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Tumor Phenotype and Gene Expression during Early Mammary Tumor Development in Offspring Exposed to Alcohol *in Utero*

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Abstract

Background—Alcohol exposure *in utero* increases susceptibility to carcinogen-induced mammary tumorigenesis in adult offspring and causes tumors with a more malignant phenotype. The present study was conducted to identify changes early in tumor development that might lead to this outcome.

Methods—Pregnant Sprague Dawley rats were fed a liquid diet containing 6.7% ethanol (alcohol), an isocaloric liquid diet without alcohol (pair-fed), or rat chow *ad libitum* (ad lib) from gestation day 7 until parturition. At birth, female progeny were cross-fostered to control dams. Pups were weaned at postnatal day (PND) 21 and fed rat chow *ad libitum* for the remainder of the experiment. Female offspring were administered N-nitroso-N-methylurea (NMU; 50 mg/kg body weight) on PND 50. Mammary glands were palpated weekly and offspring were euthanized at 16 weeks post-NMU injection.

Results—At 16 weeks post-NMU, tumor multiplicity was greater in alcohol-exposed offspring compared with control groups. Estrogen receptor- α (ER) mRNA expression was decreased in tumors from alcohol-exposed offspring and these animals developed more ER-negative tumors relative to the pair-fed group. Alcohol-exposed offspring also tended to develop more progesterone receptor (PR)-positive tumors. All tumors were HER2-negative. PR positivity was associated with higher Ki67 expression, suggesting that PR-positive tumors were more proliferative. Tumors from alcohol-exposed animals exhibited increased mRNA expression of the insulin-like growth factor (IGF) family members IGF-II and IGF-BP-5. IGF-II and DNMT1 mRNA tended to be greater in the normal contralateral mammary glands of these animals.

Conclusions—These data indicate that alcohol exposure *in utero* may shift NMU-induced tumor development towards a more aggressive phenotype and that alterations in IGF-II expression

may contribute to these changes. Additional studies should be aimed at epigenetic mechanisms that underlie IGF-II expression to further delineate how this gene is altered in mammary glands of adults exposed to alcohol *in utero*.

Keywords

mammary tumor; alcohol exposure in utero; insulin-like growth factor; tumor phenotype; hormone receptors

Introduction

The Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that an adverse environment *in utero* can pre-program offspring for increased risk of disease later in life (Barker, 2007; Wadhwa et al., 2009). In line with this hypothesis, *in utero* exposure to environmental endocrine disruptors such as bisphenol A and diethylstilbestrol or a high fat diet alters mammary gland development and increases risk of mammary carcinogenesis in offspring, suggesting that a woman's risk of breast cancer may be pre-programmed prior to birth (Fenton and Birnbaum, 2015; Hilakivi-Clarke and de Assis, 2006; Simmen and Simmen, 2011; Soto and Sonnenschein, 2015; Soto et al., 2008). An adverse fetal environment is created when women drink during pregnancy, as evidenced by the phenotype of children born with fetal alcohol spectrum disorder (FASD) and fetal alcohol syndrome, which is the most severe case of FASD (Nayak and Murthy, 2008). Alcohol exposure *in utero* alters early mammary gland development and increases susceptibility to carcinogen-induced mammary tumorigenesis in adult rats (Hilakivi-Clarke et al., 2004; Polanco et al., 2011; Polanco et al., 2010) and in MMTV-Erb-B2 transgenic mice (Ma et al., 2015). These studies support the idea that the DOHaD hypothesis could be expanded to include increased risk for breast cancer later in life following exposure to alcohol *in utero*.

The NMU carcinogen model results in predominately hormone-dependent estrogen receptor alpha (ER)-positive tumors (Manni et al., 1982; Thordarson et al., 2001) that are of mixed histology including adenomas and adenocarcinomas, thus it is an appropriate animal model for studying breast cancer risk since approximately 75% of human breast cancers are ER-positive (Allred, 2010). In humans, gene expression profile studies have identified at least two molecular subgroups within the category of ER-positive breast cancer, referred to as luminal A and luminal B (van't Veer et al., 2003; West et al., 2001). Classification of these subgroups by immunophenotype utilizes a combination of ER, progesterone receptor (PR) and HER2 status along with Ki67 expression (Tang et al., 2009). Luminal B tumors are generally more aggressive and women with these tumors have a poorer prognosis than those with luminal A tumors (Feeley et al., 2014). We have previously shown that at 23 weeks post NMU-injection, rats exposed to alcohol *in utero* develop more malignant tumors as well as more ER-negative tumors, suggesting that these tumors exhibit a phenotype that is associated with a worse prognosis when observed in humans. A goal of the present work was to determine if ER-positive tumors from alcohol-exposed offspring also exhibit a more aggressive phenotype in terms of fitting the Luminal B subtype as evidenced by increased PR or Ki67 expression. IGF-II is an imprinted gene that has been shown to be increased in cancer due to loss of heterozygosity (Rainier et al., 1993). Therefore, a second goal was to

determine if IGF-II was altered in normal mammary gland tissue or in tumors early in tumor development and to determine if changes in genes involved in epigenetic regulation might be associated with this response. Lastly, given the recent attention to nutritional status during pregnancy as it relates to the DOHaD hypothesis (Dyer and Rosenfeld, 2011), we were interested in examining the effects of the Lieber-DeCarli diet, a liquid diet often used in alcohol studies, on parameters such as maternal weight gain, litter weight and growth rate of offspring.

Materials and Methods

Animal Study

Timed-pregnant Sprague-Dawley rats (CD IGS rat, strain code: 001; gestation day (GD) 0 is day of vaginal plug) were purchased from Charles River (Wilmington, MA), received on GD 5, divided into three groups (n = 7), and individually housed in a controlled environment with a 12-hour light/dark cycle. After two days of acclimation, dams were fed one of three diets (n = 7 per group; Table 1) from GD 7 until parturition as previously described (Polanco et al., 2010). This protocol produces average blood alcohol concentrations (BACs) of 147 mg/dl (data not shown) in agreement with work of others that shows BACs between 100 and 160 mg/dl (Lan et al., 2009; Lieber and DeCarli, 1989; Miller, 1992). Pair-fed dams were fed the average volume of liquid consumed by the alcohol group the previous day. Dams were weighed every other day. Two dams from the pair-fed group died during gestation while the female offspring from a third dam in this group did not survive cross-fostering, reducing the sample size of this treatment group to four dams. Entire litters were weighed at birth; hence the litter weight data collected were not segregated by sex. At birth, female progeny were cross-fostered to control dams and litters were normalized to eight pups per dam. From 1 week after birth until study termination, individual female offspring were weighed weekly and weights were recorded. Pups were weaned at PND 21 and fed rat chow *ad libitum* for the remainder of the experiment (n = 31, 27, and 33 offspring belonging to 7, 4 and 7 litters for the alcohol, pair-fed, and ad lib groups, respectively). On PND 50, female offspring were administered a single intraperitoneal injection of 50 mg/kg body weight NMU (Sigma-Aldrich, St. Louis, MO). Mammary glands were palpated and tumors were measured with Vernier calipers weekly. Offspring were euthanized by rapid decapitation at 16 weeks post-NMU injection. Mammary tumors and contralateral mammary glands were harvested, processed and stored as previously described (Polanco et al., 2011). Animal care was performed in accordance with guidelines from the Rutgers Institutional Animal Care and Use Committee (IACUC) and complied with NIH policy.

Histology and Immunohistochemistry (IHC)

Tissues were fixed, processed and stained with hematoxylin and eosin and tumors were classified as hyperplasia, adenoma or adenocarcinoma by a toxicological pathologist as previously described (Polanco et al., 2010). For ER, IHC was performed as previously described (Polanco et al., 2010) with the following modifications: following deparaffinization, slides were placed in xylene/100% ethanol (50:50), followed by rehydration using decreasing concentrations of ethanol to water. Antigen retrieval was performed for 30 minutes, endogenous peroxidase activity was quenched for 10 minutes,

and normal goat serum (Vector Laboratories, Burlingame, CA) was used for blocking. Rabbit-ER α primary antibody (MC-20; Santa Cruz Biotechnology) was used at a dilution of 1:500. Rabbit primary antibody isotype control (Life Technologies) served as a negative control. Slides were counterstained with hematoxylin for determination of total cell counts, dehydrated using increasing concentrations of ethanol, xylene/100% ethanol (50:50) and xylene and mounted with Permount (Fisher Scientific). Images of three representative fields of view were taken for each tumor at 20x magnification. The percentage of ER-positive cells for each image was determined independently by two individuals who were blinded to treatment and these values were averaged. Cells were considered ER-positive when brown staining was specific to the nucleus. Total cell counts were determined by counting both brown and purple nuclei using ImageJ (<http://imagej.nih.gov/ij/>).

IHC to detect PR was carried out similarly with the following exceptions: after rehydration, endogenous peroxidase activity was quenched with 3% H₂O₂ made in methanol for 10 minutes prior to antigen retrieval. Slides were blocked in 50% normal horse serum (Vector Laboratories) at RT for 1 hour and incubated overnight at 4°C with a mouse monoclonal primary antibody (1:300; HPRA7, Thermo Scientific) or mouse primary antibody isotype control (Life Technologies). A biotinylated horse anti-mouse secondary antibody (Vector Laboratories) was used. For HER2, IHC was carried out as described above for ER using a rabbit HER2/ErbB2 primary antibody (1:300; 29D8, Cell Signaling, Danvers, MA). For PR and HER2 tumor status, entire tissue sections were evaluated at 10x and 20x magnifications under the microscope by two different individuals. Tumors were considered PR- or HER2-positive if 10% of cells had positive staining (Calhoun and Collins, 2015; Hammond et al., 2010).

Ki67 IHC was carried out as described for ER with the following exceptions: tissues were blocked in normal goat serum (Vector Laboratories) at RT for 1 hour and incubated overnight at 4°C with either rabbit-Ki67 primary antibody (1:100; SP6, Abcam, Cambridge, United Kingdom) or rabbit primary antibody isotype control (Life Technologies). Entire tissue sections were scanned at 10x and 20x magnifications under the microscope by three individuals. The amount of Ki67 staining was scored as either 0 (none), 1 (low), 2 (moderate), or 3 (high) and the average of the three scores was calculated for each tumor.

RNA Analysis

RNA was isolated and qRT-PCR primers were validated as previously described (Agostini-Dreyer et al., 2015) with the exception that RNA quality was determined as described in (Polanco et al., 2010). Primer sequences are listed in Table 2. qRT-PCR was performed using Power SYBR Green PCR Master Mix (Life Technologies) on 384 well plates using a ViiATM7 Real-Time PCR system. RNA from each of the treatment groups was pooled to obtain a calibrator sample for use in 2^{-C_t} calculations. β -actin was used as the reference gene.

Statistical Analysis

Gestational eight gain of dams and postnatal weight gain of offspring averaged by litter were analyzed using repeated measures ANOVA followed by post hoc Newman Keuhl's test.

Litter size, average pup weight per litter and tumor multiplicity were analyzed by one-way ANOVA followed by post hoc Tukey's test. Day of parturition was analyzed using a Fisher's exact test while Chi Square analysis was used for PR IHC analysis. Tumor incidence was analyzed using a log-rank Mantel-Cox test. IHC analysis for ER and Ki67 and qRT-PCR data were analyzed using an unpaired Student's t-test. When variances were not equal, Welch's correction was used. GraphPad Prism version 5.0 (La Jolla, CA) was used to perform statistical analyses and $p = 0.05$ was considered significant.

Results

Dam and Offspring Parameters

As shown in Figure 1A, there was a treatment ($F_{2, 15} = 55.45$; $p < 0.0001$), day ($F_{7, 105} = 175.19$; $P < 0.0001$) and treatment X day ($F_{14, 105} = 55.12$; $p < 0.0001$) effect for dam weight. Post-hoc testing revealed that all treatment groups were significantly different from each other ($p < 0.001$ for all). As anticipated, alcohol and pair-fed dams gained significantly less weight than ad lib dams beginning at gestation day 11 ($P < 0.05$ for all). In addition, the pair-fed dams gained less weight than alcohol dams beginning at gestation day 17 ($p < 0.05$ for all). Alcohol dams had a delay in parturition, with five of seven dams giving birth on gestation day 23 instead of day 22 as observed in all but one of the control dams, whose groups were combined for analysis ($p < 0.05$; Table 3). This agrees with previously published work in rodents (Abel, 1979).

Litter size was not significantly different between treatment groups (Figure 1 B); however, as shown in Figure 1 C, there was a treatment effect for average pup weight per litter ($F_{2,15} = 15.04$; $p < 0.0002$), with pup weight less for pair-fed dams than for alcohol and ad lib dams, which did not differ from each other. Beginning at cross-fostering, individual weights of female offspring were recorded biweekly until study termination (Figure 1 D). There was a treatment ($F_{2, 15} = 8.81$; $p = .003$), day ($F_{22, 330} = 3383$; $p < 0.0001$), and treatment X day ($F_{44, 330} = 4.61$ $p < 0.0001$) effect for offspring weight per litter. Post-hoc testing revealed that alcohol and pair-fed offspring had lower body weights than ad lib offspring ($p < 0.05$ for all) beginning at day 5 ($p < 0.05$ for all).

Tumor Parameters

For each dam, the number of female offspring, number of offspring with tumors, total number of tumors, and average tumors per animal are presented in Table 4. No tumors developed in any of the offspring from two of the seven alcohol-exposed dams, one of the four pair-fed dams, and one of the seven ad lib dams, thus when tumor data were analyzed by litter there were five, three and six litters, respectively. To determine the effect of alcohol exposure *in utero* on tumor incidence of the offspring, the week of first tumor appearance for each offspring of a given dam was averaged and presented as a Kaplan Meier curve (Figure 2 A). The average week of first tumor appearance was 9, 10 and 11 weeks for offspring of alcohol, ad lib and pair-fed dams, respectively. Overall there was no difference in tumor incidence between the three groups. However, tumor multiplicity tended to be greater ($F_{2, 11} = 3.532$; $p = 0.06$) for the offspring of alcohol dams. Tumor growth rate (calculated as tumor volume divided by the number of weeks the tumor was palpable) indicated no difference

between treatment groups (data not shown). Tumors contained several different types of lesions and were classified by the most advanced type of lesion present in the tumor. Many tumors exhibited extensive stromal inflammation in agreement with another report (Soares-Maia et al., 2013) describing the carcinogen-induced tumor phenotype. As shown in Figure 2 C, when all tumors across treatments were considered regardless of litter, the alcohol group developed hyperplasias, adenomas and adenocarcinomas with the majority of the tumors presenting as adenocarcinomas. No hyperplasias were observed in the pair-fed and ad lib groups, with the majority of the tumors in the pair-fed group presenting as adenomas compared to the ad lib group where approximately 60% of the tumors were adenocarcinomas. To account for the effect of litter, the first tumor that appeared from any offspring of a given dam was classified by tumor type and presented in Figure 2 D. By this analysis, the first tumor that appeared in four of the five alcohol litters and three of the six ad lib litters were adenocarcinomas. In contrast, the first tumor appearing in all three of the pair-fed litters was an adenoma.

Tumor Phenotype

In general, the tumor parameters presented above were similar for pair-fed and ad lib control groups. Since the ad lib diet differs substantially from the liquid diet, further analyses were performed with tissues from alcohol and pair-fed groups only. We previously reported that offspring from alcohol-exposed dams exhibit more ER-negative tumors relative to pair-fed controls at 23 weeks post-NMU injection (Polanco et al. 2010). For the present work, we were interested in determining if these changes would be observed at earlier time points using a more quantitative method of IHC analysis than used previously (i.e. visual observation with 10% cut-off). For this analysis, ER-positive cells were counted and expressed as percentage of total cells in the section (Figure 3 A). In general, NMU-induced tumors in both treatment groups exhibited a wide range of ER-positivity, with some tumors having little to no ER expression and other tumors containing up to 30% ER-positive cells (Figure 3 B). When data were presented per litter, two of five litters from alcohol-exposed dams had offspring with average ER values that would be characterized as ER-negative using a 10% cut-off (Figure 3 C). In contrast, offspring from all three pair-fed dams exhibited ER-positive tumors. Across all tumors there was not a relationship between ER-positivity and tumor phenotype (Figure 3 D). As shown in Figure 4 A, when all tumors were examined, the alcohol group exhibited 40% lower ER mRNA expression compared to the pair-fed group ($t = 2.150$; $df = 40$; $p = 0.038$), however, while the trend remained, this significance was lost when data were expressed per litter (Figure 4 B; $t = 0.064$; $df = 2$; $p = 0.470$). Interestingly, across all tumors, adenocarcinomas from the alcohol group tended to have lower ER α mRNA (Figure 4 C). Examination of β -actin levels indicated that this housekeeping gene did not differ by tumor phenotype (Figure 4 D).

Since NMU-induced tumors are typically ER-positive we were interested in further identifying markers of the luminal A or B subtypes using PR and Ki67 status (Figures 5 A and C). We also analyzed tumors for HER2 to rule out the triple negative phenotype (ER α , PR and HER2 negative). All tumors were negative for HER2 in both treatment groups (Figure 5 B). When all tumors were examined, the majority of tumors in the alcohol and pair-fed groups were PR negative (Figure 6 A). However, the alcohol group tended to

develop more PR-positive tumors (32%) compared to the pair-fed group (21%; $p = 0.079$). When litter was accounted for, this effect was even more pronounced (Figure 6 B), with only 8% of offspring from dams in the pair-fed group exhibiting PR positivity compared to 30% in the alcohol group ($p < 0.0001$). Ki67 expression also tended to be higher in tumors from animals exposed to alcohol *in utero* compared to the pair-fed group, although the differences were not significant (Figure 6 C and D). Interestingly, as shown in Figure 6 E, Ki67 expression was significantly increased in PR positive tumors compared to PR negative tumors ($t = 3.788$; $df = 43$; $p = 0.0005$).

Gene Expression in Tumors and Normal Mammary Gland Tissue

Our previous work showed that alterations in the IGF system in normal mammary glands occurs during early development in animals exposed to alcohol *in utero* (Polanco et al., 2011). Therefore, we examined expression of signaling molecules in the IGF pathway. There were no significant differences in tumor mRNA expression of IGF-I, IGFR, Akt1, or Akt2 mRNA among the treatment groups (data not shown). However, IGF-II mRNA expression was increased approximately 9-fold when all tumors from the alcohol group were compared to all tumors in the pair-fed group (Figure 7 A; $t = 2.226$; $df = 25$; $p = 0.035$). When litter was accounted for, the difference between the two groups was still observed (Figure 7 B) though significance was lost ($t = 2.487$; $df = 4$; $p = 0.06$). Examination of IGF-II mRNA data for all individual tumors by tumor type indicated that expression tended to be higher in the adenomas and adenocarcinomas of the alcohol group compared to each tumor type in the pair-fed group (Figure 7 C). IGFBP-5 (Figure 7 D) followed a similar pattern to that of IGF-II, with analysis of all tumors from the alcohol group demonstrating an increase in expression of both genes compared to all tumors from the pair-fed group ($t = 2.783$; $df = 36.98$; $p = 0.0084$). Again, these differences remained when the data were calculated by litter but significance was lost. Similar to IGF-II, IGFBP-5 mRNA expression tended to be greater in the adenomas and adenocarcinomas from the alcohol group compared to each tumor type in the pair-fed group (Figure 7 F).

A goal of the current study was to determine if changes in gene expression occurred in normal mammary gland tissue that could be driving tumor expression. Analysis of mammary glands that did not contain tumors from tumor-bearing animals indicated that IGF-II expression tended to be higher in offspring exposed to alcohol *in utero* (Figure 8 A: all tumors: $t = 1.924$; $df = 14$; $p = 0.0746$; Figure 8 B: by litter: $t = 1.992$; $df = 4$; $p = 0.1171$). Since IGF-II is an imprinted gene, expression of DNA methyltransferase (DNMT1) was examined. When analyzed by litter, DNMT1 mRNA expression also tended to be higher in contralateral mammary glands of alcohol-exposed offspring (Figure 8 D: $t = 2.107$; $df = 6$; $p = 0.0797$). Differences in expression of IGFBP-5 were not observed between the two treatment groups (data not shown).

Discussion

In our previous work, tumor development in animals exposed to alcohol *in utero* was followed until a plateau in tumor incidence was achieved at 23 weeks post-NMU injection (Polanco et al., 2010). The findings here support our previous study, indicating that

increased tumor multiplicity leads to an overall greater tumor burden in offspring from the alcohol group compared to the pair-fed and ad lib groups.

A large body of evidence now indicates that nutritional status of pregnant females can alter long-term disease risk in the offspring, supporting the DOHaD hypothesis (Dyer and Rosenfeld, 2011). Therefore it is important to consider the effect of diet in the experimental paradigm when studying the effects of FASD on long-term disease risk. In the current study, dams fed the liquid diet containing alcohol gained less weight throughout gestation than ad lib dams in accordance with work of others (Abel, 1979; Lan et al., 2009; Lochry et al., 1980). This is because alcohol itself decreases food intake and depresses growth (Lieber and DeCarli, 1989). Therefore the control diet is pair-fed to account for this decrease in food intake. The solid ad lib diet was originally included to determine if the liquid diet alone shifted metabolic and growth parameters. An ad lib solid diet that differs in nutrient composition has continued to be included in many studies that use a liquid alcohol diet. In our study, the liquid Lieber-DeCarli diet used for the alcohol and pair-fed groups had higher fat and lower protein than the solid diet used for the ad lib group. Given that nutritional status of the mother can affect developmental programming of the offspring, the pair-fed group is the appropriate control for the alcohol diet since the diets are isocaloric and the diet composition is similar. Also, the liquid diet does not contain phytoestrogens which also makes comparison with the phytoestrogen-containing solid diet difficult since phytoestrogens such as soy have been shown to protect against carcinogen-induced tumorigenesis in rats (Hakkak et al., 2000). Therefore only the alcohol and pair-fed groups were used for the remaining analyses. It is possible that the level of food intake and/or diet composition such as the high fat diet used here interact with alcohol to promote tumorigenesis in offspring. This is an interesting area for future research.

A caveat to the current work is that the pair-fed dams gained significantly less weight during gestation than the alcohol-fed dams, leading to smaller average pup weights per litter. While this was not enough to affect growth rate of the pups over the course of the study, it does raise the question of whether the pair-fed dams and offspring that survived were more robust. However, the agreement of the tumor parameter data with our previous study suggests this was not a major issue, although it cannot be completely ruled out.

To classify tumor subtype we performed IHC for ER, PR, and HER2. We had previously reported that at 23 weeks post-NMU injection approximately 90% of tumors from the pair-fed group were ER-positive while only 50% of the tumors from the alcohol group were ER-positive (Polanco et al., 2010). When analyzed by litter, the present results showed that at 16 weeks all of the pair-fed dams had offspring that were on average ER-positive, while offspring from two of five dams exposed to alcohol exhibited ER-negative tumors. Therefore tumors from rats exposed to alcohol *in utero* may lose ER-positivity more readily than tumors in control groups. This is supported by the finding that mammary glands of alcohol-exposed offspring had higher ER expression relative to controls, suggesting that ER-positivity may be lost as the tumors progress (Hilakivi-Clarke et al., 2004). A caveat is that colorimetric IHC is semi-quantitative since staining intensity was not accounted for in this study. Interestingly, ER mRNA expression was decreased in tumors of the alcohol group compared to the pair-fed group. The mRNA data suggest that tumors in the alcohol group

may be starting to lose ER expression and mRNA levels may not have translated to the protein level at this earlier time point. Punch biopsies collected from the same tumor over time will be needed to confirm if tumors from rats exposed to alcohol *in utero* lose ER positivity over time.

While ER status was similar between the treatment groups, the alcohol group developed more PR-positive tumors compared to the pair-fed controls when data were analyzed by litter. In our previous study we found that alcohol-exposed animals had significantly increased circulating E2 (Polanco et al., 2010). E2 induces PR expression in normal mammary glands as well as breast cancer cells (Haslam and Shyamala, 1979; Katzenellenbogen and Norman, 1990). Animal studies and clinical data indicate that the progesterone signaling pathway plays a significant role in normal mammary cell proliferation as well as the initiation and development of mammary tumors (Knutson and Lange, 2014; Obr and Edwards, 2012). The possibility that increased PR observed in tumors of the alcohol group may be promoting cell proliferation is supported by the finding that Ki67 staining was significantly increased in PR-positive tumors.

In breast cancer IGF-II is expressed mostly in the stroma as well as in malignant epithelial cells and it serves as a potent mitogen. In addition, fibroblasts derived from malignant breast tumors have increased IGF-II expression compared to fibroblasts derived from benign tumors or normal breast (Cullen et al., 1991; Osborne et al., 1989; Paik, 1992; Singer et al., 1995; Yee et al., 1988). IGF-II is an imprinted gene that is expressed on the paternal allele and epigenetically silenced on the maternal allele by DNA methylation (McCampbell et al., 2008). Loss of imprinting (LOI) of IGF-II resulting in gene expression on both paternal and maternal alleles has been found in multiple cancers including breast cancer (Cui et al., 2003; Cui et al., 2001; McCampbell et al., 2008; Ogawa et al., 1993; Okamoto et al., 1997; Rainier et al., 1993; Roy et al., 2000). Thus, it is possible that IGF-II expression is increased as a result of LOI in the tumors from animals exposed to alcohol *in utero*. The tendency for DNMT1 expression to be increased in the normal mammary glands of alcohol-exposed offspring supports this possibility and warrants further investigation.

In addition to IGF-II, IGFBP-5 was also increased at 16 weeks post-NMU injection in tumors of alcohol-exposed offspring. This protein has multiple actions and can either promote cell proliferation or apoptosis through IGF-dependent mechanisms as well as IGF-independent mechanisms (Akkiprik et al., 2008; Perks et al., 1999). In terms of tumor phenotype, animals exposed to alcohol *in utero* developed more hyperplasias than the pair-fed control group. Within the alcohol group, expression of IGF-II and IGFBP-5 was highest in the hyperplasias, suggesting that these growth factors may promote cell proliferation early on. However, both IGF-II and IGFBP-5 appeared to decrease as the tumors became more malignant (comparing hyperplasia to adenoma to adenocarcinoma). Interestingly, in our previous work, IGFBP-5 was decreased in tumors of the alcohol group at 23 weeks post-NMU injection and we proposed that this might increase the availability of IGF-I to bind to IGF receptors (Polanco et al., 2010). Therefore as the tumor progresses to adenoma or adenocarcinoma it may switch its mechanism of signaling. In keeping with the multiple mechanisms of IGFBP-5 action, studies have shown that IGFBP-5 may act as a tumor

suppressor while other studies suggest that IGFBP-5 may contribute to the development of breast cancer and serve as a prognostic factor for the disease (Li et al., 2007)

In conclusion, this study provides further evidence that alcohol exposure *in utero* enhances susceptibility to carcinogen-induced mammary tumorigenesis. Alcohol-exposed animals exhibit more malignant tumors that tend to be more ER-negative and PR-positive and express increased IGF-II and IGFBP-5 at 16 weeks post-NMU injection. The finding that IGF-II expression is also increased in the contralateral mammary gland of alcohol-exposed offspring suggests that this change may drive the enhanced tumorigenesis in these animals. Additional studies will be aimed at these particular pathways to further delineate the mechanism.

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Figure 1. Dam and offspring parameters

Data are expressed as mean $\pm$ SEM. A) Dam weight across gestation; $n = 7$ (alcohol), 4 (pair-fed), and 7 (ad lib). Repeated measures ANOVA with Newman Keuhl's posttest indicated that weights of alcohol and pair-fed dams differed from ad lib (* $p < 0.05$) beginning at day 11, while the pair-fed group differed from the alcohol group beginning at day 17 (# $p < 0.05$). B) Litter size did not differ between treatment groups. C) Average pup weight per litter at birth; ($p < 0.0002$) with Tukey's multiple comparison posttests. Letters denote significant differences. D) Weight of female offspring per litter; $n = 7$ (alcohol), 4 (pair-fed), and 7 (ad lib). Repeated measures ANOVA with Newman Keuhl's posttest indicated that alcohol and pair-fed offspring had lower body weights than ad lib offspring (* $p < 0.05$ for both) beginning at day 5 ($p < 0.05$ for all).

Figure 2. Tumor parameters and phenotype

A) Tumor incidence per litter represented as a Kaplan Meier graph. $n = 7$ (alcohol), 4 (pair-fed), and 7 (ad lib). B) Tumor multiplicity per litter at study termination; $n = 5$ (alcohol), 3 (pair-fed), and 6 (ad lib). Data are mean $\pm$ SEM ($p = 0.0654$). C) Tumor phenotype across treatment groups. D) Tumor phenotype of first tumor that appeared in any offspring of a given dam across treatment groups.

Figure 3. Estrogen receptor- α (ER α) protein expression in tumors

A) Representative image of IHC showing cells considered positive (arrows) and negative (arrowheads) for ER α . B) ER α positive cells as a percentage of total cells. C) To analyze data by litter, an average ER α value was obtained for each offspring using values from all tumors and values per offspring were averaged to determine percentage of ER α -positive cells per litter. D) Expression of individual tumor ER values presented by tumor type.

Figure 4. Estrogen receptor- α (ER α) mRNA expression in tumors

A) Relative expression of tumor ER α mRNA was analyzed by qRT-PCR using β -actin as a reference gene. Expression relative to a calibrator pool is represented as mean $\pm$ SEM (* $p = 0.0376$). B) To analyze data by litter, an average ER α mRNA value was obtained using all tumors from each offspring. Average values for all offspring were averaged by litter to determine relative expression of ER α mRNA per litter. C) ER α expression was analyzed by tumor type. D) Expression of individual tumor β -actin presented by tumor type.

Figure 5. Immunohistochemistry for progesterone receptor (PR), HER2, and Ki67 in tumors
 Representative images of A) PR-positive and PR-negative tumors using a visual 10% cut-off.
 B) HER2-negative tumors irrespective of treatment group. Rat mammary gland was used as a positive control. All tumors were classified as HER2 negative using a 10% visual cut-off.
 C) Ki67 protein expression. Staining was scored as 0 = none; 1 = low, 2 = moderate; or 3 = high.

Figure 6. Progesterone receptor (PR) and Ki67 expression in tumors

A) PR status of individual tumors expressed as a percentage for each treatment group (Chi Square analysis; $p = 0.079$). B) PR status averaged per litter (Chi Square analysis; $p = 0.0001$). C) Ki67 expression for individual tumors. D) Average Ki67 status of tumors per litter. E) Ki67 score of each tumor graphed by PR tumor status. (* $p = 0.0005$).

Figure 7. mRNA expression of IGF-II and IGFBP-5 in tumors

Data were analyzed by qRT-PCR using β -actin as a reference gene. Expression relative to a calibrator pool is represented as mean $\pm$ SEM. A and D represent data from individual tumors, B and E represent average expression per litter, and C and F represent expression in individual tumors plotted by tumor type (* $p < 0.05$).

Figure 8.

mRNA expression of IGF-II and DNMT1 in normal mammary glands of animals bearing tumors. Data were analyzed by qRT-PCR using β -actin as a reference gene and expression relative to a calibrator pool is represented as mean $\pm$ SEM. A and C represent data from individual normal mammary glands of animals bearing tumors. B and D represent average expression per offspring averaged per litter.

Table 1

Comparison of diet composition for the diets used for the alcohol, pair-fed, and ad lib groups.

Diet	Company Cat #	Protein	Fat	Carbohydrate	Ethanol
Alcohol Alcohol liquid diet, Lieber DeCarli '82	Bio-Serv Cat# F1258SP	18.0%	35.0%	11.5%	35.5%
Pair-fed Control liquid diet, Lieber DeCarli '82	Bio-Serv Cat# F1259SP	18.0%	35.0%	47.0%	–
Ad lib Solid Rat Chow	Purina/Lab Diet Cat# 5012	27.1%	13.2%	59.7%	–

Table 2

Primer sequences used for gene expression analysis by qRT-PCR.

Gene	Accession #	Primer Sequence (5' to 3')	Product Length
β -actin	NM_031144	F: CCATTGAACACGGCATTGTC R: GCCACACGCAGCTCATTGTA	82
DNMT1	NM_053354.3	F: ACCTGGGGCCAATCAATCAG R: TTGGTGCATACTCTGGGCTG	123
ER α	X61098	F: TCGGGAATGGCCTTGTTG R: AGCTGCGGGCGATTGA	65
IGF-II	NM_031511.2 (transcript variant 1) NM_001190162.1 (transcript variant 2) NM_001190163.1 (transcript variant 3)	F: AAGAGCTCGAAGCGTTCAGAGA R: CTTTGGGTGGTAACACGATCAG	63
IGFBP-5	NM_012817.1	F: TTGAGGAACTGAGGACCTCGGAA R: CCTTCTCTGTCCGTTCAACTTGCT	110

F: forward primer; R: reverse primer.

Table 3

Number of dams that gave birth on gestation days 22 and 23.

Treatment Group	Gestation Day 22*	Gestation Day 23
Alcohol	2	5
Pair-fed	4	1
Ad lib	7	0

*
p < 0.05; Fishers Exact Test for alcohol versus pair-fed and ad lib groups.

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Table 4

Tumor incidence table per litter.

Treatment	Dam	Female Offspring	# with Tumors	Total # of Tumors	Average Tumors per Animal per Litter
Alcohol	1	5	3	8	2.7
	2	3	1	2	2
	3	4	1	2	2
	4	7	6	17	2.8
	5	2	2	3	1.5
	6	6	0	0	0
	7	4	0	0	0
Pair-fed	1	9	2	2	1
	2	7	5	11	2.2
	3	4	0	0	0
	4	7	2	2	1
Ad lib	1	3	2	5	2.5
	2	5	1	1	1
	3	5	3	3	1
	4	5	2	2	1
	5	7	1	1	1
	6	5	2	2	1
	7	3	0	0	0