EFFECT OF FETAL ALCOHOL ON MAMMARY CARCINOGENESIS: THE ROLE OF THE ESTROGEN/IGF AXIS

by

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ABSTRACT OF THE DISSERTATION

Effect of Alcohol *In Utero* on Mammary Carcinogenesis: The role of the estrogen/IGF axis

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Altered fetal programming as a result of a suboptimal *in utero* environment can increase disease susceptibility in adulthood. Alcohol exposure *in utero* increases mammary cancer risk in rodents. Mammary development affects breast cancer risk, as a denser, hyperproliferative gland increases mammary cancer risk. Therefore we hypothesized that alcohol exposure *in utero* increases the action of the IGF-I/E2 system leading to hyperproliferative mammary glands and ultimately increased cancer susceptibility. We first established a model where fetal alcohol leads to increased mammary tumor susceptibility. Pregnant rats were fed a liquid diet containing ethanol (alcohol-fed), a control isocaloric liquid diet, or rat chow *ad libitum*. Alcohol-fed dams were acclimated to increasing concentrations of ethanol (2.2% and 4.4%), then fed 6.7% ethanol from day 7 to 21 of gestation. To investigate tumorigenesis, N-nitroso-N-methlyurea (NMU) was administered to induce tumor formation at postnatal day 50. Rats were palpated for

tumors weekly and euthanized at 23 weeks post-NMU injection, or in a second study at 16 weeks post-injection. At 16 weeks post-injection, tumor multiplicity was greater and tumor latency was decreased in the alcohol-fed group compared to controls. At 23 weeks post-NMU injection alcohol-fed animals developed more malignant tumors and more estrogen receptor-α negative tumors with less IGF binding protein-5 expression relative to controls, indicative of poor prognosis breast cancer in women. To determine if mammary development is altered by *in utero* alcohol exposure, a study was conducted in which animals were euthanized prepubertally and postpubertally. Mammary glands from alcohol-fed animals showed increased proliferation and aromatase expression prior to and immediately after puberty. Mammary and hepatic IGF-I mRNA levels were also higher in alcohol-fed animals. In a separate study it was determined that alcohol-fed animals also have higher circulation E2 than controls. Together, these data indicate that alcohol exposure *in utero* increases susceptibility to mammary tumorigenesis, and leads to a tumor phenotype indicative of poor prognosis breast cancer. These changes may be related to enhanced early mammary development via the IGF/E2 systems. Therefore, women born to mothers who drank alcohol during pregnancy may represent a high risk group for aggressive breast cancer.

Dedication

I'd like to dedicate this work to my family who has supported me in everything I've accomplished.

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I would like to most of all thank my mentor and advisor Dr. Wendie Cohick for sculpting me into the scientist I am today.

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E2, estradiol

NMU, N-nitroso-N-methlyurea

NBF, neutral buffered formalin

TEB, terminal end bud

IGF, insulin-like growth factor

BPA, bisphenol A

ER, estrogen receptor

IP, intraperitoneal

BrdU, bromodeoxyuricil

RNA, ribonucleaic acid

ANOVA, analysis of variance

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

EGF, epidermal growth factor

DMBA, 7,12-dimethylbenz[α]anthracene

TBS, tris buffered saline

ELISA, enzyme-linked immunosorbent assay

EIA, enzyme-linked immunoassay

AVPV, anteroventral periventricular nucleus

AI, aromatase inhibitor

CHAPTER 1

Review of the literature

Overview of Mammary Gland Development

Development of the mammary gland occurs in response to hormonal regulation during three stages of life: fetal life, puberty, and pregnancy. In rats, mammary development begins at day 10 of gestation. At this time the mammary streak can be detected and consists of a single layer of ectodermal tissue stretching from the anterior limb bud to the posterior limb bud. By day 12 of gestation, epidermal cells migrate to the site of mammary development and form several layers of cells, creating the mammary bud (1, 2). By day 14 the mammary bud has become bulb-shaped and the gonads become functional, initiating the sexual dimorphism of mammary morphogenesis. In males, fetal gonadal testosterone production desensitizes the mammary bud to estrogens, preventing further development; the mammary stalk ruptures and the rudimentary mammary gland becomes a blind duct (3). In female mice and rats, there ensues a 3 day resting period followed by rapid cellular proliferation resulting in the mammary sprout which elongates, forming ducts that penetrate the fat pad precursor. At birth there are 15-20 branching ducts embedded in the fat pad precursor (3).

During fetal development of the mammary gland, epithelial interactions with the fat pad precursor are required for developmental progress. A study in 1982 recombined embryonic mammary epithelial cells with dense mammary mesenchyme or with fat pad precursor and implanted the structure under the kidney capsule. The epithelium that was

recombined with the dense mesenchyme developed ductal hyperplasia with no elongation, whereas the epithelial cells recombined with the fat pad precursor underwent typical mammary morphogenesis including ductal elongation (4).

After birth the mammary gland continues to grow isometrically with the rest of the body until puberty when allometric growth begins. At the leading edge of each duct is a club shaped structure called a terminal end bud (TEB). The TEBs are sites of high proliferation as they create the ductal structures by elongating through the fat pad. The initial formation of the TEBs requires estradiol (E2). In female rats after gonadectomy at 5 weeks of age the TEBs regress, but when E2 pellets are implanted the TEBs reappear (5). Insulin-like growth factor-I (IGF-I) is also necessary for pubertal mammary development. In ovariectomized IGF-I -/- mice a very primitive mammary gland will form. E2 or IGF-I replacement alone will rescue development but the gland is still impaired. Only when both hormones are administered together is normal development restored (6). The TEBs also determine the branching pattern of the gland as the TEB can bifurcate into two branches, which is regulated in part by IGF-I. Specifically, local mammary IGF-I is important in branching morphogenesis. Mice with a mammary gland-specific deletion of IGF-I show significantly decreased branching patterns compared to mice with a liver specific deletion, or wild type control mice (7).

The development of the mammary gland determines the density of the gland. A gland that has developed more epithelial structure and less loose mesenchyme is considered a dense mammary gland. Increases in TEB number, ductal or TEB proliferation, ductal branching, or any combination of these could result in a denser mammary gland. TEBs are a site of high proliferation in the breast tissue, as well as the

site of initiation of most breast cancers (8). Higher breast density was first associated with an increased risk of breast cancer in women in 1976 (9) and this finding has since been replicated in many studies (10-15). Women with greater than 50% breast densities had a 3.6 times higher risk for breast cancer than women with less than a 10% breast density as reported in a 2006 study (16). Specifically, dense breast tissue has been associated with ductal carcinoma *in situ*, and invasive breast cancer (17).

During pregnancy and lactation a second level of development occurs and the gland becomes mature and fully functional. Under the influence of prolactin, E2, progesterone, insulin, cortisol, IGF-I, and epidermal growth factor (EGF) the epithelial compartment of the gland proliferates and fills the fat pad with lobular alveolar structures (18). The alveoli differentiate to produce milk proteins which are secreted into the ductal lumen and transported to the nipple. Prolactin and oxytocin are instrumental in the development of the pregnant gland and for milk secretion. Suckling stimulates the release of prolactin and oxytocin, therefore once suckling ceases the gland regresses and undergoes involution. A new hormonal milieu drives involution forcing the epithelial cells to undergo apoptosis and the alveolar structures to dedifferentiate.

The Fetal Environment and Mammary Development

The fetal environment has been shown to contribute to disease risk in many adult diseases including cancer (19-21). Studies in rodents have linked sub-optimal fetal environments such as a high fat maternal diet or maternal alcohol consumption to increased risk of mammary cancer in the offspring (22-24). In addition, animals exposed to fetal alcohol develop more tumors with a poor-prognosis phenotype compared to

unexposed controls (24). In humans fetal exposure to alcohol has been shown to increase risk for childhood leukemia, suggesting that findings in rodent models may be relevant to human disease (20).

Exposure to estrogens *in utero* either due to female twin-ship (25), or DES treatment has been well-studied in humans and is associated with a higher risk for breast cancer later in life (26). Another study suggested that circulating maternal E2 during pregnancy may play a critical role in breast cancer risk in daughters later in life (23). In rodent studies, feeding a high fat diet during pregnancy has been shown to increase circulating E2 in the dams and increase mammary carcinogenesis in response to DMBA in the offspring (22). The fetal estrogenic environment has been shown to be important in the development of the primitive mammary bud, therefore fetal estrogen could play a role in mammary development which could potentially lead to a change in susceptibility to mammary cancer (27). Many studies have been conducted to investigate the effect of *in utero* exposure to the xenoestrogen bisphenol A (BPA) on mammary development (28-31). In rodents, fetal exposure to BPA has been shown to increase branching and epithelial outgrowth in the mammary gland (28). I*n utero* exposure to BPA has also been shown to increase the sensitivity of the mammary glands to E2 after birth, possibly contributing to the hyperdevelopment of the gland (30). Therefore, changes in the estrogenic environment during fetal development could alter mammary development and lead to increased susceptibility to mammary cancer later in life.

A wide variety of toxic compounds administered during the fetal period have been shown to affect mammary gland development postnatally including nonylphenol, perfluorooctanoic, zearalenone, genestien, and atrazine (28, 31-35). The effects of

exposure *in utero* on mammary development have also been well studied. In 2005, Rayner et al. showed that in rats, atrazine exposure *in utero* led to a decrease in mammary gland length prepubertally which was lost postpubertally, without altering circulating hormones levels. In addition Rayner reported that the F2 generation born to mothers who were exposed to atrazine *in utero* weighed less than pups from control animals suggesting either a transgenerational effect, or that the animals exposed to atrazine *in utero* do not produce optimal or sufficient milk to sustain pup growth (35).

In summary, alterations in the fetal environment caused by a number of different factors can alter postnatal mammary development. Many of these changes involve alterations in the estrogen axis. While early developmental effects may resolve with maturity, they may program the gland for increased disease susceptibility later in life (36).

Estradiol and Breast Cancer

Breast cancer is the leading form of cancer in women. Due to developments in screening, diagnosis, and treatment, breast cancer mortality has dropped 25% over the past 20 years (37, 38). There has been a wealth of research conducted on the etiology and progression of the disease, but the cause of initiation in most cases is unknown. E2 is well established as a risk factor for breast cancer. It has been reported in many studies including the NYU Women's Health Study that increased serum E2 is positively associated with breast cancer risk (39-43). In addition, the relationship between a woman's endogenous E2 levels over her life time and breast cancer risk show that late age of menarche and early onset of menopause are protective factors. These conditions

result in fewer total menstrual cycles and therefore a decreased overall total exposure to E2 over a lifetime (44).

A role for E2 in the etiology and progression of breast cancer was first recognized when bilateral ovariectomy in premenopausal breast cancer patients resulted in cancer remission (45). This finding led to investigation into the role of ovarian hormones in breast cancer. It is now established that approximately 70% of all breast cancers are ER-α positive and require E2 for growth (46). E2 can promote carcinogenesis through multiple mechanisms (47). It can stimulate hyperplastic change (48) by binding ER-α and increasing cell proliferation through both classical genomic effects as well as more recently recognized non-genomic effects that involve cross-talk with peptide growth factor receptor systems at the cell membrane (49). Increasing cell proliferation indirectly leads to carcinogenesis by increasing the probability of genetic mutations. In addition to these mitogenic effects, E2 also alters cell morphology leading to a more motile population of cells. This effect on migration is mediated by FAK and c-SRC (50), and is likely due to the non-genomic effects of E2. Therefore in addition to cancer risk and initiation, E2 may be important in progression and metastasis.

In addition to the carcinogenic effects of E2 as a mitogen, cancer risk can also be due to the direct carcinogenic properties of E2 metabolites. The conversion of E2 to 2- or 4-OH-E2 results in reactive oxygen species byproducts that are carcinogenic (51). Also, the metabolites themselves can directly bind DNA and form adducts leading to mutagenesis (52, 53). The ER-α knock out/wnt-1 transgenic mouse demonstrates the importance of these metabolites in tumor initiation. In this study treating ERKO/wnt-1

transgenic mice with E2 increased mammary carcinogenesis in the absence of ER-α, thus implicating the metabolites as the culprits (54).

The great success of treating breast cancer with adjuvant therapies that deprive tumors of E2 exemplifies the importance of E2 in breast cancer etiology. Two major classes of treatments are available, selective estrogen receptor modulators (SERMs) which act by binding to and blocking ER-α action, and aromatase inhibitors (AIs) which act by inhibiting E2 production. The SERM tamoxifen has proven to reduce the risk of recurrence by 50% and reduce mortality by 25% in patients with ER-α positive breast cancer (55). While this represents a substantial improvement, the third generation AIs have proven to be more effective and less toxic than tamoxifen (56-58). More recently it has been shown that treatment with tamoxifen for 2 years followed by an AI is the best treatment strategy (59).

While therapies to block the E2 pathway have been largely successful, only 60-70% of ER-α positive tumors respond to these therapies (60). Others become tamoxifen-resistant by circumventing ER-α and utilizing other pathways, such as the IGF and EGF systems, to sustain growth (61). The IGF pathway signals through PI3K which is associated with low expression of ER-α in human breast cancers (62). In human breast cancer cells, blocking PI3K leads to increased ER-α expression, suggesting that if the IGF system is no longer functional, the cells are forced to revert back to the alternate ER-α pathway (62). The mechanism for this resistance is thought to involve the non-genomic actions of ER-α (63, 64). In this scenario, ER-α interacts with the EGFR and IGFR at the plasma membrane instead of translocating to the nucleus to activate gene expression (65-67). In tamoxifen-resistant cells, E2 has been shown to translocate to the cytosol, directly

bind and activate the IGFR (61, 66), and/or utilize the EGF family to activate downstream signaling molecules and sustain growth (65).

Signaling Molecules and Breast Cancer

In general, cancer initiation results from mutations in growth-promoting and/or tumor suppressing genes, resulting in over-stimulation of cell proliferation. The unrepressed activation of growth-promoting genes is typically controlled by growth factors, their receptors, and downstream signaling cascades. IGF-I is one such growth factor, which activates the IGFR, a transmembrane tyrosine kinase receptor (68), to stimulate cell proliferation and inhibit apoptosis (69, 70). IGF activity is modulated by a family of six IGF binding proteins (reviewed in 71). EGF also stimulates cell growth via a tyrosine kinase receptor. There are four known EGFRs, HER1 through HER4, which form homo/heterodimers upon activation (reviewed in 72). In addition to their well-established roles in normal mammary gland development (73-76), the IGF and EGF families have emerged as major factors in breast cancer etiology (71, 77). Therapeutic reagents that block the IGFR in breast cancer patients are presently in clinical trials. Similarly, HER2, a member of the EGF family, is overexpressed in 25-40% of human breast cancers. Clinical trials testing Herceptin®, a humanized monoclonal antibody that blocks HER2, began in 2000. Herceptin is currently used as an adjuvant breast cancer therapy for HER2 positive breast tumors.

While blocking HER2 has proven to be a successful treatment for breast cancers that overexpress HER2, resistance to this therapy can develop over time. This resistance is thought to be due to the cancer's ability to circumvent the EGF signaling system. The

IGFR has been shown to dimerize with HER2, which may allow the cell to regain its ability to activate downstream signaling molecules involved in cell proliferation (78, 79). These two families are also known to interact in the normal mammary gland. In non-transformed human breast epithelial cells, IGF-I is required for EGF to activate the downstream signaling target ERK, as well as to induce mitogenesis. In addition, inhibiting the EGFR does not affect the ability of IGF-I to activate Akt (80). Akt is a downstream signaling molecule in the PI3K pathway that is common to both growth factor families and is involved in resistance to breast cancer therapies (81, 82). Convergence of both pathways on this molecule has been demonstrated in normal mammary epithelial cells, where treatment with both IGF-I and EGF together activates Akt to a greater degree than either growth factor does alone (83). It is obvious that these pathway interactions play a critical role in breast cancer (84, 85), highlighting the importance of further investigation into their mode of action.

Use of *In Vivo* Models to Investigate the Role of IGF-I in Mammary Development and Cancer

The majority of circulating IGF-I is produced in the liver, while most tissues, including the mammary gland, also produce IGF-I. The importance of IGF-I in mammary development is highlighted by the finding that IGF-I null (IGF-I -/-) mice do not develop normal glands, and that giving back IGF-I partially restores mammary development (6, 74, 86). In addition, while IGFR knockout (IGFR -/-) mice are not viable, mammary rudiments from fetal IGFR -/- mice survive transplant into the cleared fat pad of 21 day old wild type mice. In IGFR -/- transplants, ducts do not elongate

through the fat pad and fewer TEBs develop compared to glands from IGFR +/+ littermates (87), demonstrating the importance of IGF signaling in mammary development.

The midi mouse, another mouse model used to investigate the role of IGF-I in mammary development, has a mutation in the IGF-I gene that diminishes its transcription (88). Midi mice present with reduced mammary tissue IGF-I, a 70% reduction in circulating IGF-I, along with impaired mammary development (7). Midi mice have fewer mammary ducts which do not extend as far into the gland as theay do in control mice at day 42; by day 80 ducts in both groups extend through the fat pad, but the midi mice have less branch points. The deficit in branching is still apparent at 144 days of age (7). These studies provide further evidence that IGF-I is necessary for mammary development. However, since both local and systemic IGF-I are reduced in the midi mouse, it cannot be distinguished which is more important.

Transgenic mouse models in which mammary IGF-I or IGFR are overexpressed have also been developed and found to result in hyperplastic mammary development and increased tumor development. (36, 89-91). In a 2000 study (89), the IGF-I analog (des(1-3) hIGF-I) was transgenically overexpressed in the mammary gland. This IGF mutant has reduced affinity for IGFBPs making it a more a potent ligand for the IGFR. All of the transgenic mice in this study developed mammary interepithelial neoplasias while all non-transgenic mice exhibited normal mammary development. In addition, 53% of the transgenic mice developed spontaneous mammary tumors, while no mice in the non-transgenic group developed tumors (89). In another transgenic model human IGF-I was overexpressed in the myoepithelial cells in the mammary gland, which led to increased

alveolar end bud formation along with increased tumor burden and decreased latency in response to DMBA (36). Mice transgenically overexpressing the IGFR or a constitutively active IGFR in the mammary gland exhibited ductal hyperplasia and spontaneous mammary tumor development (90, 91). These studies prove that increases in local IGF-I are involved in mammary development and also contribute to tumorigenesis, and provide evidence for the IGFR as an oncogene.

Rodent models have also been developed to investigate the endocrine role of IGF-I. IGF-I production in the liver is induced by growth hormone (GH) which is released from the pituitary in response to GH releasing hormone (GHRH). The little (lit) mice have a mutated GHRH receptor resulting in deficient GH release (92). Mice homozygous for the lit mutation are smaller and have 20% of the serum IGF-I of heterozygous littermates (lit/+). Xenographs of MCF-7 cells in lit mice grew half as big as xenographs in lit/+ mice. When MCF-7 cells in culture were incubated in serum from lit/+ mice, cell growth occurred in a dose-dependent manor, while cells incubated in serum from lit mice did not grow, implicating a serum factor in the mechanism behind the smaller tumor phenotype. In addition, adding back IGF-I to the serum from the lit mice restored MCF-7 cell growth to cells in the lit/+ serum (93), identifying IGF-I as the serum factor affecting cell growth. The lit mice demonstrate the role of systemic IGF-I in tumorigenesis. In addition, transgenic overexpression of GH leads to spontaneous mammary adenocarcinoma formation presumably due to increased circulating IGF-I (94).

To investigate the role of endocrine versus local IGF-I, mice with a liver-specific IGF-I deletion (LID) were created using the cre/lox system, resulting in mice with 25% of normal circulating IGF-I levels. Despite the dramatic reduction in circulating IGF-I,

ductal extension and lactation were normal (95, 96). Although no change in mammary morphology was observed, the LID mice displayed a lower tumor incidence and increased latency in both carcinogen (DMBA), and genetically induced (SV40-LTA) mammary tumors (95). These data show that while normal mammary development occurs under conditions of low systemic IGF-I, tumorigenesis is suppressed.

More recently, a model was developed to directly investigate the role of tissue IGF-I verses the role of circulating IGF-I. Hepatic IGF-I transgenic (HIT) mice which have a 3-fold increase in circulating IGF-I were crossed with IGF-I -/- mice creating HIT/IGF-I -/- mice (97). HIT mice had more TEBs at 4 weeks, and ductal branching and length were increased at 8 and 16 weeks. For all parameters, HIT/IGF-I -/- mice had similar mammary morphology to wild type controls (97). In this model, physiological levels of local IGF-I expression and elevated serum IGF-I led to increased mammary development, while elevated serum IGF-I in the face of a loss of local IGF-I allowed for normal mammary development. The elevated serum IGF-I in the HIT/IGF-I -/- mice compensated for the lack of local IGF-I and fostered normal mammary development. In contrast, the midi mice had reduced circulating and local IGF-I, therefore serum IGF-I was not able to compensate for the lack of local IGF-I resulting in impaired mammary development. Another observation implicating circulating IGF-I in normal mammary biology is that B6 mice have less ductal branching than C3H mice (7) and B6 mice also exhibit lower circulating IGF-I than C3H mice (98). Collectively, these studies suggest that levels of systemic IGF-I may determine the extent of mammary development.

The Role of IGFBP-5 in Mammary Development and Cancer

In mice, IGFBP-5 is expressed in the mammary gland from 4 to 12 weeks of age, throughout pregnancy, and during involution (99). IGFBP-5 is low during lactation, then increases 40-to 50-fold 2 days into mammary involution (100). Transgenic mice overexpressing IGFBP-5 specifically in the mammary gland have decreased ductal branching and less alveolar development during pregnancy (101), while IGFBP-5 null mice display normal mammary morphology and lactation, but delayed involution (102). Mice transgenically overexpressing IGFBP-5 in the whole body have severe growth deficits including reduced litter size and weight, increased pup mortality, and slower postnatal growth (103). Surprisingly, mice overexpressing IGFBP-5 have higher circulating IGF-I then wild type littermates, likely an attempt to rescue the growth deficit (103). In contrast, IGFBP-5 null mice grow normally and have circulating IGF-I levels comparable to wild type controls (102). These findings implicate IGFBP-5 as a growth suppressive protein important in mammary gland biology, particularly involution.

To investigate the role of IGFBP-5 in breast cancer, MDA-MB-231 tumor cells were transfected with IGFBP-5 (MDA/BP5). This resulted in increased IGFBP-5 secretion into the media and decreased cell growth compared to cells transfected with a vector control (MDA/VEC). MDA/BP5 or MDA/VEC cells were then injected into *nu/nu* mice. In the group injected with MDA/BP5 half as many mice presented with tumors and tumor growth was reduced compared to those injected with MDA/VEC. In addition, MDA/BP5 tumors exhibited reduced bax, and an increase in bcl-2 protein, indicating there was more apoptosis in the MDA/BP5 tumors (104).

A general role for IGFBP-5 in apoptosis stems from its involvement in involution

of the prostate and thyroid, atresia in ovarian follicles, and in interdigital webbing degradation during digit formation in the mouse embryo (105-109). Marshman and coworkers (110) also demonstrated a role for IGFBP-5 in apoptosis in the mammary gland as they showed that increased expression corresponded with increased apoptosis on day two of involution. While treating with exogenous IGFBP-5 did not induce apoptosis in primary mammary epithelial cells, it eliminated the ability of IGF-I to rescue cells from apoptosis induced by incubating cells in serum-free media. IGFBP-5 also blocked the ability of IGF-I to activate signaling in mouse primary mammary epithelial cells (110). In addition, MDA-MD-231 cells transfected with IGFBP-5 exhibited activation of caspase 8 & 9 and cytochrome c release from the mitochondria (111). Collectively, these studies support a role for IGFBP-5 in apoptosis.

In contrast, IGFBP-5 can also enhance the activity of IGF-I and play a role in cell growth (112). Treating mouse osteoblasts with exogenous IGFBP-5 in combination with IGF-I or without IGF-I increased cell proliferation in a dose dependent manner (113). The ability of IGFBP-5 to stimulate cell proliferation independently of IGF-I was proven when IGFBP-5 treatment increased cell proliferation in a dose-dependent manner even in osteoblasts from IGF-I -/- mice (114). The IGF-independent actions of IGFBP-5 are proposed to be mediated by its functions in the nucleus. IGFBP-5 possesses a nuclear localization sequence and has been shown to localize to the nucleus (115). In addition, chromatin immunoprecipitation assays display that in addition to localizing to the nucleus, IGFBP-5 directly binds DNA at histone 3 (116). Therefore, the IGF-I independent actions of IGFBP-5 could manifest through nuclear localization and DNA binding to alter gene expression.

Interestingly, the antiestrogen ICI stimulates IGFBP-5 expression 2- to 3- fold in DMBA- induced mammary tumors. To further investigate the interactions between IGFBP-5 and E2 studies were conducted in MCF-7 cells. E2 treatment led to a reduction in IGFBP-5 mRNA and protein expression along with an increase in proliferation, while ICI increased IGFBP-5 mRNA and protein expression and suppressed proliferation (117). Reducing endogenous IGFBP-5 with an antisense oligodeoxynucleotide led to a doubling in MCF-7 cell proliferation compared to a scrambled control, and ICI reduced cell proliferation 54% which was attenuated by IGFBP-5 antisense oligodeoxynucleotide (117).

IGFBP-5 is also involved in tamoxifen resistance. A 2010 study conducted a loss of function genetic screen and identified a short hair-pin RNA for IGFBP-5 (shIGFBP-5) in tamoxifen-resistant MCF-7 cells. Addition of exogenous IGFBP-5 to these cells enabled them to respond to tamoxifen (118). Additionally, IGFBP-5 suppression induced tamoxifen resistance *in vivo*. MCF-7 cells expressing the shIGFBP-5 or wild type MCF-7 cells were injected into nu/nu mice. The resulting tumors from the shIGFBP-5 cells did not respond to tamoxifen alone while tumors resulting from the wild type MCF-7 cells did. Treating the animals with tamoxifen plus IGFBP-5 restored the ability of the tumors from shIGFBP-5 MCF-7 cells to respond to tamoxifen (118). In addition, the IGFBP-5 status of 143 human breast tumors collected from tamoxifen-treated women was determined and women with tumors expressing low IGFBP-5 had substantially reduced survival. These studies indicate that the low IGFBP-5-expressing tumors were tamoxifen-resistant (118).

Alcohol Consumption and Breast Cancer

Epidemiological reports indicate that alcohol consumption is positively associated with an increased risk of breast cancer, showing an elevated risk of 7.1% for every 10 g of alcohol consumed daily (119). Women who consume 30 to 60 g of alcohol per day have a 41% higher risk compared to non-drinkers (120).

Mammary carcinogenesis is enhanced in rats exposed to alcohol, supporting human epidemiological studies. In a 1995 study, ethanol consumption at 15% or 20% of calories increased the number of mammary adenocarcinomas during the promotion stages of tumorigenesis (121). While it is clear that alcohol affects tumorigenesis, the mechanisms that occur in the local mammary environment to cause this effect have not been delineated. In the ER positive breast cancer cell line MCF-7, alcohol treatment results in a dose-dependent increase in cell proliferation. In contrast, no increase in proliferation is observed in ER negative cell lines. In this same study, alcohol induced the production of ER-α protein in a dose-dependent manor (122). These data suggest that ER-α may be one of the factors involved in alcohol-induced tumorigenesis. In addition, women drinking alcohol exhibit a 20% increase in serum E2 levels (123, 124). E2 levels are also higher in rodents that are consuming alcohol (125). Therefore, increased circulating E2 could contribute to the increase in mammary tumorigenesis observed in alcohol-exposed animals.

In epidemiological studies, alcohol consumption is associated with denser mammary tissue (16, 126). Denser mammary tissue is listed as a risk factor for breast cancer by the American Cancer Society. Singletary and Nelshoppen reported that in rodents, alcohol intake at 20% or 30% of calories led to a 19% and 45% increase in

terminal end bud (TEB) density, respectively (127). In a second rodent study, it was reported that alcohol exposure *in utero* resulted in a dose-dependent increase in mammary density in rats (125). These data support the conclusion that alcohol exposure leads to denser mammary tissue, and enhanced susceptibility to mammary carcinogenesis.

Evaluation of DNA labeling index shows that alcohol consumption leads to an increase in DNA synthesis in the TEBs (127). Increased proliferation could be caused by increased growth factor signaling in pathways such as the IGF-I system and could ultimately lead to higher mammary density. In 27 day old rats, six days of alcohol exposure reduces the levels of basal Akt phosphorylation in the anteroventral periventricular nucleus (AVPV) (128). In addition, IGF-I injection into the AVPV induces Akt phosphorylation, and gastric alcohol administration blocks the ability of IGF-I to induce phosphorylation of Akt (129). This indicates that alcohol can regulate signaling mechanisms in the IGF-I pathway. In the brain, alcohol inhibits IGF-I action. However, in the mammary gland alcohol may have a stimulatory affect on IGF-I action, thus enabling the increase in TEB cell proliferation mentioned above (127).

OBJECTIVES

Alcohol exposure *in utero* is known to cause fetal alcohol spectrum disorder, and in the worst cases, fetal alcohol syndrome. The high incidence of children born in the U.S. with fetal alcohol syndrome confirms reports from the Centers for Disease Control that indicate that in spite of this knowledge, expectant mothers still consume alcohol while pregnant. In addition to the direct adverse effects of fetal alcohol exposure on the offspring, a 2004 study found that alcohol exposure during fetal development leads to increased risk for mammary cancer later in life. This supports an emerging concept that the fetal environment plays a major role in determining life-long disease risk.

The first objective of this work was to establish a rodent model of fetal alcohol exposure and subsequent susceptibility to carcinogen-induced mammary cancer. We hypothesized that the IGF-I/estradiol (E2) axis was involved in the increase in tumor susceptibility, and therefore investigated IGF-I system components and circulating E2, as well as tumor histology and phenotype in this first study (Chapter II).

Mammary development is important in breast cancer risk, in that denser breast tissue in humans, as well as hyperproliferative development in rodents increases the risk of mammary cancer. To determine if early alterations in mammary development play a role in the increased tumorigenesis observed in rats exposed to alcohol *in utero*, we investigated mammary development as well as the IGF-I/E2 axis at time points that corresponded with pre-pubertal, pubertal, and mature stages of mammary development (Chapter III).

High grade breast cancers with a poor prognosis progress more rapidly than tumors with markers indicative of a more favorable prognosis. Understanding factors that increase a woman's chance of developing aggressive breast cancer could help identify a subset of women who should be screened for breast cancer more frequently. To further investigate how mammary cancer progression is affected by alcohol exposure *in utero*, we studied a time point in tumor development when the alcohol-exposed animals had increased tumor multiplicity compared to controls. Tumor phenotype and receptor status were determined to address progression of mammary tumors (Chapter IV).

CHAPTER 2

Fetal alcohol exposure increases mammary tumor susceptibility and alters tumor phenotype in rats.

Introduction

The National Cancer Institute estimated that there would be 40,170 deaths due to breast cancer (130), and the American Cancer Society predicted 192,370 new cases of invasive breast cancer among American women in 2009 (131). While considerable gains have been made in establishing new therapies for breast cancer treatment, the underlying causes of breast cancer remain poorly delineated. The idea that environmental exposures and lifestyle choices during pregnancy may affect the offspring's risk of breast cancer is a newly emerging concept (132-134). For example, studies have indicated that gestational exposure to bisphenol A, an estrogenic compound found in many consumer products, induced the development of mammary gland neoplasias in adult rats (135). Similarly, animal studies have shown that offspring from mothers consuming a high fat diet during pregnancy have an increased susceptibility to a mammary carcinogen (22). Furthermore, using rats as a model system, alcohol exposure *in utero* has been shown to increase susceptibility to chemically-induced mammary tumors in offspring (23). This is particularly alarming as the Centers for Disease Control report that 52.6% of women of child-bearing age consume alcohol (136). Even more striking is that in the United States 1 in 12 pregnant women admit drinking alcohol and 1 in 30 pregnant women admit binge drinking (five or more drinks at a time) (137).

Estradiol (E2) plays an integral role in mammary gland development as well as breast cancer progression (47, 138). It is widely accepted that a woman's lifetime exposure to both environmental and endogenous E2 affects her risk for breast cancer (139). Furthermore, epidemiological studies have shown that a higher circulating E2 level is associated with increased breast cancer risk (140). Recent data suggest that circulating E2 may be altered in offspring of rats exposed to alcohol *in utero* (141). This finding, together with the finding of Hilakivi-Clarke et al. (2004), that prenatal exposure to alcohol alters mammary morphology, suggests that E2 may contribute to the increased susceptibility to mammary carcinogens in these animals.

Considerable evidence suggests that E2 and estrogen receptor-α (ER-α) cross-talk with peptide growth factors, including insulin-like growth factor-I (IGF-I) (142, 143). The IGF system is comprised of IGF-I and -II ligands that signal through the IGF-I receptor and six binding proteins (IGFBP-1-6) and is important in breast cancer etiology (144-146). It is possible that E2 interacts with the IGF-I system either systemically or in the local environment of the mammary gland to promote tumorigenesis in animals exposed to alcohol *in utero*. Therefore, the objectives of this study were to examine the effect of alcohol exposure *in utero* on risk of mammary carcinogenesis, characterize the resulting tumor phenotype, and determine a potential role for alterations in the E2 and IGF systems in mediating this effect.

Materials and Methods

Animal Model:

Pregnant Sprague-Dawley rats were purchased from Charles River (Wilmington, MA) and individually housed in a controlled environment with a 12 h light/dark cycle. Dams were fed a liquid diet containing ethanol (alcohol-fed) (Bio-Serv, Frenchtown, NJ), an isocaloric liquid diet (pair-fed) (Bio-Serv), or *ad libitum* rat chow (ad lib-fed) (Purina Mills Lab Diet, St. Louis, MO). Dams were acclimated to the alcohol diet from d 7 to 11 of gestation by feeding a liquid diet containing 2.2% ethanol on d 7 and 8 and 4.4% ethanol on d 9 and 10. Once acclimated, dams were fed the liquid diet containing 6.7% ethanol from d 11-21, which represented 35% of total calories. At birth female pups were cross-fostered to ad lib-fed dams and litters were normalized to 8 pups per dam. Pups were weaned at 21 d of age and fed rat chow *ad libitum* for the remainder of the experiment. Animal care was performed in accordance with institutional guidelines and complied with National Institutes of Health policy.

Study 1:

Ten female pups from each treatment group were administered a single intraperitoneal (I.P.) injection of 50 mg/kg body weight N-nitroso-N-methylurea (NMU) (Sigma, St. Louis, MO) to induce mammary tumors. Five female pups from each group were administered vehicle (0.9% NaCl) to determine if spontaneous mammary tumors were observed in any treatment group. NMU and vehicle injections were given at approximately 50 days of age. Rats were weighed weekly and mammary glands were palpated twice a week to assess tumor latency. Rats were euthanized by decapitation at

23 weeks after NMU injection. A full necropsy was performed and all major organs were macroscopically examined to ensure the carcinogen was mammary-specific. Mammary tumors were harvested and half of each tumor was fixed in 10% neutral buffered formalin for histological analysis. The remaining half was snap-frozen in liquid nitrogen and stored at -80°C for RNA analysis.

Study 2:

In order to determine the effect of alcohol exposure *in utero* on circulating E2 in the offspring, a second study was conducted without NMU. Beginning at day 42 of age, the estrous cycle was monitored (n = 13 per group) by daily vaginal cytology for two to three weeks. Once animals were exhibiting normal 4-day cycles, they were euthanized during proestrus by rapid decapitation between 62 and 76 days of age. Trunk blood was collected to assess circulating E2 and IGF-I concentrations.

Histology:

Fixed tissue was dehydrated, cleared, and embedded in Paraplast using facilities located in the Histopathology Core of The Environmental Occupational Health Sciences Institute (EOHSI) at Rutgers University. Samples were sectioned at 6 μm and placed on slides. Slides were baked for 30 min at 60°C, followed by deparaffinization in xylene and rehydration in decreasing concentrations of ethanol. Slides were stained with hematoxylin-eosin and mounted with Permount (Fisher Scientific, Pittsburgh, PA). Slides were evaluated by toxicological pathologist Dr. Kenneth Reuhl, who was blinded to treatment.

Immunohistochemistry (IHC):

For ER-α staining, samples were processed as described above. Slides were baked at 60°C for 30 min, deparaffinized in xylene, and rehydrated in isopropanol. Antigen retrieval was performed by boiling slides in 0.01 M sodium citrate buffer for 10 min. After cooling, slides were submerged in 3% H_2O_2 for 5 min to quench endogenous peroxidase activity, blocked in normal horse serum (Vector Labs, Burlingame, CA) for 20 min, and incubated with rabbit ER-α primary antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. On each slide, one section was incubated with rabbit isotype IgG (Invitrogen, Carlsbad, CA) as a negative control. The next day, slides were incubated with biotinylated anti-rabbit secondary antibody (1:200) (Santa Cruz) for 40 min, then with ABC reagent from the ABC Elite Vectastain kit (Vector Labs) for 40 min. After rinsing in tris-buffered saline, slides were submerged in 3, 3'-diaminobenzidine (Sigma) for 7 min, counterstained with hematoxylin and mounted in Permount (Fisher Scientific). Three members of the laboratory viewed all tumors blindly and classified them as highly positive, positive, mostly negative, or negative. Tumors determined to be highly positive and positive were combined and termed positive, while those determined to be mostly negative and negative were combined and termed negative.

Western Immunoblot Analysis:

Frozen tissue was sheared in radioimmunoprecipitation (RIPA) lysis buffer then incubated on ice for 1 hr. The protein lysate was centrifuged at 14,000 x G for 5 min at 4°C. The upper fat layer was removed and the supernatant was transferred to a fresh tube. Protein concentration was determined using a BioRad protein assay (Hercules,

CA). Proteins were separated by SDS-PAGE, transferred, then immunoblotted using an IGFBP-5 antibody (Santa Cruz) followed by a β-actin-specific antibody (Calbiochem, Darmstadt, Germany) as previously described (Fleming et al., 2005). Protein from a single tumor sample was run on each gel as a calibrator. Densitometry was performed using an Alphaimager imaging system (Alpha Innotech, San Leandro, CA). Bands were corrected for loading, then normalized to the calibrator for comparison across gels.

Serum Analysis:

Serum was analyzed for E2 by a competitive enzyme-linked immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI) (sensitivity = 20 pg/ml), and for IGF-I using an enzyme-linked immunosorbent assay (ELISA) (Immunodiagnostic Systems, Fountain Hills, AZ) (sensitivity = 63 ng/ml) according to manufacturer's recommendations. All samples were run on one 96 well plate for each variable.

RNA Analysis:

Frozen mammary tissue was homogenized in Trizol (Sigma) and RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. RNA was treated with DNase (Qiagen) during isolation. RNA quality was verified using the Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). The High Capacity cDNA Reverse Transcription kit (Applied Biosystems (ABI), Foster City, CA) was used to reverse transcribe 2 µg of RNA. Primer sets were developed using PrimerQuest (IDT, Coralville, IA), and each primer set was validated as previously described (147). Primer sets for IGFBP-5

(forward, 5'TTGAGGAAACTGAGGACCTCGGAA3'; reverse, 5'CCTTCTCTGTCCGT TCAACTTGCT3'), and actin (forward, 5'CCATTGAACACGGCATTGTCACCA3'; reverse, 5'GCCACACGCAGCTCATTGTAGAAA3') were obtained from Sigma Genosys (St. Louis, MO). Samples were diluted 1:4 and 5 µl were amplified in a 20 µl reaction mix containing 10 µl Power SYBR Green PCR Master Mix (ABI), 4 µl ultrapure H_2O, and 0.5 µl (200 µM) of each forward and reverse gene-specific primer. Quantitative real-time PCR was performed on 384 well plates (ABI) using an ABI 7900 HT Real-Time PCR system. For each experimental sample, fold-change relative to a calibrator sample was determined using the $2^{-\Delta\Delta CT}$ method with actin as the housekeeping gene. The calibrator sample represented a pool of tumor RNA consisting of 2 RNA samples from each of the 3 treatment groups.

Statistical Analysis:

Differences in body weight, tumors per group, and tumors per animal were assessed using Kruskal-Wallis non-parametric one way ANOVA, with a Dunn's Multiple Comparison post-hoc analysis at the level of $\alpha = 0.05$. Percent of rats with tumors was analyzed using a logrank test at the level of $\alpha = 0.05$. To evaluate tumor type, a Chi Square test was performed. ER positivity was evaluated using two-way ANOVA with a Bonferroni post-test at the level of $\alpha = 0.05$. Differences in circulating E2 and IGF-I levels were assessed using one way ANOVA with a Newman-Keuls post-hoc analysis at the level of $\alpha = 0.05$. IGFBP-5 mRNA and protein values were evaluated using Kruskal-Wallis non-parametric one way ANOVA, with a Dunn's Multiple comparison post-hoc test at the level of $\alpha = 0.05$.

Results

Mammary tumorigenesis is increased in animals exposed to alcohol in utero

Mammary tumors were first detected at 8 weeks post-NMU injection, with one tumor present in the alcohol-fed group and one in the ad lib-fed control group (Fig. 1a). Thereafter, tumor number increased steadily in the alcohol-fed group, while only two tumors were detected through week 15 in the ad lib-fed control group. Tumors were not detected in the pair-fed control group until 12 weeks post injection. From this point, the rate of increase in tumor number paralleled that of the alcohol-fed group, but the absolute number of tumors remained lower in the pair-fed control group at all time points. At week 16 post-injection, the alcohol-fed group had significantly more tumors, while the number of tumors in the pair-fed control and ad lib-fed control groups did not differ from each other, indicating that tumor latency was decreased by alcohol exposure *in utero* (Fig. 1a and b). The percent of rats with palpable tumors was similar by 13 weeks post-injection in the alcohol-fed and pair-fed control groups, while remaining lower in the ad lib-fed control group (Fig. 1c). Greater overall tumor multiplicity in the alcohol-fed group at this time was due to the finding that, in general, animals in the alcohol-fed group exhibited more tumors per animal at each time point (Fig. 1d). Rats that received vehicle alone did not develop tumors.

In order to determine if overall tumor multiplicity in response to NMU was increased by exposure to alcohol *in utero*, the study was continued until tumor numbers had reached a plateau in all groups. At 23 weeks post-injection, 60 to 70% of rats in each group injected with NMU presented with tumors (Fig. 1c). At study termination, the alcohol-fed group was no longer different from the pair-fed control group, but remained

different from the ad lib-fed control group. As expected, animals exposed to alcohol exhibited decreased body weight that was maintained over the course of the study compared to either control group (Fig. 1e).

Figure 1.

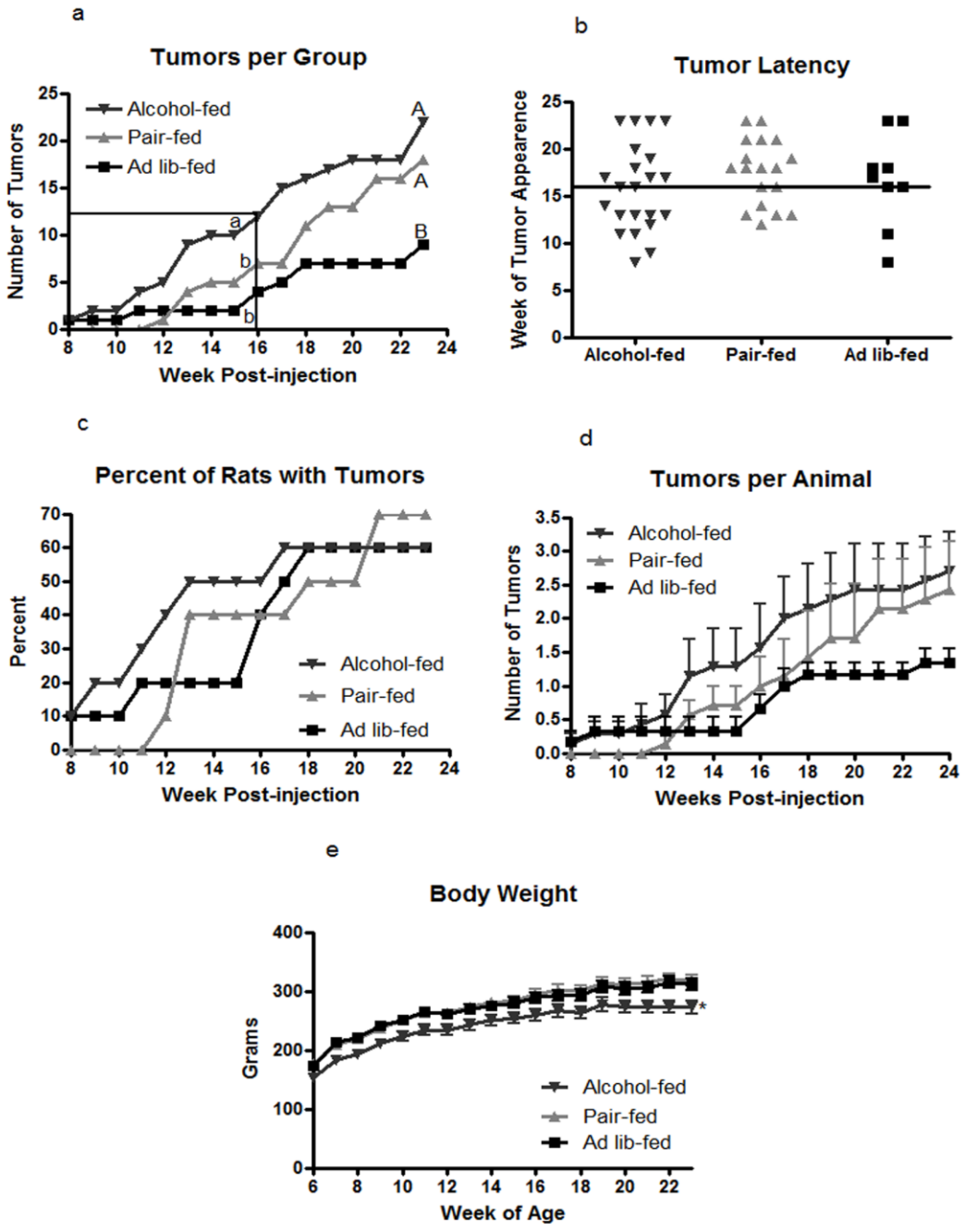

Figure 1. Alcohol exposure *in utero* enhances mammary tumorigenesis.

Animals exposed to alcohol *in utero* and control rats not exposed were administered a single I.P. injection of NMU at 50 mg/kg at approximately 50 days of age. Rats were palpated for tumors twice a week following injections. (a) Total number of tumors per treatment group each week post NMU injection. The boxed area represents when 50% tumor incidence was reached across all groups. Two time intervals were analyzed: 8-16 weeks (lower case letters, $P < 0.05$, n=10), and 8-23 weeks (upper case letters, $P < 0.05$, n=10). (b) Week of tumor appearance. Each data point represents an individual tumor. The horizontal line at week 16 post-injection represents when 50% tumor incidence occurred. (c) Percent of rats presenting with tumors each week post NMU injection, $P = 0.9494$, n=10. (d) Average number of tumors per animal in each group ± SEM. (e) Average body weight ± SEM with * denoting a significant difference ($P < 0.005$, n=10). Data in panels a, d, and e were analyzed using a Kruskal-Wallis non-parametric one way ANOVA with a Dunn's Multiple Comparison post-hoc analysis at the level of $\alpha = 0.05$, and panel c was analyzed using a log-rank test.

Tumor stage is altered in animals exposed to alcohol

The stage of progression of each tumor was evaluated by histological analysis (Fig. 2). For tumor classification, ductal/cystic hyperplasia was defined by increased proliferation of benign glandular structures, with predominantly regular cells and nuclei. Adenomas were defined by a more solid phase glandular structure with regular cells and nuclei still predominating. Adenocarcinomas presented primarily as solid-phase lesions containing many atypical and anaplastic cells, a high mitotic rate (including numerous atypical mitoses) and observable zones of tumor necrosis. Rats exposed to alcohol *in utero* developed more adenocarcinomas compared to the other two groups (Table 1). Animals in the pair-fed group developed mostly hyperplastic tumors, with few adenocarcinomas or adenomas. The ad lib-fed group contained mostly adenomas with only moderate hyperplasia and few adenocarcinomas. Treatment was found to be

associated with histological tumor type (Fig. 2a). These data suggest that *in utero* exposure to alcohol leads to a more malignant phenotype in response to a carcinogenic insult.

Figure 2.

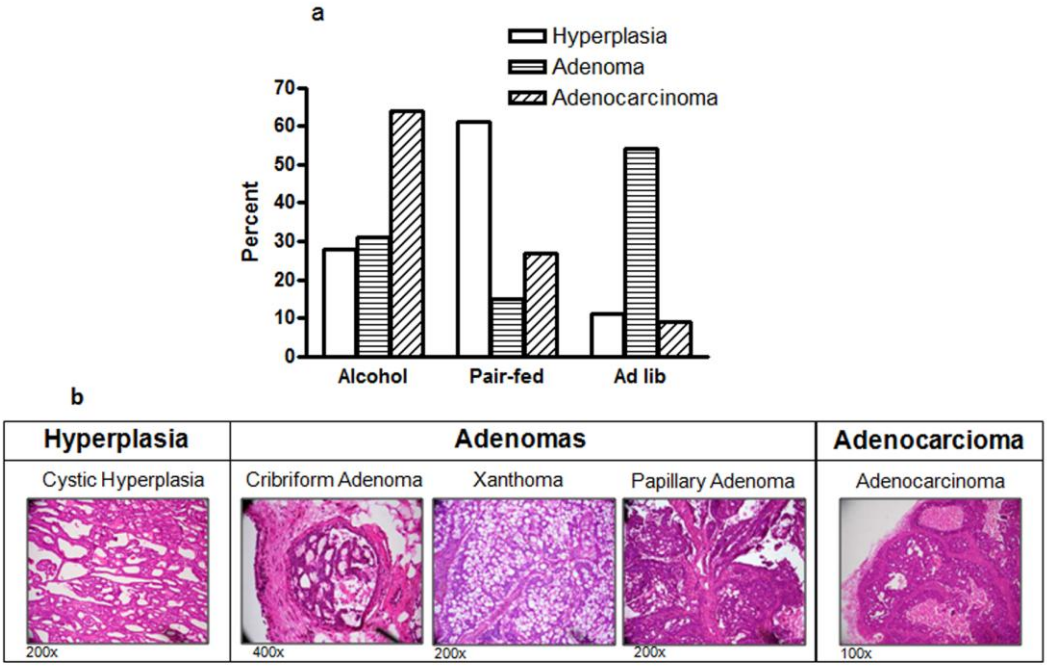

Figure 2. Alcohol exposure *in utero* results in altered tumor development.

Tumors were hematoxylin and eosin stained for histological evaluation. (a) Percent of tumors of each histological tumor type per treatment group ($P < 0.01$, n=15; Chi-square test). (b) Representative images of different histological tumor types induced in response to NMU.

Table 1. Number of Specific Histological Tumor Types per Group

	Hyperplasia			Adenoma			Adenocarcinoma		
Treatment	Number of tumors	*n*[a]	Tumors per animal	Number of tumors	*n*[a]	Tumors per animal	Number of tumors	*n*[a]	Tumors per animal
Alcohol-fed	5	3	1–3	4	3	1–2	7	3	2–3
Pair-fed	11	6	1–3	2	1	2	3	3	1
Ad lib-fed	2	1	1	7	5	1–2	1	1	1

[a]*n*, number of rats.

Animals exposed to alcohol in utero developed more ER negative tumors

IHC was conducted to determine if tumors were ER positive or ER negative. Overall, 27 tumors stained positive and 15 tumors stained negative. Animals exposed to alcohol *in utero* developed more ER negative tumors in response to NMU compared with the two control groups, which did not differ from each other (Fig. 3a). Representative tumors that were considered ER positive and ER negative are shown in Fig. 3b.

Figure 3.

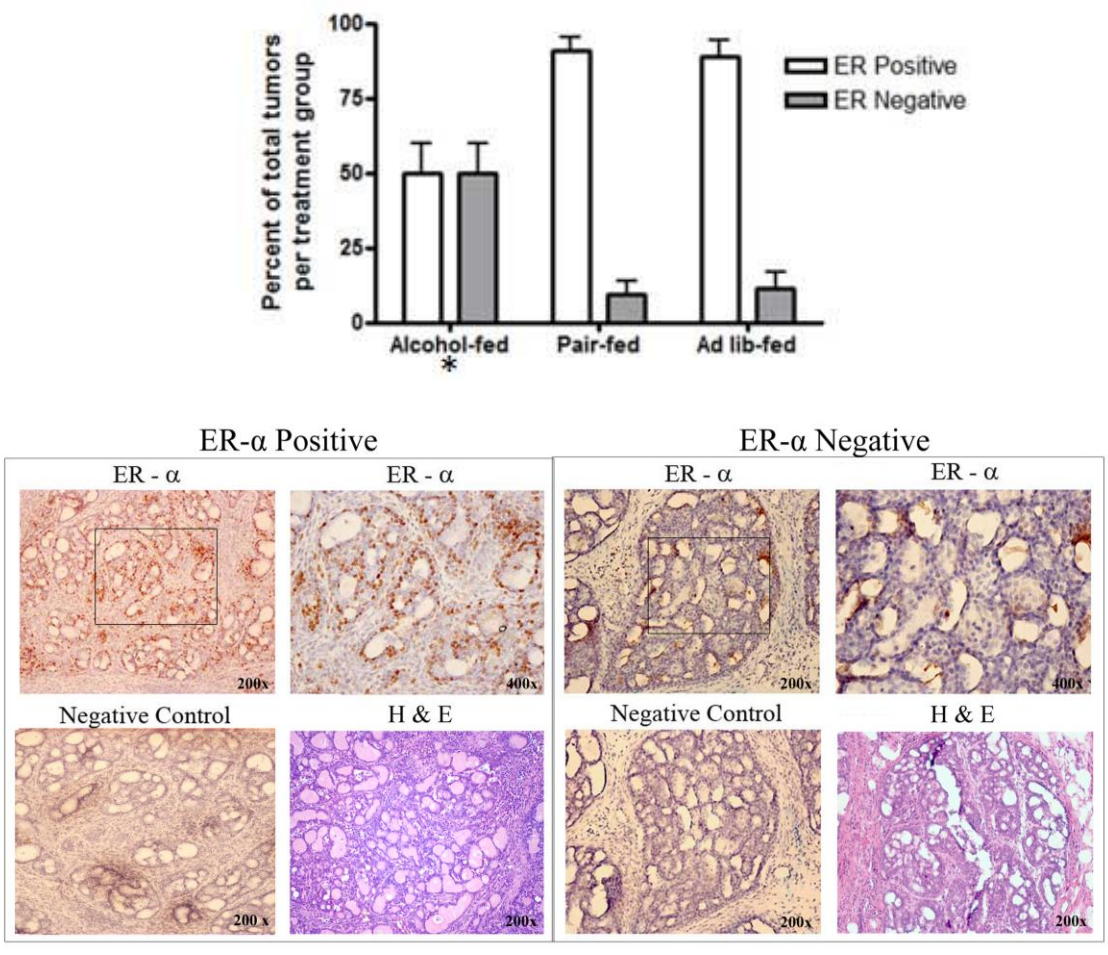

Figure 3. Alcohol exposure *in utero* results in more ER negative tumors.

Tumors were stained for ER-α as described in the Materials and Methods. (a) Percent of positive or negative tumors in each group. One tumor was arbitrarily chosen from each animal for statistical analysis. A * denotes a statistically significant difference from either control group ($P < 0.001$, n=6; two-way ANOVA with Bonferroni post-test at the level of $\alpha = 0.05$). (b) Representative images of ER-positive and ER-negative tumors are shown, the 400x magnification represents the boxed area on the 200x magnification. H & E = hematoxylin and eosin; Negative Control = non-specific IgG.

IGFBP-5 mRNA is suppressed in tumors developed in alcohol-exposed animals

The IGF system is involved in both normal mammary gland biology as well as mammary cancer. Therefore, we analyzed mRNA levels in tumor tissue of IGFR, IGF-I, IGFBP-3, and IGFBP-5. While no differences in IGFR, IGF-I, or IGFBP-3 mRNA were detected in tumors across treatment groups (data not shown), animals exposed to alcohol *in utero* developed tumors with reduced levels of IGFBP-5 mRNA compared to tumors that developed in pair-fed control animals (Fig. 4a). Western blot analysis indicated that this difference in IGFBP-5 expression was also observed at the protein level (Fig. 4b).

Figure 4.

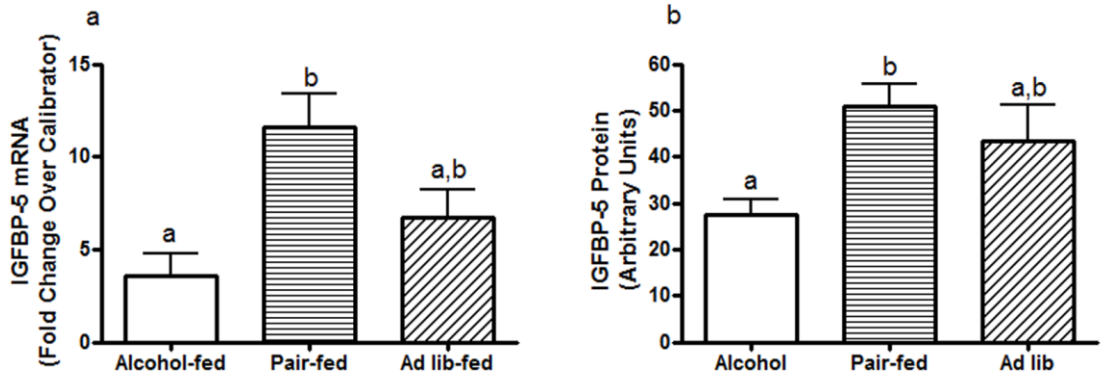

Figure 4. IGFBP-5 mRNA expression is reduced in tumors of animals exposed to alcohol *in utero.*

All mammary tumors were collected at necropsy and one tumor from each animal was randomly selected from each animal for analysis. (a) Quantitative real-time PCR was performed to determine IGFBP-5 mRNA expression. Fold-change was calculated relative to a calibrator using the $2^{-\Delta\Delta CT}$ method with actin as the housekeeping gene. The calibrator consisted of a pool of tumor RNA including 2 samples from each treatment group. ($P < 0.05$, $n = 7$; non-parametric one-way ANOVA with Dunn's post-test at the level of $\alpha = 0.05$). (b) Western immunoblot was performed using an IGFBP-5 specific antibody. Samples were corrected for loading with actin. ($P<0.01$, $n=7$; non-parametric one way ANOVA with Dunn's post-test at the level of $\alpha=0.05$).

Circulating E2 and IGF-I are altered in animals exposed to alcohol in utero

In a second study, pregnant dams were again fed the same dietary treatments (alcohol-fed, pair-fed, and ad lib-fed from day 11-21 of gestation). Offspring were sacrificed during the proestrus stage of the estrous cycle. Rats exposed to alcohol *in utero* displayed higher circulating E2 compared to the two control groups, which did not differ from each other (Fig. 5a). Circulating IGF-I was increased in the alcohol group compared to the ad lib group, while the alcohol group was not different from the pair-fed group (Fig. 5b). These data suggest that alterations in circulating E2 and possibly IGF-I may be involved in the increased tumorigenesis that is observed in animals exposed to alcohol *in utero*.

Figure 5.

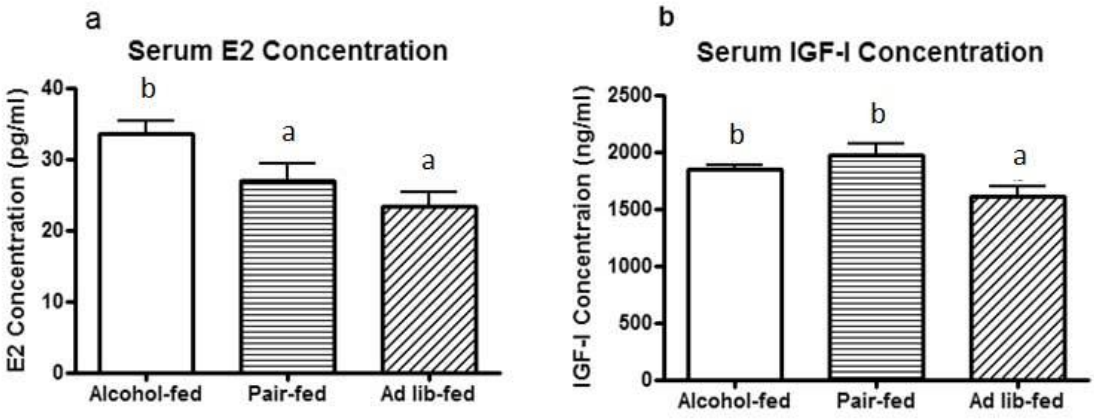

Figure 5. Circulating E2 and IGF-I are altered in pups exposed to alcohol *in utero.*

Pups exposed to alcohol *in utero* and controls not exposed were sacrificed during proestrus. Serum E2 and IGF-I were determined by EIA and ELISA, respectively. Statistical significance is denoted by different letters ($P < 0.05$, $n = 13$; one-way ANOVA with Newman-Keuls post-test at the level of $\alpha = 0.05$).

Discussion

Many diseases have been linked to a suboptimal fetal environment, including hypertension, diabetes, and heart disease (21, 148, 149). While a connection between the fetal environment and breast cancer has been proposed (132, 134, 150), only one previous study has addressed alcohol as the fetal insult (23). In the present study, pregnant dams were fed 6.7% alcohol. The expected blood alcohol levels in these animals would be 100 to 150 mg/dl (151, 152), which would be equivalent to three to five drinks in two hours for a woman (153). Offspring of the alcohol-fed dams exhibited an increase in tumor multiplicity in response to NMU at 16 weeks post-injection. A similar result was observed by Hilakivi-Clarke et. al (23) using a more moderate level of alcohol, though no effect on tumor multiplicity was observed when dams were fed a low level of alcohol. Thus, the results of these two studies indicate that both moderate to high alcohol intake

during pregnancy may significantly impact breast cancer susceptibility in offspring.

Hilakivi-Clarke et al. (23) reported that circulating maternal E2 increased when dams were drinking alcohol and suggested that this may play a role in the increased risk for mammary tumors in the offspring. While E2 levels were not measured in the dams in the present study, we did find that circulating E2 levels were significantly higher during proestrus in the alcohol-exposed offspring. It is widely accepted that a woman's endogenous E2 levels over her lifetime can affect her breast cancer risk, therefore, this could be a contributing factor to the increase in mammary tumor susceptibility observed in the offspring in the present study. Recently, Lan and co-workers found that E2 was higher during proestrus compared to other phases of the cycle in both rat offspring exposed to alcohol as well as pair-fed controls relative to ad lib-fed controls (141). However, in the Lan et al. study, alcohol-exposed and pair-fed pups remained with their dams after birth, with both groups weighing less throughout the study relative to ad-lib fed controls. Therefore, the authors concluded that these differences were largely due to nutritional status. In the present study, all animals nursed from dams that did not receive alcohol. The offspring of the two control groups were of similar weight throughout the study, with only the alcohol-exposed offspring showing a reduced body weight and an increase in circulating E2 levels.

Increased circulating E2 levels could contribute to mammary tumorigenesis through multiple mechanisms (154). E2 metabolites such as 2- and 4-OH-E2 have been shown to directly bind DNA and introduce DNA adducts, thus inducing mutagenesis (52, 53). In addition, the conversion of E2 to 4-OH-E2 during E2 metabolism results in the formation of reactive oxygen species that are also carcinogenic (51). E2 may contribute

to tumor susceptibility by activating ER-α and promoting cell proliferation. This can occur through both classical genomic effects as well as more recently recognized non-genomic effects (155). The classical ligand-dependent mode of E2 action involves binding of the E2/ER-α complex to specific estrogen response elements (EREs) and enhancers located within the regulatory regions of target genes (156). In addition, this complex can upregulate expression of genes that lack classical EREs by binding to other DNA-bound transcription factors that tether the activated ER to DNA (157). In contrast, the non-genomic effects of ER may involve cross-talk with tyrosine kinase receptors including IGFR at the level of the plasma membrane (158, 159).

In addition to cross-talk with the IGF signaling pathway, E2 can also upregulate expression of individual components of the IGF signaling pathway (158). For example, E2 can increase IGFR expression, as well as that of IRS-1 in breast cancer cells (160, 161). This regulation is also observed *in vivo* in mammary tissue of ovariectomized rats and mice (162, 163). While we found no differences in mRNA levels of IGFR, IGF-I, or IGFBP-3 between tumors from the three treatment groups in the present study, we found that IGFBP-5 mRNA and protein were significantly reduced in the tumor tissue from alcohol-exposed rats when compared to pair-fed controls.

A large body of evidence supports a role for IGFBP-5 as a growth-inhibitory and/or pro-apoptotic factor in the mammary gland. Many studies have shown a strong positive relationship between IGFBP-5 expression and the occurrence of cell death during mammary involution (164-166). Transgenic overexpression of IGFBP-5 in mammary tissue resulted in increased apoptotic death of epithelial cells and reduced invasion of the mammary fat pad, while addition of exogenous IGFBP-5 to murine

mammary epithelial cells suppressed IGF-I mediated survival (101, 110). Furthermore, addition of exogenous IGFBP-5 to human breast cancer cells inhibited growth *in vitro* (104). Many of these pro-apoptotic, growth-inhibitory effects have been thought to be related to the ability of IGFBP-5 to sequester IGF-I and prevent its pro-survival and growth-stimulatory effects. Therefore, in the present study the decrease in IGFBP-5 expression in tumors of rats exposed to alcohol *in utero* may have allowed more free IGF-I to access the IGFR and promote tumorigenesis. However, we cannot rule out IGF-independent roles for IGFBP-5, which have also been suggested (167).

Interestingly, previous work has shown an inverse correlation between E2 and IGFBP-5 expression in breast cancer cells. In MCF-7 cells, E2 decreased IGFBP-5 expression while the anti-estrogen ICI 182780 (ICI) increased IGFBP-5 expression (117, 168). Similarly, ICI stimulated IGFBP-5 expression in DMBA-induced mammary tumors (117). These authors also showed that reducing IGFBP-5 expression in MCF-7 cells with IGFBP-5 antisense oligonucleotides increased DNA synthesis in both untreated and ICI-treated MCF-7 cells. They therefore suggested that E2-stimulated proliferation involved reducing the inhibitory effect of IGFBP-5. These findings support the present study where increases in circulating E2 and decreases in tumor IGFBP-5 expression were observed in animals that exhibited an overall increase in tumorigenesis in response to NMU. It is interesting to speculate that this may represent an IGF-independent mechanism of growth inhibition by IGFBP-5.

Interestingly, at study termination the alcohol-exposed animals had more ER negative tumors as well as more adenocarcinomas. In the polyoma middle T oncoprotein (PyMT) mouse model of mammary tumor progression, Lin et al. (2003) showed that

mammary tumors in these mice lost ER positivity as they progressed to the more malignant phenotype (169). These data support our findings that mammary tumors that arise in alcohol-exposed offspring are more likely to progress to a malignant phenotype compared to animals that are not exposed to alcohol *in utero*.

The results of the present study show that alcohol exposure *in utero* leads to an increased susceptibility to mammary carcinogenesis in adulthood. Furthermore, the resulting tumors are characterized by a more malignant phenotype with increased ER-negative status. The idea that alcohol exposure *in utero* leads to tumors with a worse prognosis is a novel finding and warrants future investigation into the mechanisms that underlie this tumor phenotype. Our data also show that E2 and components of the IGF system may play a role in this enhanced susceptibility. Further studies will be aimed at delineating the mechanisms by which the E2 and IGF systems are altered by alcohol exposure *in utero.*

CHAPTER 3

Fetal alcohol exposure advances mammary gland development by altering local IGF-I and estradiol.

Introduction

Exposure to alcohol *in utero* results in a range of adverse outcomes collectively referred to as fetal alcohol spectrum disorder, the most severe cases manifesting as fetal alcohol syndrome (FAS). Despite this information, the Centers for Disease Control reports that 1 in 8 women drink and 1 in 50 women binge drink while pregnant (136). This is substantiated by reports that 0.28-0.46% of newborns in the United States and Europe are diagnosed with FAS (170). In addition to the effects of alcohol exposure observed early in life, considerable evidence suggests that a sub-optimal *in utero* environment can increase an offspring's risk of disease in adulthood, such as diabetes, cardiovascular disease, and cancer (19, 171). Alcohol exposure *in utero* increases susceptibility to carcinogen-induced mammary tumors in rodent offspring (23, 24). In addition, these animals develop more tumors with phenotypic characteristics of poor-prognosis breast cancer (24). A recent epidemiological study showed that fetal alcohol exposure increases the risk for acute myeloid leukemia in children (20), which suggests that findings in animal models may be relevant to human disease.

Early developmental changes in the mammary gland could affect susceptibility to breast cancer later in life (172). Development of the mammary gland begins at day 10 of gestation in the rat. At birth, a primordial gland embedded in a fat pad precursor is

observed. The gland undergoes isometric growth until puberty when growth becomes allometric. Terminal end buds (TEBs) lead ductal elongation through the fat pad creating a network of branching ducts decorated with alveolar buds (173). This process is regulated by estradiol (E2) and insulin-like growth factor-I (IGF-I), which are both necessary for normal mammary gland development (6) and play integral roles in breast cancer etiology (174). We have previously shown that circulating E2 is higher in adult offspring that were exposed to alcohol *in utero* (24). In addition, Hilakivi-Clarke et al. (23) found that fetal alcohol exposure leads to increased estrogen receptor-α (ER-α) expression in the mammary glands of 22 week-old offspring. Since the E2 and IGF signaling systems exhibit cross-talk (142, 143), we predicted that fetal alcohol exposure might lead to enhanced expression of E2 and IGF system components that would ultimately advance mammary gland development. The current study was conducted to determine if alcohol exposure *in utero* alters normal mammary gland development through enhanced IGF and E2 action, which might contribute to increased susceptibility to mammary cancer in adulthood.

Materials and Methods

Animal Model:

Pregnant Sprague Dawley rats were purchased from Charles River (Wilmington, MA) and housed in a controlled environment. Dams were acclimated to the environment for 2 days, then divided into 3 groups (n = 7) and fed a liquid diet containing ethanol (alcohol-fed; Bio-Serv, Frenchtown, NJ), an isocaloric liquid diet without ethanol (pair-fed; Bio-Serv), or *ad libitum* rat chow (ad lib; Purina Mills Lab Diet, St. Louis, MO) from

d 7 to 21 of gestation. Alcohol-fed dams were acclimated to increasing amounts of ethanol (2.2% (v/v) d 7-8 and 4.4% (v/v) d 9-10) and began receiving 6.7% (v/v) alcohol on day 11 of gestation. At birth, all female pups were cross-fostered to ad lib-fed dams and litters were normalized to eight pups per dam. Animals were weighed weekly until euthanasia. On post-natal day (PND) 20, offspring from each of the three *in utero* treatment groups were euthanized by rapid decapitation. The remaining pups were weaned at 21 days of age, fed rat chow *ad lib* for the remainder of the experiment, and euthanized on PND 40 or 80 (n = 9-13 offspring per treatment group per time point). Animals were injected intraperitoneally 2 and 4 h before euthanasia with BrdU (70 mg/kg body weight/injection; Acros Organics, Geel, Belgium). A second study was conducted using the same experimental design to confirm changes in mammary morphology observed at PND 20 (n = 10 per treatment group). Animal care was performed in accordance with institutional guidelines and complied with National Institutes of Health policy.

Sample Collection:

Livers, trunk blood, and mammary glands from the fifth inguinal gland were harvested from each animal. Half of each mammary gland was fixed in 10% neutral buffered formalin (NBF) (Richard Alan Scientific, Kalamazoo, MI) for histological analyses. The liver and the remaining half of the mammary gland were frozen in liquid nitrogen and stored at -80°C for RNA and protein analyses. The contralateral fifth inguinal mammary gland was left adhered to the skin and fixed in 10% NBF for whole mount analysis.

Whole Mount Analysis:

After fixing in 10% NBF for 2 days, glands were dissected away from the skin, placed on slides, and allowed to air dry for 30 min. Glands were then rehydrated in 70, 50, and 25% ethanol for 15 min each, placed in H_2O for 5 min, and stained in carmine alum overnight. Carmine alum stain was made fresh by boiling 2 g carmine and 5 g aluminum potassium sulfate (Sigma-Aldrich, St. Louis, MO) in 800 ml H_2O for 20 min, filling to 1 L volume, and filtering through no. 1 Whatman paper. After staining, slides were dehydrated in 70 and 95% ethanol for 15 min each followed by xylene 2 x 30 min. PND 40 and 80 glands were cleared in toluene for 1 week after dehydration to remove excess fat. Glands were dried for 1 h before mounting in SealPAK pouches (Kapak, Minneapolis, MN) with cedarwood oil (Sigma-Aldrich). PND 20 mammary glands were imaged using a Nikon SMZ1000 dissecting scope (Nikon, Melville, NY) with ACT-1 software (Nikon) for high magnification images. High magnification images were used to quantitate TEB size, number, and epithelial structure area (i.e. mammary density) with Image Pro Plus (Media Cybernetics, Bethesda, MD) at the Facility for Confocal Imaging at Rutgers University. PND 20, 40 and 80 were imaged using a Nikon DS-Fi1 camera (Nikon) with NIS Elements software (Nikon) to capture the entire gland at lower magnification. Mammary gland length was measured from the center of the lymph node to 3 different points on the leading edge of the gland.

Serum Analysis:

Trunk blood was kept on ice for 4 h and spun at 5,000 rpm, then serum was collected and stored at -80°C. Serum IGF-I levels were detected using an ELISA

(Immunodiagnostic Sytems, Fountain Hills, AZ) (sensitivity = 63 ng/ml) as per kit instructions. Serum IGFBP-3 levels were detected using ligand blot analysis. Serum E2 was detected by a competitive enzyme-linked immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI) (sensitivity = 20 pg/ml).

Immunohistochemistry:

Fixed mammary gland tissue was dehydrated, cleared, embedded in Paraplast, and sectioned onto slides at the Histopathology Core of The Environmental Occupational Health Sciences Institute at Rutgers University. Slides were immuno-stained as previously described (24) using BrdU (1:200; BD Biosciences, San Jose, CA), and CYP19 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies. The ABC elite Vectastain kit (Vector Labs, Burlingame, CA) was used for primary antibody detection. Slides were stained in 3,3'-diaminobenzidine (Sigma), counterstained with hematoxylin (Sigma) and mounted in Permount (Fisher Scientific, Pittsburgh, PA) as described previously (24). For BrdU analysis, all epithelial cells and BrdU stained cells were counted and presented as a percent of total epithelial cells stained for BrdU. For CYP19 analysis, three representative ductal areas at 200x magnification were counted and data were presented as the number of stained cells per ductal area.

Apoptosis Analysis:

Slides were prepared as described above and stained for DNA fragmentation using the *In Situ* Cell Death Detection kit (Roche, Basel, Switzerland) as per kit instructions. Sections were counterstained with Hoechst dye (Invitrogen, Carlsbad, CA)

and imaged using an Olympus BX41 fluorescent scope (Olympus, Center Valley, PA) with MetaMorph software (Molecular Devices, Sunnyvale, CA). For analysis, labeled cells in 4 fields at 200x magnification were counted and data were presented as the number of apoptotic cells per field.

Ligand Blot Analysis:

Proteins in serum (4 μl) were separated under non-reducing conditions by SDS-PAGE, transferred to nitrocellulose membranes, then ligand-blotted with ^{125}I-IGF-I as previously described (175). Membranes were exposed to phosphorimaging screens and scanned using a Storm 860 (GE Healthcare, Piscataway, NJ). Densitometry was performed using ImageQuant 5.2 software (GE Healthcare). Bands were normalized to a calibrator sample which was run on each gel for comparison across gels.

RNA Analysis:

Mammary tissue (100 mg) was homogenized in Trizol (Sigma) and RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA) including DNase treatment according to the manufacturer's specifications. RNA quality was verified using the Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). The High Capacity cDNA Reverse Transcription kit (Applied Biosystems (ABI), Foster City, CA) was used to reverse transcribe 2 μg of RNA. Primer sets for IGF-I (forward: 5'CTGTGCAGTTCGCCCATTGTTTGA3'; reverse: 5'ACATTTGGACACCCAGGCAGGTAT3'), IGFBP-5, and β-actin (24) were developed using PrimerQuest (IDT, Coralville, IA), purchased from Sigma-Aldrich, and validated as

previously described (175). Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (ABI) on 384 well plates (ABI) using an ABI 7900 HT Real-Time PCR system as described previously (24). For each experimental sample, fold-change relative to a calibrator sample was determined using the $2^{-\Delta\Delta ct}$ method with β-actin as the housekeeping gene. The calibrator sample was a pool of mammary gland RNA consisting of 2 RNA samples from each of the 3 treatment groups.

Statistical Analysis:

Treatment differences were assessed using one-way or two-way ANOVA with a Newman-Keuls or Bonferroni post-hoc analysis at the level of $\alpha = 0.05$. GraphPad Prism 5 was used to perform statistical analysis.

Results

Mammary gland morphology

Mammary gland morphology was assessed by whole mount analysis at PND 20, 40, and 80, which correspond with pre-pubertal, postpubertal, and mature developmental time points, respectively (Fig 1a). The number of TEBs was highest at PND 40 when the ducts are actively elongating through the fat pad (Fig 1b). At PND 20, alcohol-exposed offspring had significantly more TEBs than either control group, which were not different from each other (Fig 1b). This effect was not observed at PND 40 or 80 (Fig 1b). There was no effect of *in utero* alcohol exposure on the size of TEBs, the length of the ductal tree, or the density of the mammary parenchyma at any time point (data not shown).

Figure 1.

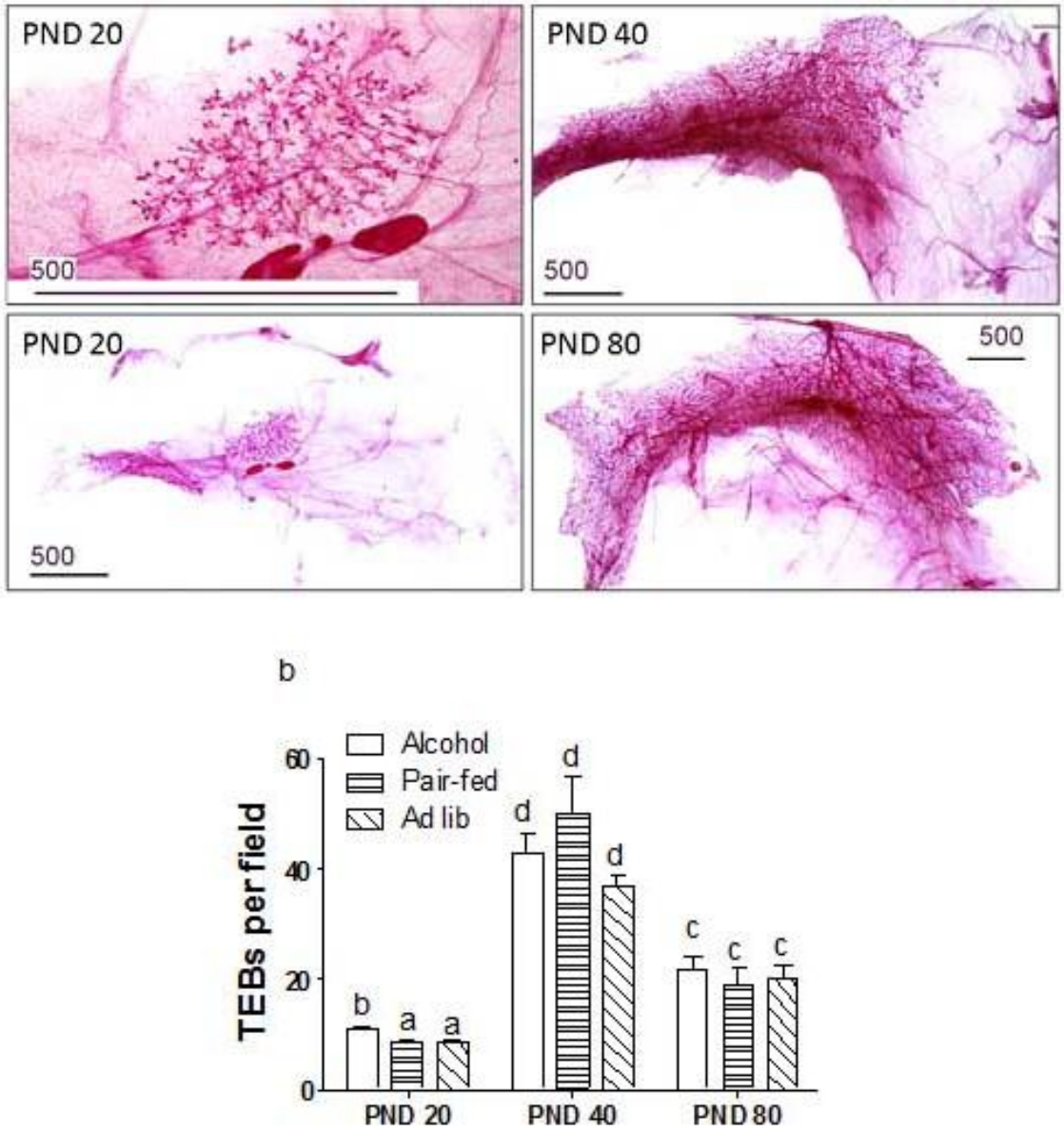

Fig. 1 Alcohol exposure *in utero* leads to an increase in TEB number at PND 20

Pups exposed to alcohol *in utero* and controls not exposed were euthanized at PND 20, 40, or 80. The fifth inguinal mammary gland was processed for whole mount analysis and TEBs were counted in 3 fields of view at 10x magnification for each gland. (a) Representative whole mount images from each time point. (b) Number of TEBs per field of view. Bars represent mean ± standard error (SE) with different letters

denoting significant differences (n = 20 for PND 20 and n = 10, 9, 13 for alcohol, pair-fed, and ad lib groups, respectively for PND 40 and 80). A second experiment was conducted at PND 20 to examine TEB number. In the first experiment including all time points, the mean TEB numbers for PND 20 were 12.5, 10.8, and 10.0, and in the second experiment including only PND 20 the means were 9.2, 7.4, and 6.2 for alcohol, pair-fed, and ad lib, respectively. A one-way ANOVA was performed to test if there was an interaction between treatment and experiment at PND 20. There was no interaction between treatment and experiment, with a significant effect of treatment, and a significant effect of experiment. Either experiment alone did not have a significant treatment effect. When both experiments were analyzed together alcohol-exposed animals had more TEBs. For all three time points, a two-way ANOVA was performed with a Bonferroni post-test at the level of α=0.05.

Mammary epithelial cell proliferation

Cell proliferation was determined by injecting BrdU prior to euthanasia (Fig 2a). Proliferation was highest at PND 20 and decreased with time (Fig 2b). BrdU incorporation was greater in animals exposed to alcohol *in utero* compared to either control group at PND 20 and compared to pair-fed controls at PND 40, indicating that mammary epithelial cells were more proliferative in alcohol-exposed animals at these time points (Fig 2c). There were no effects of treatment on proliferation at PND 80 (Fig 2c). At PND 20 the apoptotic index was not affected by *in utero* alcohol exposure (Fig 2d).

Figure 2.

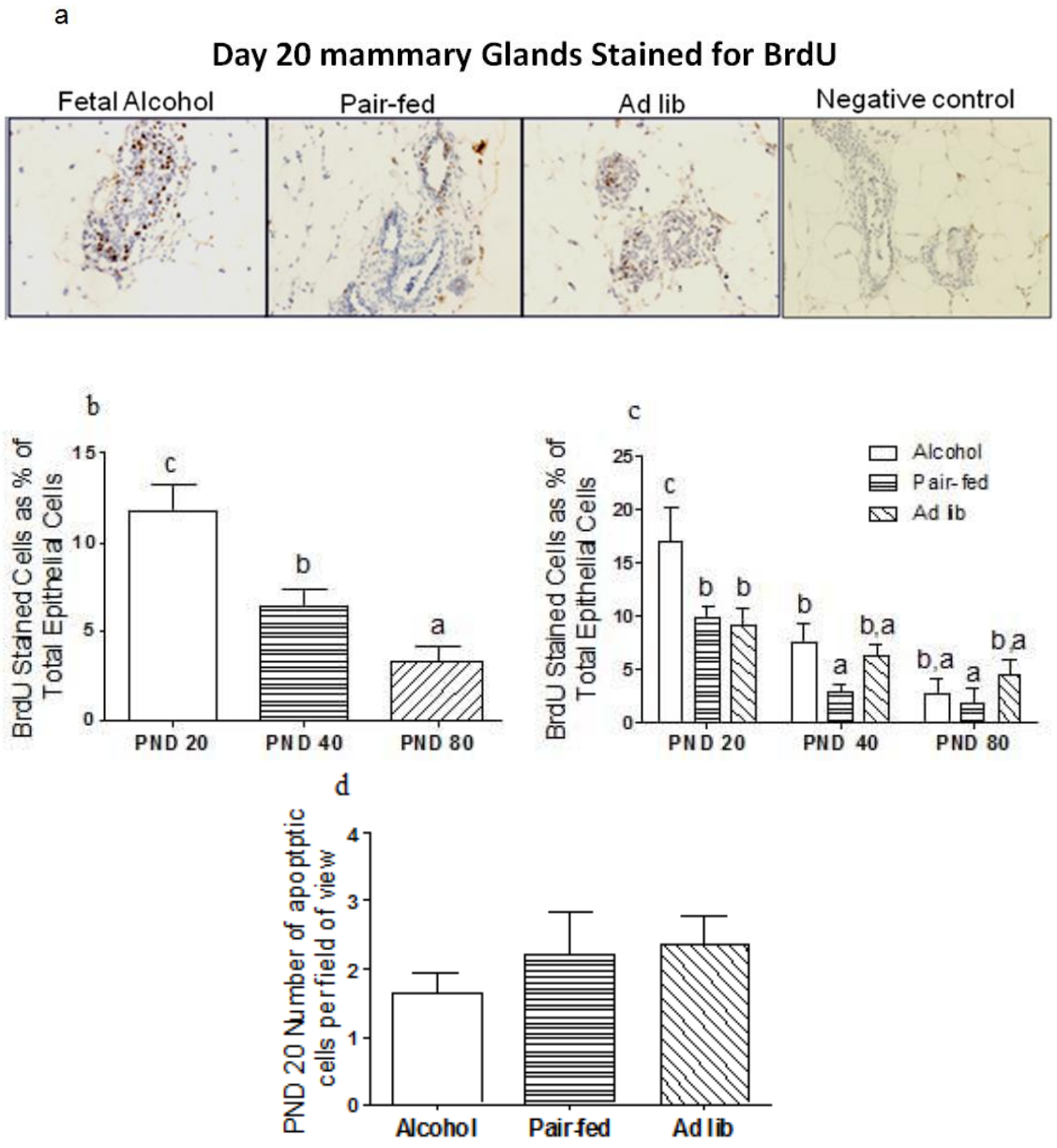

Fig. 2 Mammary epithelial cell proliferation is higher in animals exposed to alcohol *in utero* at PND 20 and 40

Mammary glands were prepared and stained for BrdU by immunohistochemistry as described in Materials and Methods. Total stained and unstained epithelial cells were counted and BrdU stained cells as a percent of total epithelial cells is presented. (a) Representative sections stained for BrdU. (b) Percent of total

epithelial cells stained for BrdU. Each bar includes samples from all 3 treatment groups ($P < 0.0001$, $n = 30$). (c) Percent of total epithelial cells stained for BrdU with treatment groups separated ($P < 0.05$, $n = 10, 9, 13$ for alcohol, pair-fed, and ad lib groups, respectively). (d) Number of apoptotic cells per field of view. Bars represent mean ± SE with different letters denoting a significant difference. A one-way ANOVA was performed with a Newman-Keuls post-hoc test at the level of $\alpha=0.05$ in panel b. A two-way ANOVA was performed with a Bonferroni post-hoc test at the level of $\alpha=0.05$ in panel c.

Systemic IGF-I effects

As shown in Fig 3a, hepatic IGF-I mRNA expression was significantly increased in animals exposed to alcohol *in utero* compared to both control groups at all time points. In contrast to the changes observed in hepatic mRNA levels, circulating IGF-I levels did not change with treatment, but did increase significantly with time (Fig 3b). Ligand blot analysis indicated that circulating IGFBP-3 increased in a similar manner to IGF-I concentrations (Fig 3c). Similar to our previous study, alcohol-exposed animals weighed significantly less than either control group (Fig 3d), although this decrease in weight was not enough to affect circulating IGF-I levels.

Figure 3.

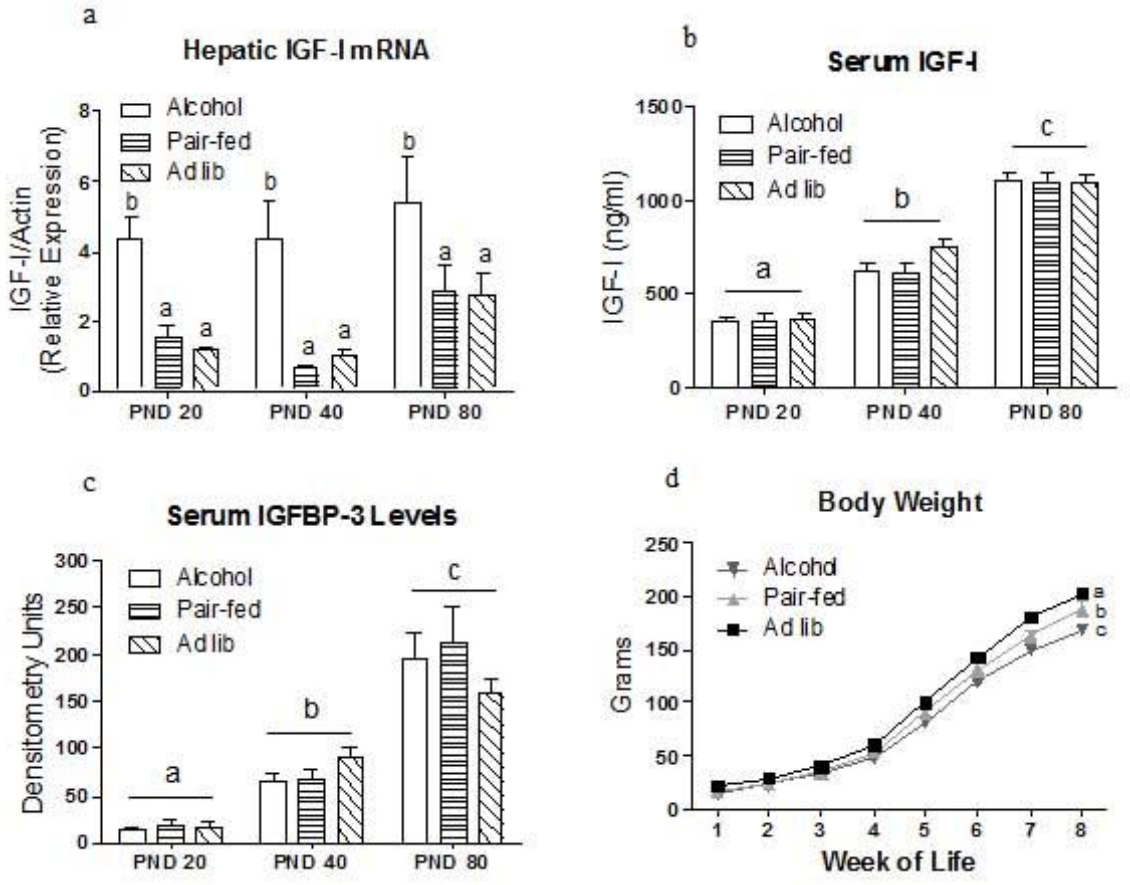

Fig. 3 Hepatic IGF-I mRNA is increased in animals exposed to alcohol *in utero,* while serum IGF-I and IGFBP-3 increase over time in all treatment groups

Hepatic tissue and serum was collected from animals exposed to alcohol and controls not exposed and prepared as described in Materials and Methods. (a) Hepatic IGF-I mRNA expression was assayed by quantitative RT-PCR ($P < 0.05$). (b) Circulating IGF-I levels were assayed by ELISA ($P < 0.001$). (c) Serum IGFBP-3 levels were assayed by ligand blot ($P < 0.0001$). (d) Weekly body weight recordings ($P < 0.0001$). Mean ± SE are represented with different letters denoting significant differences. A two-way ANOVA was performed in panels a, b, and c with a Bonferroni post-test at the level of $\alpha = 0.05$ (n = 10, 9, or 13 for alcohol, pair-fed, and ad lib groups, respectively at each time point). A repeated-measures one-way ANOVA was performed in panel d with a Newman-Keuls post-test at the level of $\alpha=0.05$ (during weeks 1-4 n = 30, 28, and 40, during weeks 5 & 6 n = 20, 18, 27, and during weeks 7 & 8 n = 10, 9, 13 for alcohol, pair-fed, and ad lib groups, respectively).

Mammary gland IGF-I and IGFBP-5 mRNA expression

At PND 20, IGF-I mRNA levels in the mammary gland were significantly higher in alcohol-exposed animals compared to control animals (Fig 4a). In contrast, IGF-I mRNA levels did not change over time in the mammary glands of either control group (Fig 4a). IGFBP-5 mRNA expression in the mammary gland increased between PND 20 and 40 in control animals (Fig 4b). However, the rise in IGFBP-5 mRNA expression was delayed in alcohol-exposed animals and at day 40, IGFBP-5 mRNA levels were lower in alcohol-exposed animals relative to pair-fed controls (Fig 4b).

Figure 4.

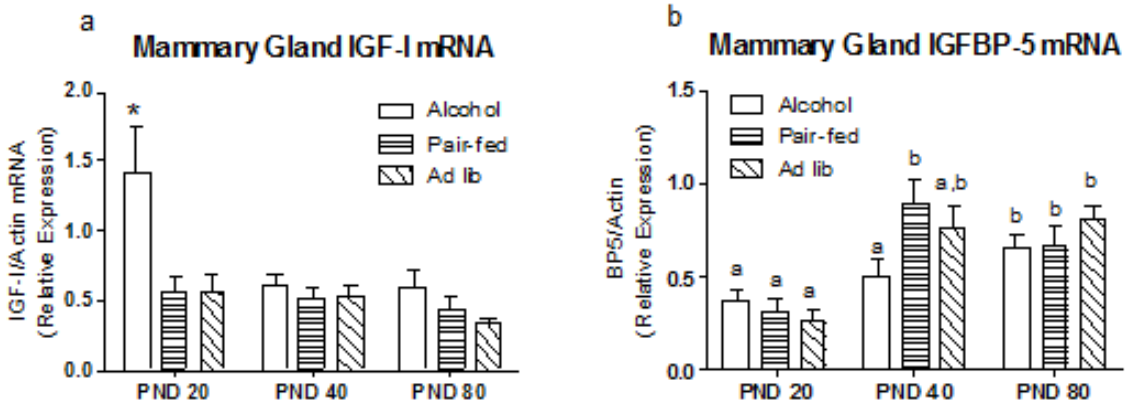

Fig. 4 IGF-I mRNA is significantly higher at PND 20 and IGFBP-5 mRNA increased later in the mammary glands of alcohol exposed animals

Mammary gland tissue was collected, RNA was isolated and quantitative PCR was performed as described in Materials and Methods. (a) Mammary gland IGF-I mRNA expression with * denoting significant difference ($P < 0.001$). (b) Mammary gland IGFBP-5 mRNA expression with different letters denoting significant differences ($P < 0.05$). Bars represent mean ± SE. A two-way ANOVA was performed with a Bonferroni post-test at the level of $\alpha = 0.05$ ($n = 10$, 9, or 13 for alcohol, pair-fed, and ad lib groups, respectively at each time point).

Mammary gland aromatase expression

At PND 20 and 40, there was significantly more aromatase expressed in mammary glands of alcohol-exposed animals compared to either control group (Fig 5b). Circulating E2 was measured at PND 20 before the onset of estrous cycles, and was not altered by alcohol exposure *in utero* (Fig 5c). In the mammary gland, testosterone is converted to E2 by the P450 enzyme aromatase. Therefore, these data suggest that while circulating E2 is not changed (Fig 6c), there may be more E2 in the local mammary gland environment in animals exposed to alcohol *in utero* (Fig 5b).

Figure 5.

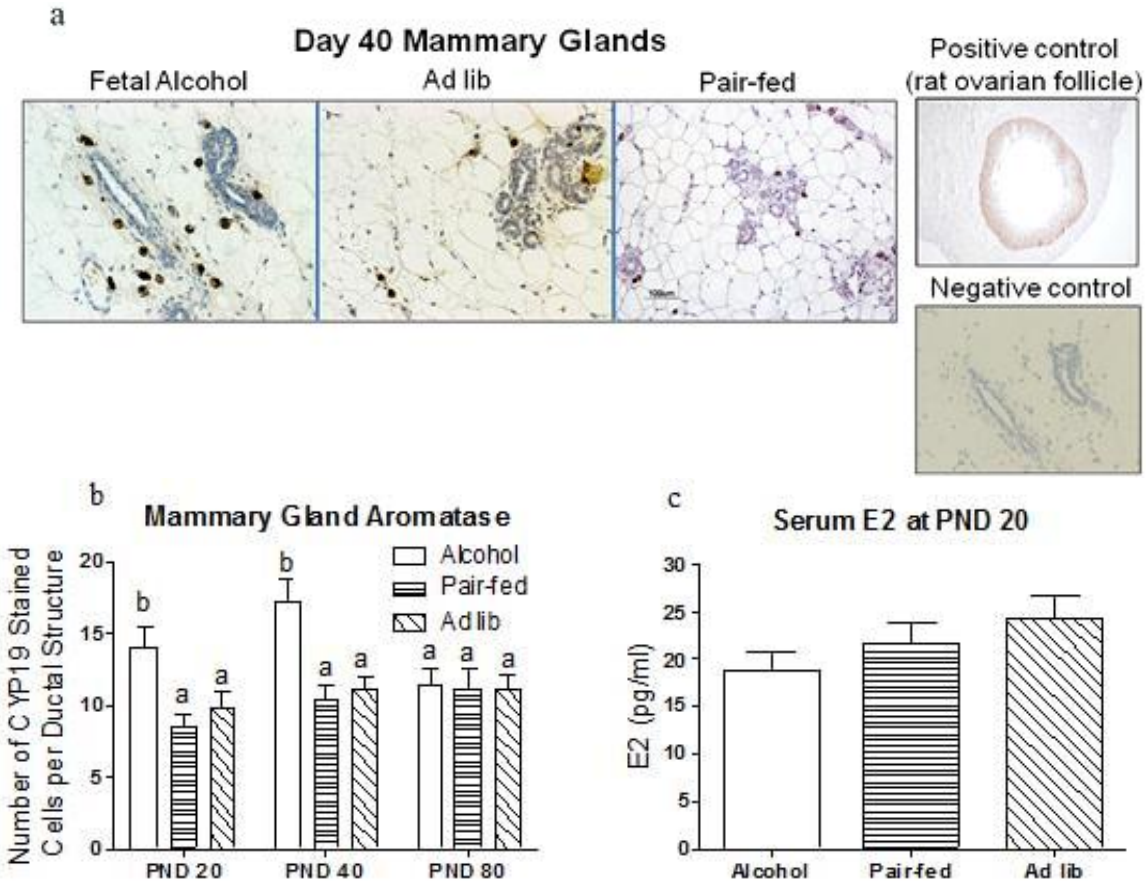

Fig. 5 Aromatase expression is increased in the mammary glands of animals exposed to alcohol at PND 20 and 40, while circulating E2 does not change.

Mammary glands were prepared and stained for aromatase as described in Materials and Methods. Three fields of view at 400x magnification were counted per slide. (a) Representative sections of aromatase-stained cells. (b) Number of aromatase-stained cells per ductal area. (c) Circulating E2 concentrations at PND 20 as measured by EIA. Bars represent mean ± SE with different letters denoting significant difference ($P < 0.05$). A two-way ANOVA was performed with a Bonferroni post-hoc test at the level of $\alpha = 0.05$ ($n = 10$, 9, or 13 for alcohol, pair-fed, and ad lib groups respectively at each time point) for panel b. A one-way ANOVA was performed for panel c ($n = 10$, 9, 13 for alcohol, pair-fed, and ad lib groups respectively).

Discussion

A sub-optimal fetal environment has been shown to contribute to a variety of adult diseases, including cancer (19-21, 24). However, the mechanisms that explain how early developmental exposure affects disease susceptibility in adulthood have not been determined. Many studies suggest that epigenetic alterations in gene expression early in life define the individual's response to later events that may enhance their risk for cancer, diabetes, or cardiovascular disease (176, 177). In terms of mammary gland development, fetal exposure to a wide variety of environmental chemicals such as diethylstilbestrol, genistein, nonylphenols, zearalenone, atrazine, and bisphenol-A, have been shown to affect mammary gland development postnatally (28-32, 34, 35). These endocrine disrupting compounds act by altering estrogen or androgen action. There is evidence that fetal alcohol exposure may also alter mammary gland development or cancer susceptibility by altering the estrogen system (23, 24). Since the estrogen system is intricately entwined with the IGF system, we hypothesized that fetal alcohol exposure

might alter the IGF-E2 axis to affect early mammary gland development. These alterations might predispose the gland to enhanced susceptibility to carcinogenic exposure later in life.

In the current study, animals were exposed to alcohol *in utero* and euthanized at three postnatal time points that correspond with different developmental stages of the gland. PNDs 20 and 40 correspond with periods of active proliferation and expansion of TEBs, while PND 80 represents a period of dormancy after the ductal structure reaches maturation. Across all treatment groups, the number of TEBs increased from PND 20 to 40, while the number of actively proliferating epithelial cells in the gland decreased over this time interval. This is explained by a change in the morphology of the gland at these time points. At PND 20 there is a relatively small epithelial tree and the proportion of epithelial cells proliferating to those non-proliferating is high, while at PND 40 the epithelial tree has quadrupled in length and the proliferation is only at the edge of the gland, where the TEBs are located. Therefore, at PND 40 the ratio of proliferating epithelial cells to non-proliferating epithelial cells is much lower. In the present study, alcohol exposure *in utero* altered early mammary gland development as evidenced by increased epithelial cell proliferation and TEB numbers at PND 20. These increases in TEB number and proliferation could be a result of increased mammary IGF-I since IGF-I mRNA was elevated at this time point in animals exposed to alcohol *in utero*. de Ostrovich et al. (36) found that transgenic mice expressing IGF-I in the myoepithelial cells of the gland exhibited increased epithelial cell proliferation pre-pubertally (4 – 5 weeks of age), but that this increase was lost after puberty (7 – 8 weeks of age) even though transgene expression of IGF-I was still elevated. Similarly, Rayner et al. (35)

found that atrizine exposure *in utero* led to a decrease in mammary length pre-pubertally that was lost postpubertally. Even though the change in mammary morphology was no longer detectable, the subsequent lactation was suboptimal resulting in stunted pup growth. These studies indicate that early changes in mammary gland morphology may correspond with biological outcomes later in life even though the mature gland is not morphologically altered.

Of interest was the finding that hepatic IGF-I mRNA was elevated in animals exposed to alcohol *in utero* at all time points. However, these changes did not translate to an increase in circulating IGF-I concentrations. Studies that have reported circulating IGF-I in alcohol-exposed offspring have examined animals immediately prior to parturition and report conflicting results. Singh et al. (178, 179) reported that in rats, alcohol exposure *in utero* led to stunted growth and lower circulating IGF-I levels in the fetus, while in both rats and sheep (180) serum IGF-I tended to be higher in alcohol-exposed fetuses (181). Children born with FAS display no difference in circulating IGF-I under the age of 2, while circulating IGF-I is higher in FAS children at 3-4 years of age. By age 5, the difference in circulating IGF-I is lost, while their heights are never significantly different (182). An increase in hepatic IGF-I expression without an increase in circulating IGF-I could be due to the highly regulated homeostatic mechanisms that control circulating IGF-I and could reflect increased uptake of IGF-I by tissues, including the mammary gland. Work with different genetic mouse models support roles for both circulating as well as local IGF-I in the development of the mammary gland (7, 183). Results of the present work suggest that IGF-I could potentially be increased in mammary glands of alcohol-exposed offspring through both mechanisms.

The availability of IGF-I in the mammary gland could also be increased by a decrease in IGFBP-5 expression, which was observed at PND 40 in alcohol-exposed animals. This could result in more unbound IGF-I available to activate the IGFR, which could contribute to the increased proliferation seen in fetal alcohol-exposed animals at this time point. Human breast cancer cells (MDA-MB-231) transfected with IGFBP-5 (MDA/BP5) exhibit decreased growth compared to cells transfected with a vector control (MDA/VEC) (104). In addition, nude mice injected with MDA/BP5 cells develop smaller tumors compared to mice injected with MDA/VEC cells (104). While a decrease in IGFBP-5 could also decrease apoptosis, TUNEL stain was very low in the present study and was not altered by fetal alcohol exposure, suggesting that increased availability of IGF-I mainly influenced cell proliferation verses apoptosis.

E2 and IGFBP-5 have been reported to be inversely related. For instance, in MCF-7 cells, E2 treatment decreases IGFBP-5 expression, while treatment with the anti-estrogen ICI 182780 increases IGFBP-5 expression (184, 185). These data support our finding at PND 40, where there was increased aromatase expression in the mammary gland (Fig 6), suggestive of higher levels of local E2 production, and decreased expression of IGFBP-5 (Fig 5). We have also previously reported a significant increase in circulating E2 levels in alcohol-exposed animals, and significantly lower levels of IGFBP-5 mRNA and protein in tumors which develop in these animals in response to a carcinogen (24). Therefore, our previous data together with the current study support the hypothesis that IGFBP-5 expression is altered in fetal alcohol-exposed animals possibly via alterations in E2, which could contribute to the changes observed in mammary gland development.

In conclusion, animals exposed to alcohol *in utero* displayed advanced pre-pubertal development of the mammary gland. This advanced development was demonstrated by an increase in TEB number, and epithelial cell proliferation in the glands of animals exposed to alcohol *in utero* at PND 20. Changes in local expression of IGF-I, IGFBP-5 and aromatase in these animals corresponded with the enhanced proliferative state, supporting the hypothesis that changes in the IGF/E2 axis in early mammary gland development may contribute to the increase in susceptibility to mammary carcinogenesis observed in alcohol-exposed animals (24).

CHAPTER 4

Animals exposed to alcohol *in utero* exhibit increased tumorigenesis A second look

Introduction

Breast cancer is the most commonly diagnosed cancer among women, and the second leading cause of cancer-related death in women in the United States (186). While many primary breast cancers are treatable, highly aggressive, poor prognosis breast tumors grow rapidly and are often less responsive to chemotherapy (187). Determining the factors that put a woman at higher risk for aggressive breast cancer would help in determining which women would benefit from more frequent screening. In addition, understanding how these highly aggressive tumors progress could lead to the development of treatments as well as preventative therapies.

In rodents, exposure to alcohol during fetal development leads to increased tumor multiplicity and decreased tumor latency in response to a carcinogen (22). Even more alarming is the finding that alcohol exposure *in utero* results in carcinogen-induced tumors that have characteristics of a poor prognosis phenotype in humans, such as a greater number of ER-α negative tumors, and more malignant adenocarcinomas (24). To further investigate the development of this aggressive phenotype, a second study was conducted similar to our first study (24) with the difference that the study was terminated earlier, at 16 weeks post-NMU injection, instead of at 23 weeks. This represented the time that 50% of maximal tumor burden was reached in the first study.

Materials and Methods

Animal Model:

Pregnant Sprague-Dawley rats were purchased from Charles River (Wilmington, MA) and individually housed in a controlled environment with a 12 h light/dark cycle. Dams were fed a liquid diet containing ethanol (alcohol-fed) (Bio-Serv, Frenchtown, NJ), an isocaloric liquid diet (pair-fed) (Bio-Serv), or *ad libitum* rat chow (ad lib-fed) (Purina Mills Lab Diet, St. Louis, MO) (n = 7 dams per diet). Dams were acclimated to the alcohol diet from d 7 to 11 of gestation by feeding a liquid diet containing 2.2% ethanol on d 7 and 8 and 4.4% ethanol on d 9 and 10. Once acclimated, dams were fed the liquid diet containing 6.7% ethanol from d 11-21, which represented 35% of total calories. At birth female pups were cross-fostered to ad lib-fed dams and litters were normalized to 8 pups per dam. Pups were weaned at 21 d of age and fed rat chow *ad libitum* for the remainder of the experiment. On day 50 of life 30 female pups from each *in utero* treatment group were injected with a single injection of 50 mg/kg of NMU I.P to induce mammary tumors. Rats were weighed and palpated for tumors once a week. Animals were euthanized 16 weeks post-NMU injection by rapid decapitation. A full necropsy was performed and all major organs were macroscopically examined to ensure the carcinogen was mammary-specific. Trunk blood, tumors, and mammary glands were collected at study termination. Trunk blood was kept on ice for 4 hr then serum was harvested. Mammary tumors, mammary glands, and livers were each divided into three parts: two of the three pieces were flash frozen in liquid nitrogen and stored at -80°C for RNA and protein analysis, the third was fixed in 10% NBF (Richard-Allan Scientific, Kalamazoo, MI) for histological analysis. Animal care was performed in accordance

with institutional guidelines and complied with National Institutes of Health policy.

Histology:

Fixed tissue was dehydrated, cleared, and embedded in Paraplast using facilities located in the Histopathology Core of The Environmental Occupational Health Sciences Institute (EOHSI) at Rutgers University. Samples were sectioned at 6 μm and placed on slides. Slides were baked for 30 min at 60°C, followed by deparaffinization in xylene and rehydration in decreasing concentrations of ethanol. Slides were stained with hematoxylin-eosin and mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Immunohistochemistry (IHC):

For immunostaining, samples were processed as described above, and slides were baked at 60 °C for 30 min then deparafinized in xylene and isopropanol. Antigen retrieval was performed, and endogenous peroxidase activity was quenched with 3% H_2O_2. Slides were blocked in normal horse serum (Vector Labs, Burlingame, CA), and incubated with ER-α (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA), or HER2 (1:200) (Cell Signaling, Danvers, MA) primary antibodies overnight at 4 °C. On each slide, one section was incubated with rabbit or mouse isotype IgG (Invitrogen, Carlsbad, CA) as a negative control. The next day, slides were incubated with a biotinylated anti-rabbit secondary antibody (1:200) (Santa Cruz), then with ABC reagent from the ABC Elite Vectastain kit (Vector Labs), stained in 3,3'-diaminobenzidene (Sigma), counterstained with hematoxylin and mounted in Permount (Fisher Scientific, Pittsburgh, PA). To assess ER-α and HER2 positivity, three members of the laboratory viewed all tumors

blindly and classified them as highly positive, positive, mostly negative or negative. Tumors determined to be highly positive and positive were combined and termed positive, and those determined to be mostly negative and negative were combined and termed negative.

RNA Analysis:

Frozen mammary or tumor tissue was homogenized in Trizol (Sigma) and RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. RNA was treated with DNase (Qiagen) during isolation. RNA quality was verified using the Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). The High Capacity cDNA Reverse Transcription kit (Applied Biosystems (ABI), Foster City, CA) was used to reverse transcribe 2 µg of RNA. Primer sets were developed using PrimerQuest (IDT, Coralville, IA), and each primer set was validated as previously described (147). Primer sets for ER-α (F: 5'TCGGGAATGGCCTTGTTG3'; R: 5'AGCTGCGGGCGATTG A3'), IGFBP-5 (F: 5'TTGAGGAAACTGAGGACCTCGGAA3'; R: 5'CCTTCTCTGTCCGTTCAACTT GCT3'), and actin (F: 5'CCATTGAACACGGCATTGTCACCA3'; R: 5'GCCACACGC AGCTCATTGTAGAAA3') were obtained from Sigma Genosys (St. Louis, MO). Samples were diluted 1:4 and 5 µl were amplified in a 20 µl reaction mix containing 10 µl Power SYBR Green PCR Master Mix (ABI), 4 µl ultrapure H_2O, and 0.5 µl (200 µM) of each forward and reverse gene-specific primer. Quantitative real-time PCR was performed on 384 well plates (ABI) using an ABI 7900 HT Real-Time PCR system. For each experimental sample, fold-change relative to a control sample was determined using

the $2^{-\Delta\Delta ct}$ method with actin as the housekeeping gene. The calibrator sample represented a pool of tumor RNA consisting of 2 RNA samples from each of the 3 treatment groups.

Statistical Analysis:

Differences in body weight, tumors per group, and tumors per animal were assessed using a repeated measures one way ANOVA, with a Newman-Keuls post-hoc test at the level of $\alpha = 0.05$. Percent of rats with tumors was analyzed using a logrank test at the level of $\alpha = 0.05$. ER-α and HER2 positivity was evaluated using two-way ANOVA. mRNA and protein values were evaluated using a one way ANOVA or t-test.

Results

Tumor development

Animals exposed to alcohol *in utero* and control rats not exposed were administered a single I.P. injection of NMU (50 mg/kg body weight) at 50 days of age. In order to investigate an earlier time point in tumor development rats were euthanized at 16 weeks post-NMU injection. At this time 50% of maximal tumor incidence was reached across all groups in our previous study. In the present study, 30 to 40% of animals across all treatment groups presented with a tumor. At study termination animals exposed to alcohol had significantly more tumors than animals from either control group, which did not differ from each other in terms of overall tumor number (Fig 1a). The percentage of animals presenting with tumors was also significantly higher in alcohol-exposed animals than in either control group (Fig 1b), while tumor multiplicity was higher in alcohol-exposed animals compared to ad lib controls, but not pair-fed controls

(Fig 1c). Body weights differed between the treatment groups with the pair-fed animals weighing the least, and the ad lib animals weighing the most (Fig 1d). Tumor latency was decreased in the animals exposed to alcohol *in utero*, in that there were more tumors presenting earlier than in either control group (Fig 1e).

Figure 1.

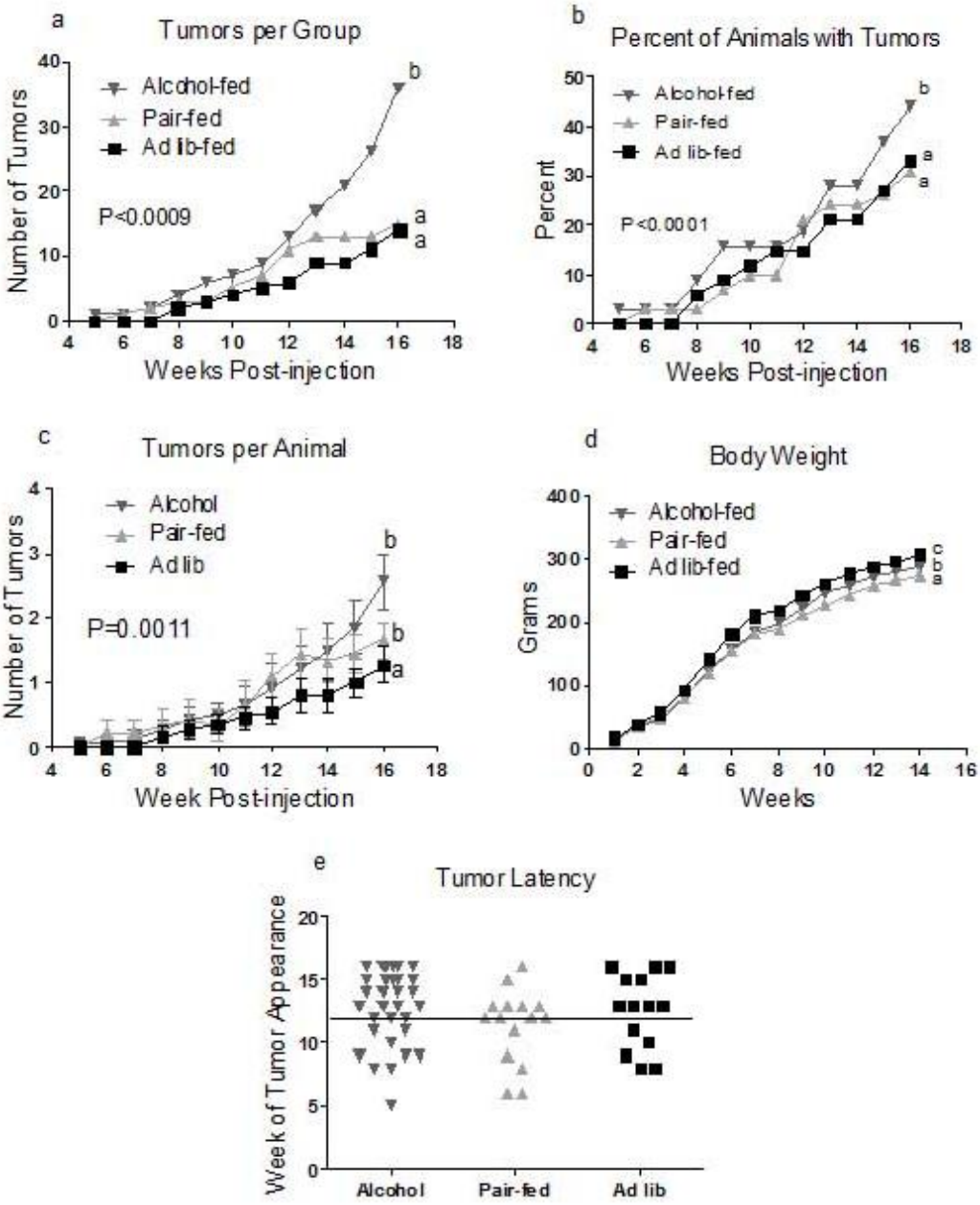

Figure 1. Animals exposed to alcohol *in utero* display increased tumor multiplicity and decreased latency.

Animals exposed to alcohol *in utero* and controls not exposed were administered a single NMU injection (50 mg/kg body weight) at day 50 of life. Subsequently all animals were palpated weekly for tumors. (a) Total number of tumors per treatment group each week post injection. Each point on the line represents the number of tumors in each treatment group ($P = 0.0009$). (b) Percent of rats presenting with a tumor in each treatment group each week post-injection ($P < 0.0001$). (c) Lines represent the mean number of tumors per animal in each group ± SE ($P = 0.0011$). (d) Weekly mean body weight ± SE for each treatment group ($P < 0.0001$). (e) Week of tumor appearance. Each data point represents an individual tumor. The horizontal line at week 12 post-injection represents when 50% of tumor incidence occurred. A one-way repeated measures ANOVA was performed for panels a - d with a Newman-Keuls post-test at the level of $\alpha = 0.05$ ($n = 32$, 29, and 33 for alcohol, pair-fed, and ad lib, respectively).

ER-α expression in tumors and mammary glands

In order to determine the ER-α status of the tumors, IHC was performed. Overall, 40 tumors stained positive and 23 tumors stained negative for ER-α. At this time point there was no effect of *in utero* alcohol exposure on the ER-α status of the tumors (Fig 2a). Alcohol exposure *in utero* did not affect ER-α mRNA expression in tumor tissue (Fig 2b). When ER-α mRNA expression in tumors collected at 23 weeks post-NMU injection (from a previous experiment) was compared to ER-α mRNA expression in tumors collected 16 weeks post-NMU injection (the current experiment) there was a decrease in mRNA levels in tumors that were allowed to develop to week 23 post-NMU injection (Fig 2c).

Figure 2.

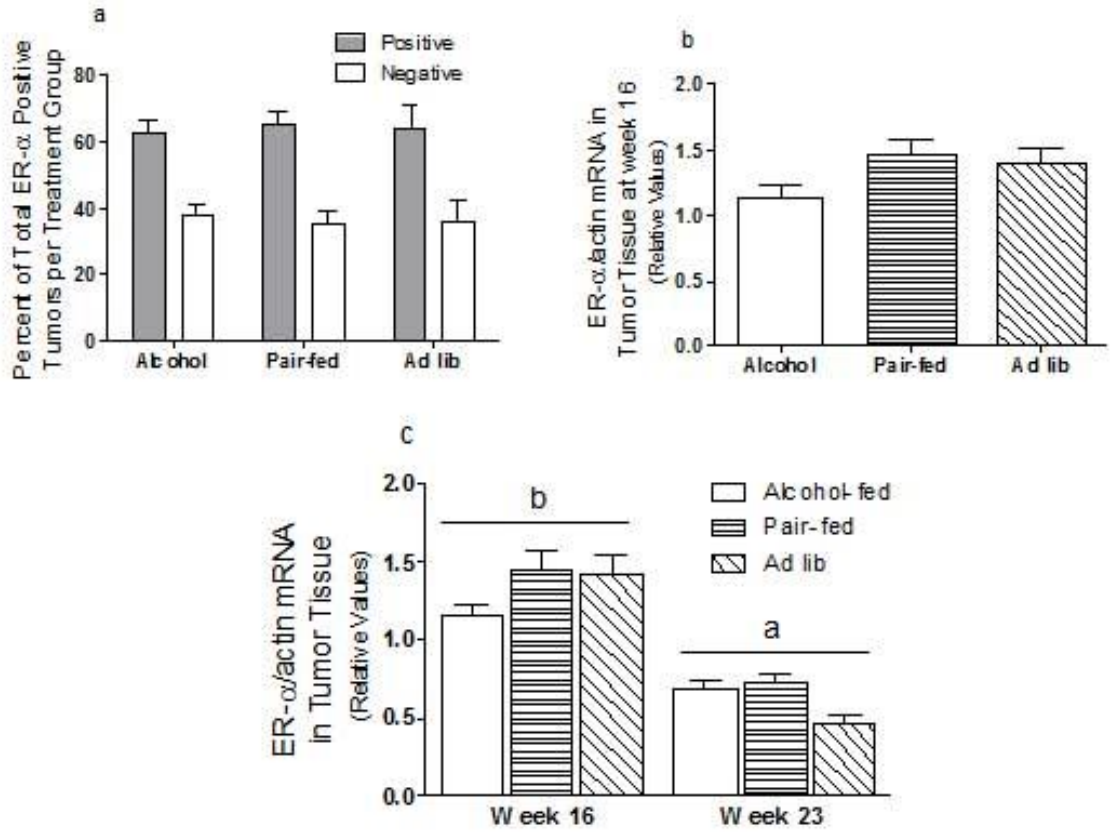

Figure 2. alcohol exposure *in utero* does not affect ER-α status in tumors 16 weeks post-NMU injection.

Tumors were collected 16 weeks post-NMU injection, stained for ER-α by IHC, or RNA was isolated for quantitative PCR as described in the Materials and Methods. (a) Percent of positive or negative tumors in each treatment group. A two-way ANOVA was performed (n = 30, 19, and 14 for alcohol, pair-fed, and ad lib, respectively). (b) Tumor ER-α mRNA expression. A one-way ANOVA was performed (n = 29, 13, and 11 for alcohol, pair-fed, and ad lib, respectively). (c) Tumor mRNA expression from a previous experiment was compared to tumor mRNA from the current experiment. Two 384 well quantitative PCR plates were run with the same calibrator on each plate. A two-way ANOVA was performed with a Dunn's multiple comparison post-test at the level of $\alpha = 0.05$ ($P < 0.0001$, n = 29, 13, and 11 for week 16, and 36, 23, and 11 for week 23, for alcohol, pair-fed, and ad lib, respectively). Bars represent mean ± SE.

HER2 tumor status

HER2 status in tumor tissue was determined by IHC (Fig 3c). Overall 22 tumors stained positive (35%) and 41 stained negative (65%). Alcohol exposure *in utero* did not significantly affect HER2 tumor status (Fig 3a). A similar result was seen in tumors from a previous experiment which was terminated at week 23 post-NMU injection (Fig 3b).

Figure 3.

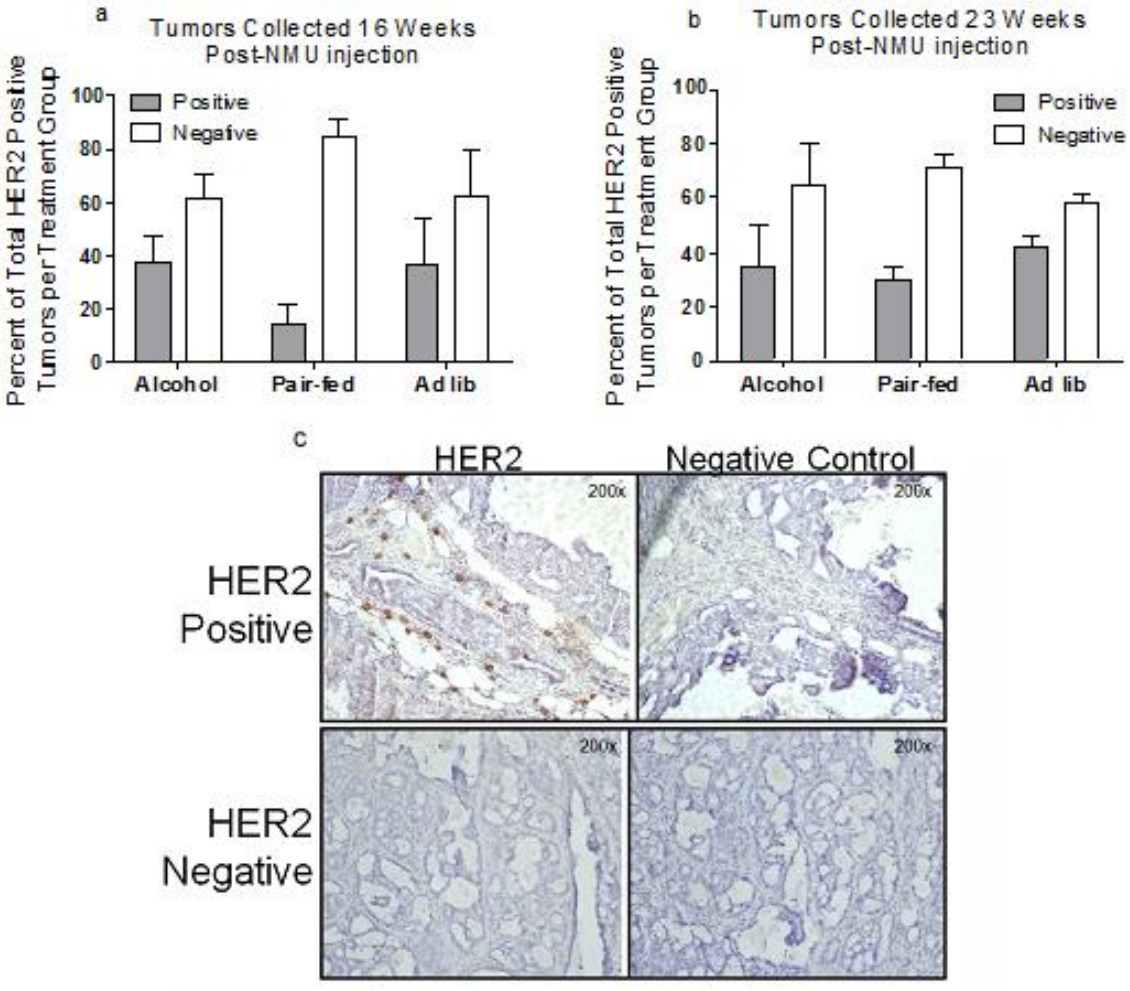

Figure 3. Alcohol exposure *in utero* does not affect HER2 tumor status.

Tumors were collected at study termination, processed and stained for HER2 by IHC as described in Materials and Methods. (a) Percent of tumors stained positive or negative for HER2 in each group (n = 30, 19, 14 for Alcohol, Pair-fed and ad lib, respectively). (b) Percent of HER2 positive and negative tumors in each treatment group from a previous experiment terminated 23 weeks post-NMU injection. (c) Representative images of HER2 positive and HER2 negative tumors are shown at 200x magnification; Negative Control = non-specific IgG.

Tumor histology

At study termination tumors were collected, processed, and H & E stained for determination of tumor phenotype. Tumors were termed hyperplastic if they were highly differentiated and presented as an overproliferation of ductal structures. Adenomas were more solid-form tumors, still well-differentiated with a low mitotic index. The malignant adenocarcinomas were highly undifferentiated with large areas of necrosis and a high mitotic rate. Approximately 70% of all adenocarcinomas presented in the alcohol-exposed animals compared to 10 and 20% in the ad lib and pair-fed groups, respectively (Fig 4). Over 40% of the hyperplasias presented in the alcohol-exposed group with 20 and 30% in the ad lib and pair-fed groups, respectively, while a similar percentage of adenomas presented in each treatment group (Fig 4). Therefore the animals exposed to alcohol *in utero* were more susceptible to presenting with the malignant adenocarcinoma tumor type than either control group.

Figure 4.

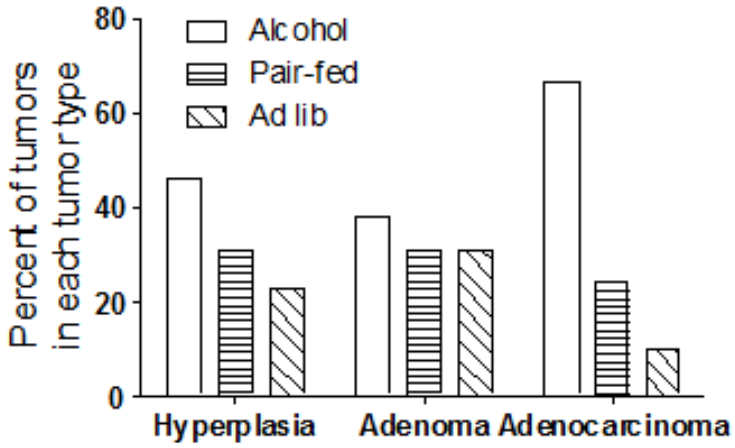

Figure 4. Animals exposed to alcohol *in utero* are more susceptible to a malignant tumor phenotype. Tumors were collected at 16 weeks post-NMU injection, processed and stained for H & E as described in Materials and Methods. Each entire tumor was inspected for malignant features, particularly mitotic index, degree of differentiation, and abundance of atypical nuclei and cell shape. The percentage of each tumor type in each treatment group is presented.

IGFBP-5 mRNA expression in tumors

Tumors were collected 16 weeks post-NMU injection. Alcohol exposure *in utero* did not affect IGFBP-5 mRNA expression in NMU-induced tumors (Fig 5a). IGFBP-5 mRNA expression was significantly decreased in tumor tissues compared to non-diseased mammary tissues (Fig 5b).

Figure 5.

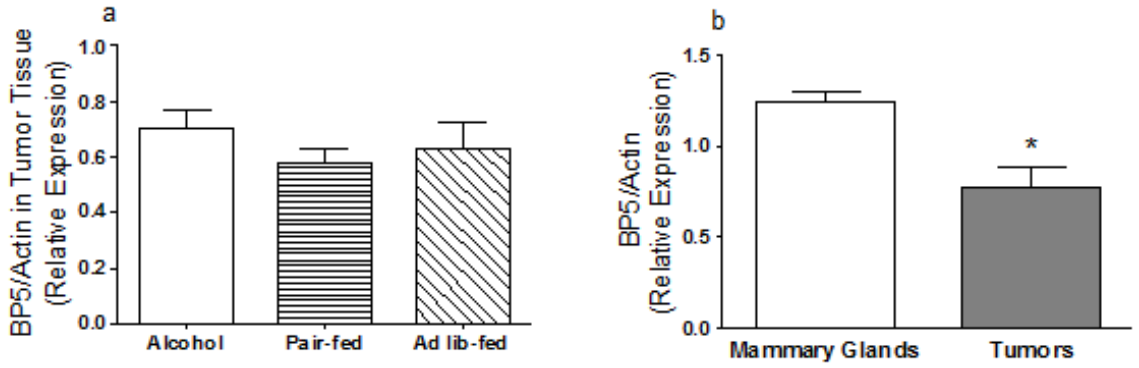

Figure 5. IGFBP-5 mRNA was not affected by alcohol exposure *in utero* or time of tumor collection, but was decreased in tumors compared to non-diseased mammary gland tissue.

Tumors were collected 16 weeks post-NMU injection, RNA was isolated, and quantitative PCR was performed as described in Materials and Methods. (a) IGFBP-5 mRNA expression in tumor tissue by *in utero* treatment (n = 29, 13, 11 for alcohol, pair-fed, and ad lib, respectively). (b) IGFBP-5 mRNA expression in mammary gland and tumor tissues collected 16 weeks post-NMU injection. A t-test was performed and a significant difference is denoted by a * ($P < 0.0001$, n = 90 and 53 for mammary glands and tumors respectively). Bars represent mean ± SE in each panel.

Discussion

Rodent and human studies have demonstrated that a variety of insults during fetal development can affect cancer risk in adulthood, such as a maternal high fat diet, toxin exposure, or E2 exposure (19, 20, 22, 30, 39, 133, 135). One study besides our own has investigated fetal alcohol as the *in utero* insult affecting mammary cancer risk using a relatively low dose of alcohol (125). Women who are alcoholics might be more likely to continue to drink throughout pregnancy, while many women who discover they are pregnant will abstain from alcohol for the duration of their pregnancy. Therefore we

chose a dose more representative of the alcohol intake of an alcoholic (24).

The current study investigated an earlier time point in NMU-induced tumor progression in response to alcohol exposure *in utero* in relation to our previous study (see Chapter 2). At termination of the previous study, the alcohol-exposed group did not differ from the pair-fed group in tumor multiplicity. However, it was determined that at 16 weeks post-NMU injection the alcohol-exposed group had significantly more tumors than either control group which were not different from each other. Therefore, we hypothesized that if the IGF-I or E2 systems were involved in tumor progression to a poor prognosis phenotype in the alcohol-exposed group then this time point would be of particular interest since the alcohol-exposed group was different from both controls in terms of tumor multiplicity.

Alcohol exposure *in utero* again led to an increase in tumor multiplicity and a decrease in tumor latency, while the ER-α tumor status was not affected. Expression of ER-α is thought to decrease over time with tumor progression. Spontaneous tumors arise by 4 weeks of age in mice with polyoma virus middle T oncoprotein transgenically overexpressed specifically in the mammary epithelium and most mice present with adenocarcinomas by 14 weeks of age (188). In this model, hyperplastic lesions in 4 week old mice express high levels of ER-α while adenocarcinomas in 18 week old animals express minimal levels of ER-α (169). These data support our finding that ER-α mRNA decreased from 16 weeks post-NMU injection to 23 weeks post-NMU injection. At 23 weeks post-NMU injection the animals exposed to alcohol *in utero* were more likely to develop ER-α negative tumors (24) indicating that they progress to an ER-α negative tumor phenotype faster that control animals. HER2 tumor status was not affected by

alcohol-exposure *in utero* at either time point, and in both studies approximately 65% of all tumors were HER2 negative.

The finding that more tumors were ER-α negative in the alcohol-exposed group and that more tumors overall were HER2 negative rasises the possibility that more basal-like mammary tumors develop in the alcohol-exposed group. Basal-like tumors typically encompass triple negative tumors, which do not respond to hormone-based therapies, and account for 15% of all breast cancers (189, 190). Basal-like tumors more often affect younger women (i.e. they have a decreased latency compared to other tumor subtypes). In a study of 486 tumors, 63% of triple negative tumors presented in women under 50 years old, while 45% of non-triple negative tumors presented in women under 50 (191), and in a study using 1,601 patients age of diagnosis was significantly lower in basal-like cases (192). The basal-like subtype presents as high histological grade cancers with high mitotic rates and poor overall survival rates, and typically metastasizes to the brain and lung (191, 192). Over 90% of metastatic breast cancers are of the basal-like subtype (190). These studies indicate that tumors in the basal-like category are particularly aggressive and often present as early onset tumors.

Interval cancers are cases that are detected by a patient, who then presents the problem to a doctor. These cancers are diagnosed between regular yearly screenings due to their high rate of growth (192), and are more likely to be of the basal-like subtype than cancers that are discovered with annual screenings (193). These cancers have increased Ki67 staining demonstrating their increased growth rate compared to tumors discovered at yearly screenings (194). Interval cancers present with an increased rate of recurrence, decreased overall survival, and a high rate of lung and brain metastasis, and are less

responsive to chemotherapy when compared to tumors discovered at yearly screenings (194, 195). These cancers are also more likely to be stage III cancers with positive lymph-node status than cancers discovered at yearly screenings (192). Therefore, tumors of the basal-like subtype which have a poor prognosis are more likely to progress rapidly and present as late stage at detection. More frequent screening for women at risk for developing these cancers could work to detect these tumors at an earlier stage which is vital to survival.

At both time points the animals exposed to alcohol *in utero* developed more malignant adenocarcinomas versus benign adenomas or hyperplasias. These data together indicate that the animals exposed to alcohol *in utero* develop tumors similar to a poor prognosis phenotype, supporting the idea that these women may have more basal-like tumors. These data could provide a rationale for women born to alcoholic mothers to be screened for breast cancer sooner and/or more frequently than typical women, especially since these aggressive cancers are likely to present between screenings (196). Our studies indicate that fetal alcohol is a potential risk factor for cancers phenotypically similar to interval/basal-like cancers and further studies should be pursued.

SUMMARY & CONCLUSIONS

According to the American Cancer Society, 1.4 million women will be diagnosed with breast cancer and almost half a million women worldwide will die from the disease annually (197). With such a high prevalence it is surprising that so little is known about what causes this disease. While there is a list of risk factors, there is no guarantee that eliminating them will prevent breast cancer. It is now becoming clear that the fetal environment plays a role in determining disease risk. Alcohol exposure during fetal development leads to increased mammary tumor susceptibility in rats (23). Fetal alcohol exposure can lead to fetal alcohol syndrome which is diagnosed in 0.28-0.46% live births in The United States and Europe. Since women consume alcohol while pregnant it is important to understand how this leads to increased cancer risk to more appropriately screen at risk women. A significant finding of this work is that rats exposed to alcohol *in utero* develop more adenocarcinomas and more tumors that are ER-negative in response to the carcinogen NMU. Poor prognosis tumors typically progress faster and are more aggressive then tumors that present with markers indicative of a more positive prognosis. Understanding the mechanism by which alcohol *in utero* leads to higher risk of poor prognosis cancer could help to identify women who might benefit from better preventative measures such as more frequent screenings. Therefore we investigated the mechanism by which fetal alcohol exposure leads to increased mammary cancer risk.

Our data demonstrate a role for alcohol exposure *in utero* in mammary cancer development and progression. One previous study showed that alcohol exposure *in utero* led to increased tumor multiplicity and an increase in TEB number (23). However, our

studies are the first to investigate tumor progression in response to alcohol exposure *in utero*. In addition to developing tumors with poor prognosis characteristics, tumors from the alcohol-exposed animals also expressed less IGFBP-5. IGFBP-5 is largely associated with apoptosis and reduced cell growth, and therefore could be acting as a tumor suppressor gene which is repressed in tumors from animals exposed to alcohol *in utero* (24). The animals exposed to alcohol *in utero* also exhibited increased circulating E2. Since IGFBP-5 expression is suppressed by E2 (117) the increased circulating E2 may play a role in suppressing IGFBP-5 expression in tumors from alcohol-exposed animals.

To further investigate tumor progression in our model, we studied an earlier time point in tumor development when the alcohol-exposed group had increased tumor multiplicity. At this time point the alcohol-exposed animals still had more malignant adenocarcinomas than the controls. ER-α tumor status did not change with treatment at this time point, but ER-α mRNA did decrease at the later time point. This indicates that ER-α is lost over time with tumor progression. To prove that this finding is not due to experimental differences, a single study needs to be conducted where the animals are treated with alcohol or control diets *in utero*, injected with NMU, and euthanized at 16 or 23 weeks post-NMU injection. This would provide a direct comparison between the time points definitively showing if ER-α is lost with time.

Dense mammary tissue in humans is listed as a risk factor for breast cancer by the American Cancer Society. In addition, advanced mammary gland development, such as increased TEBs or hyperplastic ductal development, leads to increased incidence of mammary cancer (47, 145). In the present work, alcohol exposure *in utero* led to an increased number of TEBs at PND 20, and increased proliferation in the mammary gland

at PND 20 and 40. IGF-I and E2 are integral in the development of the mammary gland (6, 47, 86). Therefore we investigated if IGF-I and E2 play a role in the alterations in mammary development observed in animals exposed to alcohol *in utero*. Since the majority of circulating IGF-I is produced in the liver we looked at hepatic IGF-I mRNA and circulating IGF-I concentrations to determine if *in utero* alcohol exposure affected systemic IGF-I. We found that alcohol-exposed animals had higher hepatic IGF-I mRNA expression compared to controls, while circulating levels were not affected by *in utero* treatment. It is possible that in alcohol-exposed animals, the liver produces more IGF-I and releases it into the circulation to be taken up by tissues such as the mammary gland. In addition, mammary IGF-I mRNA expression was higher in the alcohol-exposed animals at PND 20, suggesting that the pool of local IGF-I might be increased in the mammary environment. IGFBP-5 mRNA levels were lower in the alcohol-exposed group at PND 40, which would provide more unbound IGF-I to activate the IGFR. Since IGFBP-5 is associated with apoptosis and growth suppression, its absence could also be promoting cell proliferation independent of IGF-I.

Alcohol exposure did not affect circulating E2 pre-pubertally (PND 20), but at PND 20 and 40 aromatase protein expression in the mammary gland was elevated. This suggests that more E2 may be produced in the mammary gland and could be contributing to the increase in TEB number and cell proliferation, and the decrease in IGFBP-5 expression.

These data together demonstrate that the IGF-I/E2 axis plays a role in the increased susceptibility to mammary cancer in animals exposed to alcohol *in utero* and additionally increases the risk for poor prognosis tumor development in part by creating a

more proliferative gland during pubertal development. These findings indicate that women born to mothers who drank alcohol during pregnancy may benefit from more frequent screenings in an attempt to identify fast growing poor prognosis tumors as early as possible.

SUPPLEMENTARY DATA

Rationale and Methods

To determine which growth factor signaling pathways were involved in the mechanism underlying the increase in mammary tumor susceptibility and the enhanced mammary development observed in animals exposed to alcohol *in utero*, many molecular parameters were investigated. Protein and mRNA for specific signaling molecules listed in tables 1 & 2 were analyzed by western immunoblot, ligand blot, or quantitative RT-PCR as described in Materials & Methods. Primer sets not listed in previous chapters include IGFR (forward: 5'AGAGCGAGCTTCCTGTGAAAGTGA3'; reverse: 5'TGCCA CGTTATGATGATGCGGTTC3'), and PR (forward, 5'CGGACGCATTCGTCTGTAGT CT3'; reverse, 5'CTGGCAGGACCGAGAGAAGA3'). Interestingly, while these parameters were not affected by *in utero* treatment, many were altered in tumor tissue compared to non-diseased mammary tissue (Fig 1 & 2)

Results

Alcohol exposure in utero did not affect many parameters

The following tables depict factors which were not affected by alcohol exposure *in utero*. Tumors and mammary glands were collected 23 weeks post-NMU injection (Table 1). Mammary glands were collected from a separate study at PND 20, 40, and 80 (Table 2).

Table 1. Protein and mRNA levels not affected by alcohol exposure *in utero* at 23 weeks post-NMU injection.

Tumors		Mammary Glands		Serum
mRNA	**Protein**	**mRNA**	**Protein**	**Protein**
IGFR	IGFR	EGFR	IGFR	IGFBP-3
EGFR	EGFR	PR	EGFR	IGFBP-2
PR	PR	ER	PR	IGFBP-4
ER	IGFBP-2	IGF-I	ER	
FABP	Total akt	IGFBP-5	Total akt	
IGF-I				

Table 2. Protein and mRNA levels not affected by alcohol exposure *in utero* during mammary gland development.

Day 20 MG		Day 40 MG		Day 80 MG	
mRNA	**Protein**	**mRNA**	**Protein**	**mRNA**	**Protein**
IGFR	IGFR	EGFR	IGFR	IGFR	IGFR
EGFR	EGFR	ER	EGFR	EGFR	EGFR
ER	PR		PR	ER	PR
	ER		ER		ER
	pEGFR		pEGFR		pEGFR
	pAkt		pAkt		pAkt
	Total Akt		Total Akt		Total Akt

The IGF and ER systems differ between tumor and non-diseased mammary tissue

Non-diseased mammary tissue and tumors were collected at 23 weeks post-NMU injection. One tumor from each animal was arbitrarily selected to be included in the analysis. IGFR mRNA levels were increased in tumor tissue compared to mammary tissue (Fig 6a), while IGF-I mRNA levels were increased 140-fold in mammary tissue compared to tumor tissue (Fig 6b). IGFBP-5 as well as ER-α mRNA was decreased in tumor tissue compared to mammary tissue approximately 50% (Fig 6c & d), while

progesterone receptor (PR) mRNA was increased 3.5-fold in tumor tissue compared to mammary tissue (Fig 6e).

Figure 1.

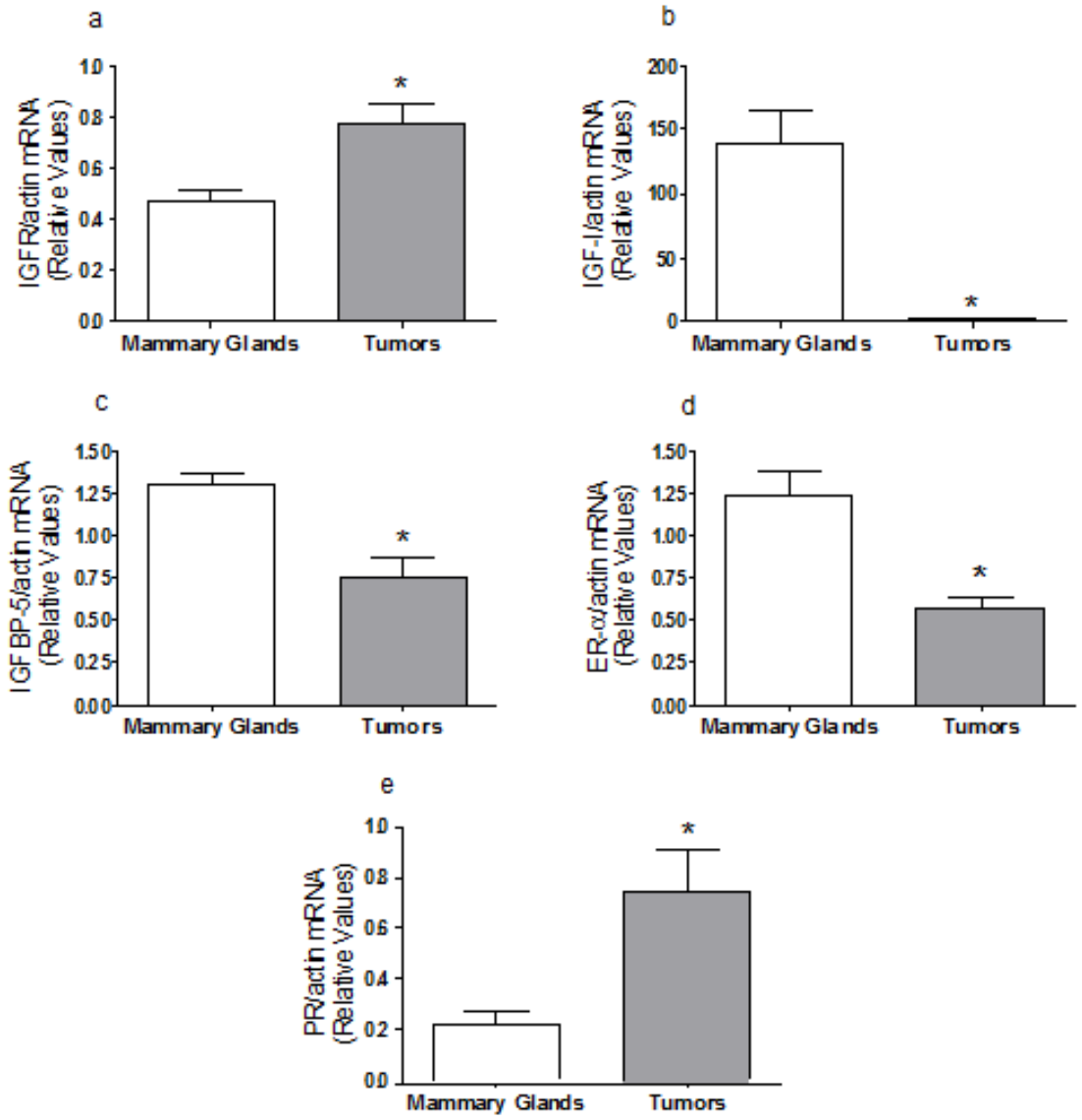

Figure 1. Tumors differ from mammary glands in expression of components of the IGF and E2 system.

Mammary and tumor tissue were collected at necropsy. Quantitative real-time PCR was performed to determine IGFR, IGF-I, IGFBP-5, ER-α, and PR mRNA expression. Fold-change was calculated relative to a calibrator using the $2^{-\Delta\Delta CT}$ method with actin as the housekeeping gene. The calibrator consisted of a

pool of tumor RNA including 2 samples from each treatment group. Bars represent mean ± SE. An unpaired T-test was used to analyze these data ($P < 0.05$, n = 16 and 22 for panel a, 16 and 20 for panel b, 14 and 20 for panel c, 16, and 21 for panel d, and 14 and 21 for panel e for mammary glands and tumors, respectively).

IGFR and ER-α protein levels in tumors and non-diseased mammary tissue

Non-diseased mammary tissue and tumors were collected at 23 weeks post-NMU injection. One tumor from each animal was arbitrarily selected to be included in the analysis. IGFR protein levels were increased in tumor tissue, while ER-α protein levels decreased in tumor tissue compared to non-diseased mammary tissue (Fig 7a & b). These data agree with the mRNA levels for each of these molecules (Fig 6a & d).

Figure 2.

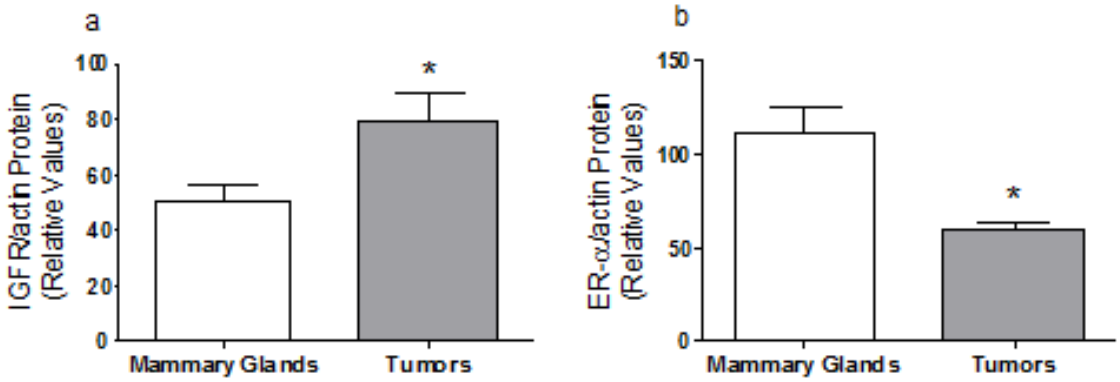

Figure 2. IGFR Protein is increased in tumor tissue, while ER-α is decreased in tumor tissue compared to non-diseased mammary gland tissue.

Mammary glands and tumors were collected from each animal at necropsy. Western immunoblot was performed using an IGFR or ER-α specific antibody. Samples were corrected for loading with actin. Bars represent mean ±SE. An unpaired t-test was used to analyze these data ($P < 0.05$, n = 13 in panel a, and n = 19 and 20 in panel b for mammary glands and tumors, respectively).

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PEER REVIEWED PUBLICATIONS

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