater proportion of mammary stem/progenitor cells ($p<0.05$).

3.4.4: CORT125134 Decreases Gene Expression of Pathways involved in Metabolism

To determine the mechanistic basis of the mixed agonist/agonistic effects of CORT125134 in rat mammary glands, a comprehensive RNA-sequence analysis using RNA eluted from the right pectoral mammary gland in all experimental conditions, group housed + vehicle, socially-isolated + vehicle, and socially-isolated + CORT125134. The RNA-sequence analysis generated significant ($p<0.05$) differentially expressed genes in the conditions: socially-isolated + CORT12134 vs socially-isolated + vehicle and group housed + vehicle vs socially-isolated + vehicle. These differentially expressed gene sets were input into an Ingenuity Pathway Analysis (IPA) to determine differential canonical pathways, diseases and functions, signal pathway interactions, and genes and networks of upstream regulators [209].

The IPA software showed of slew of modulations changing in response to the vehicle and CORT125134 injections. When comparing socially-isolated + CORT125134 to socially-isolated + vehicle, there was a significant reduction in expression of genes involved in lipid metabolism, inflammatory response, and energy production (**Figure 3.5A**). There was a concurrent upregulation of PPARα/RXRα receptor pathways, which reduces the levels of circulating triglycerides and free fatty acids (**Figure 3.6A**). To further hone in on pathways of interest, IPA

3.5A Socially-Isolated + CORT125134 vs Socially-Isolated + Vehicle



3.5B Group Housed + Vehicle vs Socially-Isolated + Vehicle

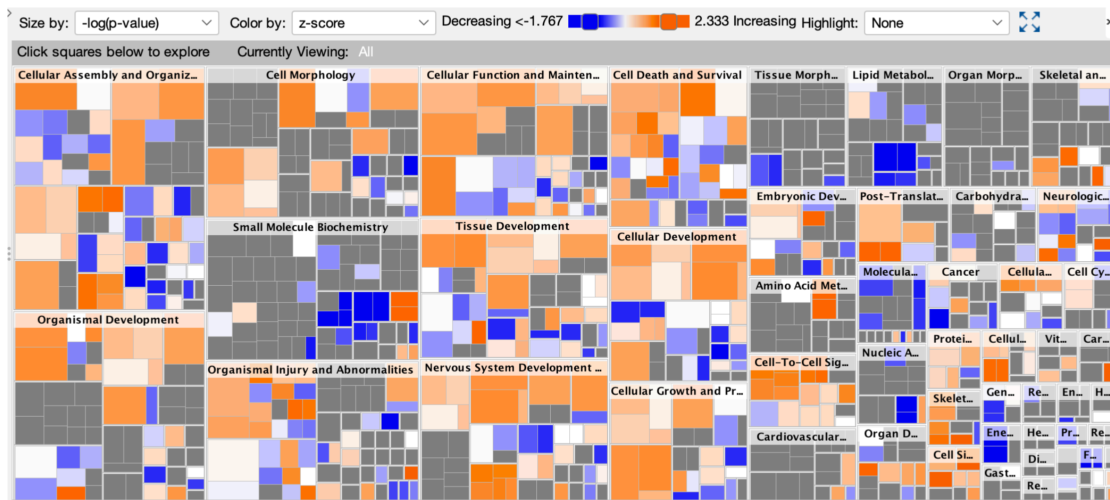
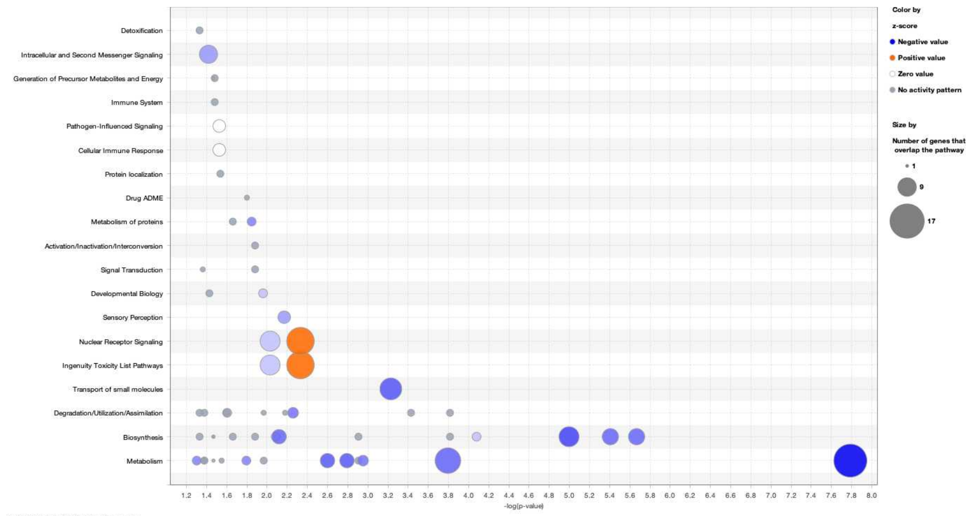


Figure 3.5. Diseases and Functions from Ingenuity Pathway Analysis configured from bulk RNA-Seq A) Diseases and Functions inhibited or activated in socially-isolated + CORT125134 vs socially-isolated + vehicle B) Diseases and Functions inhibited or activated in group housed + vehicle vs socially-isolated + vehicle (n=5 animals per experimental condition).

3.6A **Socially-Isolated + CORT125134 vs Socially-Isolated + Vehicle**



3.6B **Group Housed + Vehicle vs Socially-Isolated + Vehicle**

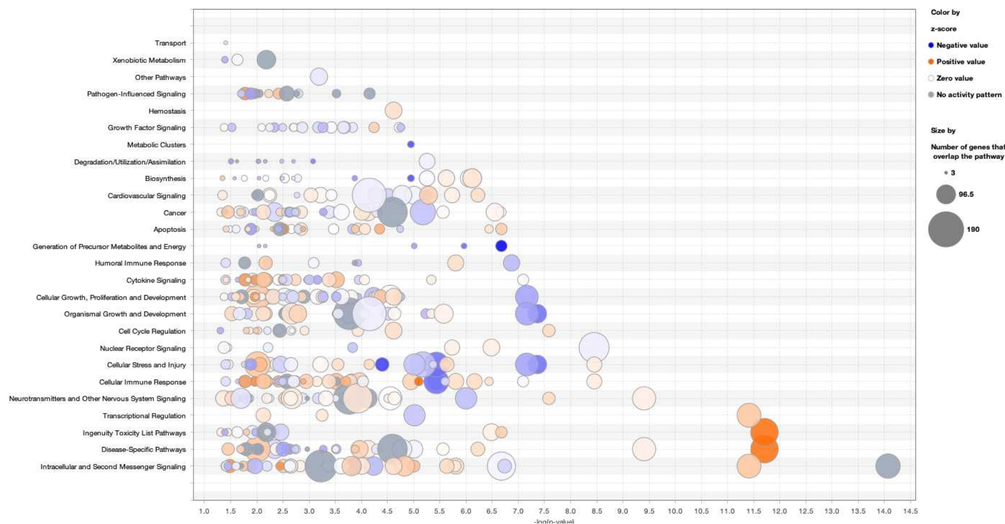
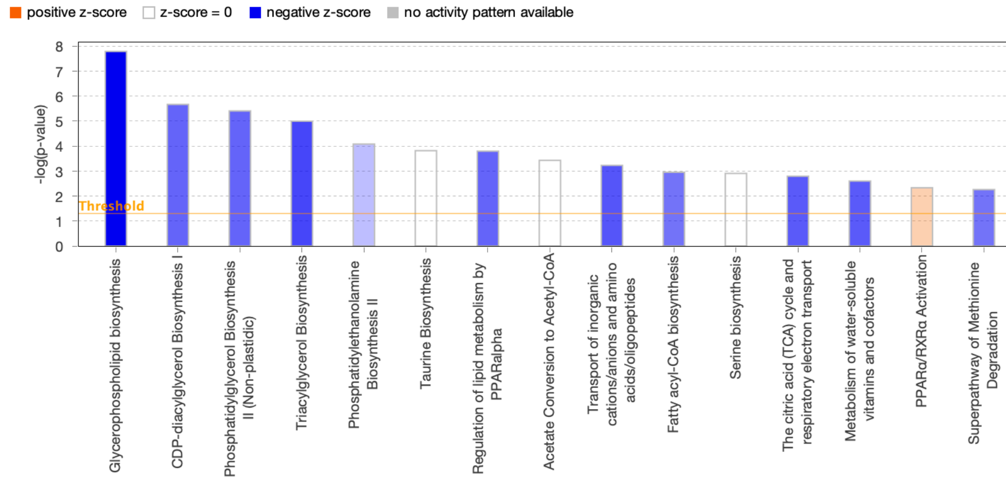


Figure 3.6. Bubble Chart from Ingenuity Pathway Analysis configured from bulk RNA-Seq A) Bubble chart of pathways inhibited or activated in socially-isolated + CORT125134 vs socially-isolated + vehicle B) Bubble chart of pathways inhibited or activated in group housed + vehicle vs socially-isolated + vehicle (n=5 animals per experimental condition).

3.7A **Socially-Isolated + CORT125134 vs Socially-Isolated + Vehicle**



3.7B **Group Housed + Vehicle vs Socially-Isolated vs Vehicle**

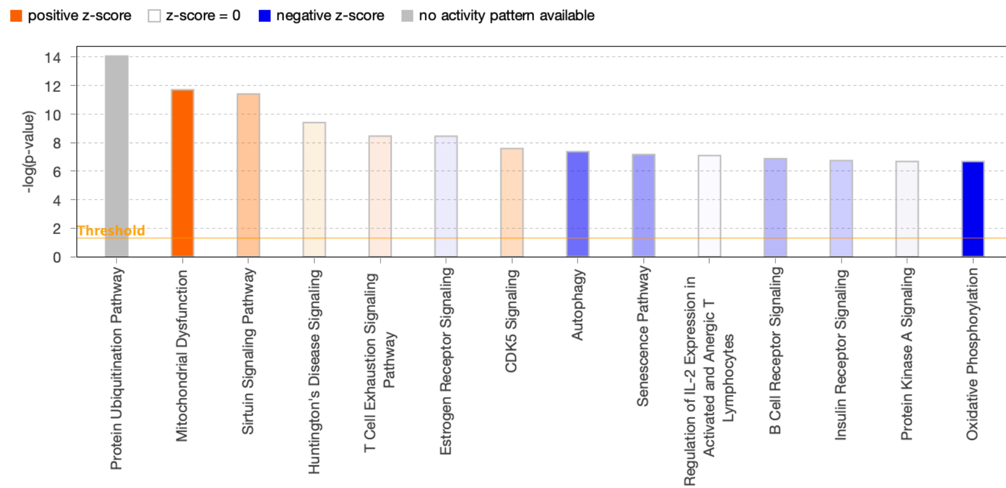


Figure 3.7. Canonical Pathways configured in Ingenuity Pathway Analysis from bulk RNA-Seq
A) Canonical pathways inhibited or activated in socially-isolated + CORT125134 vs socially-isolated + vehicle B) Canonical pathways inhibited or activated in group housed + vehicle vs socially- isolated + vehicle (n=5 animals per experimental condition)

generated canonical pathways of most and greatest significance being glycerophospholipid biosynthesis and triacylglycerol biosynthesis (**Figure 3.7A**). The observed downregulation suggests antagonist effects of CORT125134, as previous research has demonstrated increased glucocorticoid exposure in socially-isolated animals result in upregulation of pathways involved in metabolism and fatty acid synthesis [159]. However, when CORT125134 is administered it results in the downregulation of metabolism pathways involved in lipogenesis and fatty acid synthesis.

When comparing group housed + vehicle to socially-isolated + vehicle, there was a significant reduction in expression of genes involved in lipid metabolism and increased expression of genes involved in tissue development, nervous system development, and cell function and maintenance (**Figure 3.5B and 3.6B**). To hone in on pathways of interest, IPA generated mitochondrial dysfunction and sirtuin signaling as the canonical pathways that were upregulated in the group housed+ vehicle, and pathways involved in estrogen receptor signaling, insulin receptor signaling, and oxidative phosphorylation were downregulated **(Figure 3.7B)**

It is intriguing to observe the significant contrast in the genes and pathways affected between socially-isolated and group-housed animals that did not receive any injections (previous cohorts; data shown in Chapter 2), compared to socially-isolated and group-housed animals that received vehicle injections. When comparing the top 10 canonical pathways affected between both groups (socially-isolated vs group housed no injections and socially-isolated vs group housed vehicle injections) there was no overlap. Thus, we can conclude the vehicle injections as a confounding factor, as the findings lack commonality with previous reports (data shown in Chapter 2). We postulate that vehicle injections administered three times a week for 8 weeks,

potentiates an additional stressor for the animals. Suggestions for mitigating the effects of vehicle injections and additional experimental arms will be outlined in the Discussion.

3.5: Discussion

In our study, we utilized a presumed GR antagonist, CORT125134, to explore the impact of reduced glucocorticoid exposure on mammary gland development. Our initial hypothesis stemmed from previous research indicating that heightened glucocorticoid reactivity and increased exposure to glucocorticoids may impede mammary ductal development and differentiation. This hypothesis was supported by the expression of GR in the mammary gland epithelium, and observations that socially-isolated animals exhibit heightened glucocorticoid reactivity and diminished mammary development [158]. Consequently, we postulated that blocking glucocorticoid reactivity via the antagonist would facilitate the restoration of mammary ductal development and differentiation.

However, our findings revealed unexpected complexities regarding the effects of CORT125134. Through measurements of mammary development including ductal extension, distal ductal area, mammary stem/progenitor proportion, and gene expression, we observed mixed agonistic and antagonistic effects of the compound. Notably, socially-isolated animals treated with CORT125134 exhibited the most delayed ductal development and differentiation, alongside a higher proportion of mammary stem/progenitor cells compared to group housed + vehicle and socially-isolated + vehicle.

The decline in weight and lack of subsequent weight gain in treated animals suggest ingestion of the drug. This is consistent with previous studies demonstrating that GR antagonists can prevent and reverse weight gain in various models (private communication from Corcept).

Moreover, our study aligns with prior research indicating that selective GR antagonists exhibit tissue-specific effects. For instance, these compounds have shown beneficial effects in metabolic disease models by activating brown adipose tissue [214]. In our model, CORT125134 partially mirrored these effects by significantly decreasing pathways related to lipogenesis, fatty acid synthesis, and insulin receptor signaling, akin to the actions of CORT125281 in reducing corticosterone-induced hyperinsulinemia [215] .

Selective GR antagonists like CORT125134 have been shown to have no effect on HPA activity and stress-induced corticosterone levels. For instance, a study demonstrated that mice treated with CORT125281 and untreated mice displayed similar corticosterone levels following a novelty stressor [215]. Our findings, indicating delayed mammary ductal development and differentiation in socially isolated animals administered with CORT125134, suggest that the antagonist may have no effect on HPA activity. However, the effects of the drug appear to be tissue-dependent and do not substantially alter the impact of chronic psychological exposure during late puberty and early adulthood on mammary gland development.

We hypothesized that inhibiting glucocorticoid receptor (GR) activity with an antagonist could mimic the effects of rehousing, an environmental intervention. Rehousing socially-isolated animals from late puberty to early adulthood (14-22 weeks) led to significant outcomes, including the restoration of mammary gland development and differentiation, as well as a decrease in the proportion of mammary stem/progenitor cells. However, administering CORT125134 to socially-isolated animals from 14-22 weeks did not block or diminish the effects of increased exposure to chronic psychological stress during this period. These results offer two possible interpretations.

Firstly, in the mammary gland, CORT125134 may act as a mixed agonist/antagonist. Despite previous characterization of CORT125134, prior experimentation did not explore its effects in the mammary gland. As noted, selective GR antagonists exhibit tissue-specific effects [214]. Given the heterogeneities of the mammary gland, it is conceivable that CORT125134 may exert mixed antagonist and agonist effects, as evidenced by impeded mammary development and differentiation.

The second interpretation involves the possibility that CORT125134, if acting as a pure antagonist, contributes to a U-shaped effect on GR activity and subsequent mammary development. GR's role in the developing mammary gland has been implicated; for example, studies using Cre-LoxP models with deletion of the GR gene underscored its essential role in cell proliferation during lobuloalveolar development [113] . Thus, it is conceivable that GR activity exhibits a U-shaped effect on mammary gland development. It could be postulated that heightened GR activity due to social isolation impedes mammary development, while blocked GR activity similarly hinders it. Conversely, physiologic levels of GR activity, as observed in group housing, may result in more developed and differentiated mammary glands.

In addition to CORT125134 displaying interesting results, the group housed + vehicle and socially-isolated + vehicle groups exhibited similar mammary development measures, including ductal extension, distal ductal area, and mammary stem/progenitor proportion. This contrasts with previous findings showing a significant reduction in mammary development and differentiation in socially-isolated animals compared to group housed animals. Additionally, gene expression and canonical pathways modulated between socially-isolated vs group housed administered vehicle injections compared to socially-isolated vs group housed no injections (data shown in Chapter 2) is markedly contrast. Based upon these findings, we hypothesize that the

subcutaneous injections administered three times a week may act as a stressor. Previous evidence has shown that repeated animal handling via subcutaneous injections can induce stress due to the physical handling and discomfort associated with the injection process [216-219]. For instance, one study demonstrated that physical restraint and injections increased corticosterone and plasma amphetamine levels by 50% compared to animals not handled [216]. In another study, similar results were observed, where rats that received subcutaneous saline injections showed a fourfold increase in serum corticosterone levels compared to rats that were not handled [219]. The impact of subcutaneous injections indicates a stress response affecting behavior, physiology, and overall experimental outcomes.

To mitigate the effects of injections and obtain a true assessment of the impact of CORT124134 on mammary development, we propose some alterations to the experiment. Firstly, would be to add more arms to the experimental design. These arms would include group housed and socially isolated conditions without vehicle subcutaneous injections, as well as group housed + CORT125135, and group housed + dexamethasone. Such additions would help mitigate confounding factors such as the impact of stress on vehicle injections. Furthermore, the use of a GR antagonist (CORT125134) and a GR agonist (dexamethasone) in group housed conditions could elucidate which effects of social isolation are GR-mediated. For instance, in the group housed + dexamethasone condition, we would hypothesize a mammary developmental phenotype similar to that of socially-isolated animals.

To mitigate the stress of injections in animals, alternative methods to administer drug (CORT125134) should be explored. For example, delivering CORT125134 in the animal's chow. A study looking at the effects of glimepiride (antidiabetic medication) on glucose tolerance in mice, administered glimepiride in chow [220]. They observed that chow

administration not only minimized stress, but also its route of drug absorption was most akin to humans ingesting a pill. It is important to note that as rats eat multiple times a day, dosage would be spread out over a longer period versus a single bolus.

In summary, these findings offer intriguing insights into the use of selective GR antagonists and suggest preferred methods of drug administration. This study also serves as a catalyst for exploring additional pathways and mechanisms by which increased exposure to chronic psychological stress results in decreased mammary development. As the rehousing study revealed the eIF2α pathway as the most significantly modulated in socially-isolated vs group housed animals, and its subsequent phosphorylation in socially-isolated. It provides another mechanistic avenue to explore the effects of increased exposure to chronic psychological stress during puberty and early adulthood on mammary gland development and mammary cancer risk in late adulthood.

Chapter 4 : Conclusions and Future Directions

The research described in previous chapters was built upon the initial findings that social isolation and exposure to chronic psychological stress induces the formation of more aggressive and malignant spontaneous tumors at an earlier onset in Sprague Dawley Rats. Furthermore, it was demonstrated that exposure to chronic psychological stress via social isolation during the critical developmental window of puberty through early adulthood results in delayed mammary development and differentiation. Delayed mammary development and differentiation during puberty through early adulthood is associated with increased breast cancer risk in adulthood. This is demonstrated through previous studies indicating that women at high risk for breast cancer often exhibit less differentiated ductal trees [24].

Given the novelty of these findings, the mechanisms underlying how increased exposure to chronic psychological stress during puberty through adulthood leads to delayed mammary development and differentiation remained elusive. However, in our current studies, we've delved deeper, providing mechanistic insights into this phenomenon. By identifying a specific pathway and gene sets modulated by social isolation, we've shed light on potential mechanisms elucidating the impact of chronic psychological stress exposure on delayed mammary gland development and differentiation.

Furthermore, our research has demonstrated how these modulations induced by social isolation can be reversed through environmental interventions, such as rehousing socially-isolated animals from late puberty to early adulthood. The implications of our findings extend beyond mere mechanistic insights; they serve as a data-driven rationale for interventions aimed at vulnerable human populations. While our transdisciplinary research has addressed numerous questions, many still linger. In the following chapter, we'll explore potential future directions,

spanning from in vitro and in vivo experimentation to human population studies and interventions.

We have identified the eIF2α pathway as the most significantly modulated between socially-isolated and group housed animals, as well as between socially-isolated and rehoused animals. As previously mentioned, the eIF2α pathway is integral to the integrated stress response. Upon exposure to external stressors, upstream kinases phosphorylate eIF2α, leading to the attenuation of global protein synthesis. We propose that this attenuation during puberty through early adulthood contributes to the observed delayed mammary development and differentiation. Notably, the covalent modification of eIF2α phosphorylation persists for 8 weeks in this context, suggesting it as an adaptive response to chronic stressors.

To further understand the significance of this covalent modification and its long-term effects, we propose a methodology involving the induction of a phosphomimetic mutation that mimics the effect of eIF2α phosphorylation in group-housed (control) animals. By studying the functional consequence of constitutive phosphorylation of eIF2α, we aim to gain insights into its biological role in mammary development and differentiation. Additionally, we propose conducting a knockout of phospho-eIF2α in socially-isolated animals, either through mutation on the serine site of eIF2α or by targeting PERK, one of the kinases responsible for phosphorylating eIF2α.

These subgroups of animals would be subjected to the same experimental conditions: socially isolating the phospho-eIF2α knockout animals from early puberty (5 weeks) through early adulthood (22 weeks) and keeping the animals with induced phosphorylation of eIF2α in group housing from 5-22 weeks. We hypothesize that animals with induced phosphorylation of eIF2α would exhibit halted mammary development and differentiation, regardless of housing

condition. To assess this, we would measure mammary ductal extension and distal ductal area, progenitor/stem cell population, and perform subsequent single-cell sequence analysis.

This experiment could extend into late adulthood, coinciding with the time when Sprague Dawley rats develop palpable tumors , to examine the effect of persistent eIF2α phosphorylation on mammary development. Additionally, it would be intriguing to explore the long-term effects of exposure to chronic psychological stress during puberty and early adulthood on mammary cancer incidence through this induced or knockout model of eIF2α phosphorylation. We speculate that this adaptive response may represent a form of "antagonistic pleiotropy," whereby eIF2α phosphorylation allows socially-isolated animals to adapt to chronic psychological stress while slowing mammary gland development. However, prolonged attenuation of protein translation could lead to the earlier formation of aggressive mammary tumors in adulthood.

In addition to the in vivo manipulation of eIF2α, gaining a deeper understanding of the upstream signaling events that trigger eIF2α phosphorylation would be invaluable for identifying optimal strategies to target this pathway effectively. Utilizing an agnostic approach, bulk RNA-seq analysis followed by IPA revealed a convergence of gene sets within the eIF2α pathway. This analysis unveiled not only the downregulation of ribosomal genes but also the concurrent downregulation of CCND1 and upregulation of MAPK1. In the developing mammary gland, CCND1 plays a key role through promoting mammary epithelial cell proliferation and ductal elongation and branching [211, 212]. MAPK1 can modulate the activity of kinases involved in eIF2α phosphorylation. Specifically, the interrelationship between PERK and MAPK1 has been explored, but no elucidated [221]. Thus, it would be beneficial to provide insights into the upstream signaling.

Employing organoids derived from animals subjected to social isolation during puberty through early adulthood could offer valuable insights into the underlying mechanisms of this pathway, eliminating the need for in vivo injections or experimentation. We have previously generated organoids from control animals that remained in group housing from puberty through early adulthood (data not shown). These organoids were then exposed to physiologic levels of corticosterone (400 ng/mL), a concentration consistent with levels measured in serum post-restraint tests [222]. Remarkably, the organoids treated with physiologic levels of corticosterone exhibited reduced growth and proliferation compared to controls, as observed in a 7-day growth assay conducted using Incucyte.

The proposed experiment involves culturing organoids derived from animals socially isolated from puberty through adulthood and subjecting them to three experimental conditions. Given our hypothesis that phosphorylation of eIF2α leads to delayed mammary gland development, the organoids will be treated with a drug that further induces eIF2α phosphorylation (Rapamycin) and a drug hypothesized to reduce eIF2α phosphorylation (trazodone hydrochloride). Rapamycin indirectly induces eIF2α phosphorylation by inhibiting mTORC1 activity, while trazodone hydrochloride has been shown in mouse studies to reduce eIF2α protein-mediated translational attenuation [223, 224].

Under these three conditions—control, organoids treated with Rapamycin, and organoids treated with trazodone hydrochloride—growth curve and proliferation assays will be performed to gain insight into the mechanism of upstream eIF2α phosphorylation and its mechanistic premise on delayed mammary development. Our hypothesis is that organoids treated with Rapamycin will exhibit the least growth over the course of 7 days, while those treated with trazodone hydrochloride will display the greatest overall growth.

Furthermore, RNA will be derived from these organoids, followed by RNA-seq and IPA analyses, enabling a better understanding of the mammary gland microenvironment and the upstream signaling pathways affected. This comprehensive approach will provide valuable insights into the mechanisms underlying delayed mammary development and its potential implications.

Taking a step back, it would be intriguing to conduct a long-term experiment to observe the impact of social isolation during early puberty through early adulthood on mammary cancer development in adulthood. Although Sprague Dawley Rats are genetically predisposed to develop mammary tumors, tumors become palpable at around 67+ weeks [152, 225]. The experiment would involve three conditions: animals group housed throughout their lifetime (5-67 weeks), animals socially-isolated from 5-22 weeks and then returned to group housing until sacrifice at 67 weeks, and animals socially-isolated from early to late puberty (5-13 weeks) but reintegrated into group housing at the onset of late puberty (13 weeks) until sacrifice at 67 weeks (see experimental schema).

Given the discovery that prolonged social isolation from weaning until death leads to the development of more aggressive and malignant tumors at an earlier onset [226], it is hypothesized that animals socially-isolated from early puberty through early adulthood would exhibit a greater tumor burden at an earlier onset in late adulthood. Conversely, based on observations that rehousing during late puberty restores behavior, mammary development, and genetic phenotype, it is hypothesized that animals reintegrated into group housing during late puberty would develop less aggressive and more benign spontaneous mammary tumors. These anticipated outcomes align with those expected from animals group housed throughout their lifetime. As puberty is considered a window of susceptibility, it is proposed that exposure to

chronic psychological stress during this period results in the development of more aggressive tumors at an earlier onset in late adulthood.

This study would serve to consolidate the findings of the laboratory and provide a comprehensive understanding of mammary cancer outcomes. While previous research has extensively explored the effects of social isolation during puberty and its subsequent impact on mammary development, this study would bring together these results, offering a cohesive narrative on the subject.

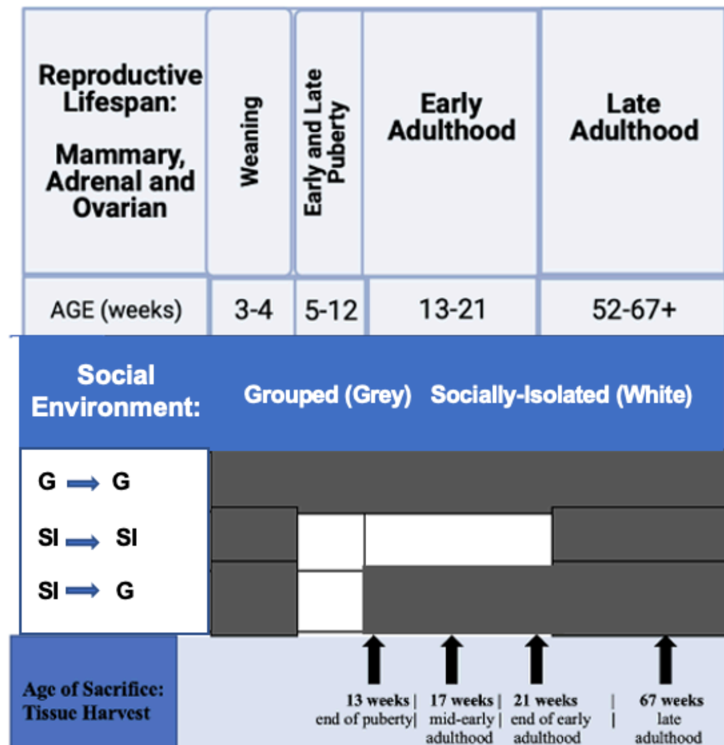


Figure 4.1. **Experimental schema of long-term cohort of animals**. Used to determine the effect of exposure to chronic psychological stress during puberty and early adulthood on mammary cancer incidence later in life.

The hypothesis suggesting that halted mammary development and differentiation during puberty leads to an increased incidence of mammary cancer in late adulthood finds support in the stem cell theory. According to this theory, it is proposed that undifferentiated mammary

stem/progenitor cells have the potential to transform into mammary cancer stem cells over time, giving rise to aggressive subtypes of mammary cancer in adulthood. Literature reinforces the stem cell theory, demonstrating that luminal progenitor cells can give rise to tumors resembling HR+ breast cancer [227]. Moreover, there is evidence indicating that luminal stem/progenitor cells may serve as the origin of basal-like, triple-negative breast cancers [10, 228].

Given that socially-isolated animals exhibit a higher proportion of mammary stem/progenitor cells from early puberty through early adulthood and subsequently develop more aggressive cancer subtypes in late adulthood, this theory gains further validation. While existing evidence supports this hypothesis, it is essential to address any potential gaps in understanding.

To investigate whether the population of preserved mammary stem/progenitor cells during puberty contributes to more aggressive mammary tumors, lineage tracing offers a promising methodology. Drawing from the lab's extensive experience in isolating CD24+CD29+ cells enriched for mammary stem/progenitor cells, a similar approach would be employed. This involves isolating and culturing mammary stem/progenitor cell populations from both socially-isolated and group-housed animals through FACS sorting.

Following isolation, these cells would undergo expansion and be transduced with GFP for labeling. Subsequently, the GFP-labeled CD24+CD29+ cells from both socially-isolated and group-housed animals would be implanted into the mammary fat pad of immunodeficient female Sprague Dawley Rats ("SRG") at 5 weeks of age. The animals would then be sacrificed at 67 weeks, which is the age when palpable tumors begin to form [152, 225].

Upon sacrifice and excision of tumors, fluorescence microscopy would be utilized to visualize the GFP-labeled cells within the tumor microenvironment. The hypothesis predicts that SRG animals transduced with CD24+CD29+ cells derived from socially-isolated animals would

develop more aggressive mammary tumors, and lineage tracing could reveal that tumors arise from this specific cell population. While this approach holds promise, it's crucial to acknowledge the existing gaps in this hypothesis. However, given previous evidence demonstrating the emergence of aggressive cancer subtypes from luminal progenitor cells, this methodology provides a targeted means to delve deeper into the mechanisms underlying mammary tumor development.

To comprehensively investigate the potential link between the preservation and epigenetic modulation of mammary stem/progenitor cells during puberty and early adulthood and the subsequent development of aggressive mammary tumors in late adulthood, it is imperative to explore additional epigenetic mechanisms that may contribute to the transformation of these cells into cancer stem cells. Extensive literature suggests that epigenetic modifications, such as DNA methylation and histone modifications, play crucial roles in reprogramming normal stem/progenitor cells into cancer stem cells [229].

Epigenetics, which governs how environmental and behavioral factors influence genetic regulation, underscores the connection between social environments and the dysregulation of stem/progenitor cells through epigenetic reprogramming [230]. Notably, research has established a causal relationship between health disparities and social adversity via epigenetic alterations, particularly DNA methylation [231]. Furthermore, studies demonstrate that life experiences can directly impact epigenetic patterns at specific genomic loci [232]. Therefore, it is hypothesized that factors such as social isolation and chronic psychological stress during critical developmental periods may lead to the inappropriate preservation of mammary stem/progenitor populations and subsequent additional epigenetic alterations, ultimately promote cancer stem cell formation.

To elucidate the role of various social determinants of health, such as nutrition, healthcare access, and neighborhood environments, in influencing epigenetic reprogramming and breast cancer risk, a longitudinal study design is proposed. This study would track individuals from puberty through adulthood, assessing their exposure to chronic psychological stress and other social determinants while monitoring breast cancer incidence. By examining how these factors cumulatively contribute to breast cancer risk and development, this longitudinal approach aims to provide a comprehensive understanding of the interplay between social environments, epigenetic regulation, and cancer pathogenesis.

In summary, investigating the nexus between mammary stem/progenitor cell preservation, epigenetic modulation, and breast cancer development necessitates a multifaceted approach that considers both biological and social factors. A longitudinal study encompassing diverse social determinants of health can offer valuable insights into the complex interactions shaping breast cancer risk across the lifespan.

While doing a deep dive into human epidemiological data, there is scant data on longitudinal effects of exposure to chronic psychological stress during puberty and breast cancer development in adulthood. A major limitation in measuring stress in humans is method of measurement. Existing tools and measures for assessing stress, such as self-reported questionnaires, physiological monitoring devices, or behavioral observations may not capture the full complexity of the stress experience. A proposed non-invasive method to measure cortisol would through the collection of saliva. In response to a stressor, cortisol is released in the bloodstream. A portion of this cortisol is transported into saliva through passive diffusion where it remains in its biologically active form [233].

To explore the potential link between exposure stress during puberty and breast cancer incidence in adulthood, we could utilize saliva-based cortisol measurements as a non-invasive method to assess stress levels. Recruitment would involve individuals who experienced varying degrees of stress during puberty and early adulthood, considering factors like social isolation, socioeconomic status, neighborhood, traumatic events experience, family dynamic, etc. Saliva samples would be collected at multiple time points during puberty and adulthood, with participants spitting into collection tubes or using absorbent materials placed in the mouth. ELISA or LC-MS/MS would be used to accurately measure cortisol levels. Longitudinal analysis would then be conducted to examine the relationship between cortisol levels during puberty and early adulthood and breast cancer incidence later in life. Control variables, such as genetics and lifestyle factors, would be considered, and ethical guidelines would be followed throughout the study. Ultimately, this approach could provide valuable insights into the potential role of stress in breast cancer risk, informing preventive strategies and interventions aimed at mitigating stress-related health outcomes.

The results of the environmental intervention of rehousing, show a restorative effect on the effect of exposure to chronic psychological stress serves as a data-driven rationale for vulnerable human populations. While “rehousing” human populations located in stressful environments is not feasible there could be programs put in place to reduce the amount of chronic psychological stress placed on adolescents in puberty through adulthood. A proposed comprehensive intervention is the "Youth Resilience Program" designed to promote mental health and well-being among vulnerable adolescents in urban communities. The program could be implemented in collaboration with local schools, community organizations, and mental health professionals. To address all needs, this comprehensive intervention would be multifaceted.

Mental health education and awareness workshops would be conducted in schools to educate about stress management, coping strategies, and the importance of seeking aid for mental health issues. These workshops would be facilitated by trained counselors and psychologists, who will provide information and resources to students. The goal would be to have trained counselors and psychologists sourced from the community to aid in comfortability and allowing the youth to have pillars of trust. They would offer individual and group counseling sessions for all students. Using evidence-based techniques such as cognitive-behavioral therapy (CBT) [234], would allow students to learn coping skills and resilience-building strategies.

To address socioeconomic factors contributing to stress, the program could collaborate with local organizations to provide resources and support for families in need. This would include access to food assistance programs, financial literacy workshops, and employment opportunities for parents. Finally, the comprehensive intervention could involve ongoing evaluation and feedback to assess its effectiveness and make necessary adjustments. Regular surveys and focus groups would be conducted to measure changes in participants' mental health outcomes and identify areas for improvement. Overall, the "Youth Resilience Program" seeks to empower vulnerable adolescents with the knowledge, skills, and support systems needed to effectively manage stress and build resilience in the face of adversity. By addressing mental health needs within schools and communities, the program aims to improve the overall well-being of adolescents and promote positive outcomes for future generations.

The research conducted in the previous chapters offers significant implications for understanding the impact of stress on breast cancer risk and development. The novelty of our findings suggests promising avenues for exploration across mechanistic, clinical, and epidemiological domains. This underscores the transdisciplinary nature of our project,

emphasizing the diverse perspectives and methodologies employed to advance our understanding of stress-related breast cancer risk and incidence. In conclusion, our research opens doors to multifaceted investigations that hold potential for informing both scientific understanding and clinical practice in the field of breast cancer research.

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