Research Article

Cancer Prevention Research

Exposure to Excess Estradiol or Leptin during Pregnancy Increases Mammary Cancer Risk and Prevents Parity-Induced Protective Genomic Changes in Rats

Sonia de Assis, Mingyue Wang, Lu Jin, Kerrie B. Bouker, and Leena A. Hilakivi-Clarke

Abstract

Using a preclinical model, we investigated whether excess estradiol (E2) or leptin during pregnancy affects maternal mammary tumorigenesis in rats initiated by administering carcinogen 7,12-dimethylbenz(a) anthracene (DMBA) on day 50. Two weeks later, rats were mated, and pregnant dams were treated daily with 10 μg of 17β-estradiol, 15 μg of leptin, or vehicle from gestation day 8 to 19. Tumor development was assessed separately during weeks 1 to 12 and 13 to 22 after DMBA administration, because pregnancy is known to induce a transient increase in breast cancer risk, followed by a persistent reduction. Parous rats developed less (32%) mammary tumors than nulliparous rats (59%, $P < 0.001$), and the majority (93%) of tumors in the parous rats appeared before week 13 (vs. 41% in nulliparous rats), indicating that pregnancy induced a transient increase in breast cancer risk. Parous rats exposed to leptin (final tumor incidence 65%) or E2 (45%) during pregnancy developed mammary tumors throughout the tumor-monitoring period, similar to nulliparous control rats, and the incidence was significantly higher in both the leptin- and E2-exposed dams after week 12 than in the vehicle-exposed parous dams ($P < 0.001$). The mammary glands of the exposed parous rats contained significantly more proliferating cells ($P < 0.001$). In addition, the E2- or leptin-treated parous rats did not exhibit the protective genomic signature induced by pregnancy and seen in the parous control rats. Specifically, these rats exhibited downregulation of genes involved in differentiation and immune functions and upregulation of genes involved in angiogenesis, growth, and epithelial-to-mesenchymal transition. *Cancer Prev Res; 6(11); 1194–211. ©2013 AACR.*

Introduction

Pregnancy affects a woman's breast cancer risk by first inducing a transient increase in risk, lasting for 5 to 7 years (1–4), and then either permanently reducing or increasing the risk, depending upon the age of the woman. Women who gave birth before age 20 decrease their breast cancer risk by half compared to women who were over 30 when they had their first child (5). The latter, in turn, have a significantly higher lifetime risk of breast cancer than nulliparous women (6, 7). The protective effect of early pregnancy is limited to estrogen and progesterone receptor positive (ER+ and PR+) breast cancers (8, 9), whereas late first pregnancy can increase the risk of developing either ER+ or ER− cancers (9, 10).

Several theories have been offered to explain the protective effects of early pregnancy on breast cancer risk (11, 12). Importantly, parous women and animals exhibit permanent changes in gene expression patterns, resulting in a pregnancy-induced protective genomic signature. This signature involves genes that can prevent malignant transformation, including those that reduce mammary epithelial cell proliferation and increase differentiation (13–15). It is less clear why a late first pregnancy increases breast cancer risk, but it may be caused by an aging-related increase in the presence of transformed mammary epithelial cells that can start proliferating when exposed to a high pregnancy hormonal environment. Accumulating evidence indicates that women who had the highest circulating estrogen levels during pregnancy (16, 17) or were exposed to the synthetic estrogen diethylstilbestrol (DES; refs. 18 and 19) are at highest risk of developing breast cancer. In addition, giving birth to an infant with high birth weight is associated with a high maternal estriol/α-fetoprotein ratio and increased breast cancer risk (20).

The possibility that elevated leptin levels during pregnancy also may increase breast cancer risk has not been explored. Serum leptin concentrations increase during pregnancy, peaking during the second trimester (21, 22), although the increase is not nearly as dramatic as with estrogens. Pregnant women who gain an excessive amount of weight have high leptin levels (23–25) and are significantly more likely to develop breast cancer after menopause

Authors' Affiliation: Lombardi Cancer Center and Department of Oncology, Georgetown University, 3970 Reservoir Road, NW, Washington, DC 20057

Corresponding Author: Leena A. Hilakivi-Clarke, Georgetown University Medical Center, NRB, Room E407, 3970 Reservoir Road, NW, Washington, DC 20057. Phone: 202-687-7237; Fax: 202-687-7505; E-mail: clarkel@georgetown.edu

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than women whose weight gain during pregnancy does not exceed the recommendations provided by the Institute of Medicine (IOM; ref. 26). In preclinical studies, excessive weight gain induced by feeding pregnant dams an obesity-inducing high-fat diet increases pregnancy leptin levels and subsequent mammary tumorigenesis (27). Importantly, leptin interacts with estradiol (E2) and the ER. Leptin has been shown to activate ER-alpha, likely through its ability to stimulate aromatase and/or mitogen-activated protein kinases (MAPK; refs. 28 and 29). Furthermore, leptin decreases ER-alpha ubiquitination and increases ER-alpha half-life, potentially leading to increased ER-alpha activity (30). E2, in turn, can interfere with leptin's actions by regulating the expression of the leptin receptor (31). Similar to E2, leptin promotes the growth of ER+ human breast cancer cells in culture (32, 33), but it also induces proliferation of ER− breast cancer cells (34).

In this study, we sought to establish experimentally whether treating pregnant dams with excess E2 or leptin during pregnancy increases later mammary tumorigenesis in rats. Our results indicate that in the vehicle-treated control rats pregnancy induced a transient increase in mammary cancer risk that lasted until mammary glands had undergone involution and returned to a nonpregnant and nonlactating stage. When back to this stage, the risk of developing breast cancer was dramatically reduced, resulting in a lower lifetime risk than what was seen in nulliparous rats. Rats exposed to an excess of either E2 or leptin during pregnancy exhibited a sustained increase in mammary tumorigenesis, similar to nulliparous rats. Higher breast cancer risk in the parous E2 or leptin rats than in vehicle-treated parous control rats may be related to a persistent increase in cell proliferation in their mammary glands, and absence of parity-induced protective changes in the genome. Thus, our preclinical study suggests that an exposure to excess E2 or leptin during pregnancy increases risk by preventing pregnancy-induced reduction in breast cancer risk and the protective changes in genomic signaling pathways seen in the parous mammary gland.

Materials and Methods

Animals

Five-week-old Sprague-Dawley rats were obtained from Charles River and fed AIN93G diet upon arrival. Animals were housed in a temperature- and humidity-controlled room at the Georgetown University Resource Animal Facility under a 12-hour light–dark cycle. All animal procedures were approved by the Georgetown University Animal Care and Use Committee, and the experiments were performed following the National Institutes of Health guidelines for the proper and humane use of animals in biomedical research.

Carcinogen exposure

At 50 days of age, a total of 223 female rats were administered 10 mg of the mammary carcinogen 7,12-dimethylbenz(a)anthracene (DMBA; Sigma Chemical Co.) by oral gavage. Carcinogen was dissolved in peanut oil and given in a volume of 1 mL.

Mating and hormonal exposures

Two weeks after DMBA exposure, female rats were mated by housing 2 female rats and 1 male rat together. Positive vaginal plug was used to determine the first day of pregnancy. On gestation day 8, pregnant females were divided into 3 experimental groups: control dams receiving subcutaneous vehicle injections ($n = 43$), E2 dams receiving subcutaneous injections of 10 μg of 17β-estradiol (Sigma Chemical Co.; $n = 42$), or leptin dams receiving subcutaneous injections of 15 μg of leptin (R&D systems; $n = 40$). Injections were given daily until gestation day 19. The doses were chosen based upon a pilot study that indicated that neither 10 μg E2 nor 15 μg leptin affected weight development during pregnancy. After giving birth, dams were allowed to nurse their offspring for 3 weeks, and then the pups were weaned.

Exposure of nulliparous rats to hormones

An additional set of 78 DMBA-exposed female rats, 3 weeks after the carcinogen exposure (to match with day 8 of gestation), were divided to 3 groups and given subcutaneous injections of vehicle ($n = 29$), 10 μg of E2 ($n = 41$), or 15 μg of leptin ($n = 28$). Injections were given daily for a total of 2 weeks.

Monitoring tumorigenesis

Four weeks post-DMBA administration, we began checking rats weekly for mammary tumors by palpation. Tumor growth was measured using a caliper and the length, width, and height of each tumor were recorded. Animals were sacrificed if any tumor reached a size of 25 to 30 mm in diameter. The remaining animals, including those that did not develop tumors, were sacrificed 17 weeks after pregnancy ended/22 weeks after DMBA administration. Endpoints for this study were time to tumor appearance (tumor latency), the number of tumors per animal (tumor multiplicity), and the percentage of rats that developed tumors per experimental group (tumor incidence).

Pregnancy hormone measurements

Concentrations of circulating leptin and E2 were determined in serum collected by tail bleeding on gestation day 19 ($n = 5$–7 per group), using a rodent leptin EIA kit from Assay Designs Inc. and a rodent E2 EIA kit from Cayman Chemical Company, respectively, following the manufacturers' instructions.

Immunohistochemical detection of cell proliferation

At the end of the tumor-monitoring period (22 weeks post-DMBA exposure), all rats were sacrificed and their mammary tissues and tumors were obtained. Cell proliferation in the mammary tissue was assessed by immunohistochemistry staining for PCNA in 6 rats per group. The second and third glands were used and they were fixed in 10% buffered formalin, embedded in paraffin, and

sectioned (5 μm). Sections were deparaffinized in xylene, hydrated through graded alcohols, and incubated with 3% H_2O_2 for 10 minutes to block endogenous peroxidases. Nonspecific binding was blocked with normal rabbit serum from the Vectastain Elite ABC Kit (Vector Laboratories, Inc.) for 20 minutes. Tissue sections were incubated overnight with the primary antibody against PCNA at a 1:500 dilution (Santa Cruz Biotechnology Inc.). After several washes, sections were treated with the secondary antibody (biotinylated anti-goat IgG from the Vectastain Elite ABC Kit; Vector Laboratories, Inc.) for 30 minutes at room temperature, followed by treatment with an avidin and biotinylated horseradish peroxidase complex from the Vectastain Elite ABC Kit (Vector Laboratories, Inc.) for 30 minutes at room temperature. Sections were washed and stained with 3,3′-diaminobenzidine (DAB; Vector Laboratories, Inc.) for 1 minute, washed, and counterstained with Vector's Hematoxylin QS Nuclear Counterstain (Vector Laboratories, Inc.) for 45 seconds. Proliferation index was determined by calculating the percentage of cells with positive PCNA staining in at least 1,000 cells per mammary gland section. Slides were evaluated using the Metamorph software, without knowledge of treatment group.

Detection of apoptosis

Apoptosis was assessed in the same mammary gland sections used to determine proliferation ($n = 6$ per group) by *in situ* oligo ligation (ISOL) assay with an ApopTag Kit (Serologicals Corporation) following the manufacturer's instructions. Briefly, sections were deparaffinized in xylene and hydrated in a series of graded alcohols. The sections were then treated with 20 μg/mL of Proteinase K for 15 minutes. Endogenous peroxidases were quenched with 3% H_2O_2 for 5 minutes. Sections were washed with equilibration buffer (ApopTag Kit) and incubated with the Ligase enzyme for 16 hours at 16°C to 22°C. The reaction was stopped and sections were incubated with a streptavidin–peroxidase conjugate at room temperature. Sections were again washed, incubated with the peroxidase substrate for 10 minutes, and counterstained with 0.5% methyl green (Vector Laboratories, Inc.) for 10 minutes. Apoptotic index was determined by calculating the percentage of cells that were apoptotic through both positive staining and histologic evaluation among 1,000 cells per mammary gland section. All sections were evaluated using the Metamorph software, without knowledge of treatment group.

Microarray analysis

Array hybridization and scanning. The fourth mammary glands that contained no palpable growth or nonpalpable microtumors were obtained from 5 rats per group (control, E2, and leptin exposed), sacrificed 22 weeks after DMBA exposure. Six micrograms of purified total RNA was used to synthesize cDNA and then generate cRNA, which was labeled with biotin according to techniques recommended by Affymetrix. Labeled cRNA was fragmented at 94°C for 35 minutes in a fragmentation buffer and then hybridized to Affymetrix Rat U34 A GeneChips, which contained approximately 7,000 full-length sequences and 1,000 EST clusters. After washing, the chips were stained with strepavidin–phycoerythrin conjugate and then scanned using the Affymetrix GeneChip Scanner 3000 (Hewllet-Packard Co.). Raw data were generated using Affimetrix GeneChip 3.1 software.

Data normalization. In Affimetrix GeneChip experiments, variations in the amount and quality of target hybridized to the array may contribute to an overall variability in hybridization intensities. To reliably compare data from multiple probe arrays, differences of nonbiological origin must be minimized. We accomplished this by normalizing the data using the MicroArray Suite 5.0 (Affymetrix) software to average the intensities for each GeneChip and to calculate a normalization factor. The normalized intensities were obtained from each chip by multiplying raw intensities by the normalization factor.

Identification of gene expression profiles. Normalized results obtained from each group were used to calculate the ratio (control/treated) for each gene. Hybridization signal intensities of relative fold changes, which ranged from $\leq$0.5 for downregulation or $\geq$2-fold for upregulation, were considered to be significant and were reported. The level of significance was set at $P < 0.05$. Dimensionality reduction (elimination of noninformative data) was performed by filtering out genes with low threshold (intensity < 0.1 in both groups) and low fold change (<2.0). In addition, comparisons made had to be significantly different in at least 1 of 3 statistical tests (i.e., equal and unequal variance *t* tests, equal and unequal variance *t* tests on log transformed data, Wilcoxon test).

Data visualization. We calculated the 3-dimensional projections of multidimensional gene expression microarray datasets by using principal component analyses and discriminant component analyses.

Generation and testing of a neural network. To determine whether the model could accurately predict the leptin/E2 exposure, a neural network was trained, independent of gene expression profile selection.

Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was used to confirm the differential expression of selected genes between the control and high-risk groups shown in the microarray analysis. The 4th mammary glands were obtained from a different set of rats ($n = 6$–8 per group) than the ones used for microarray analysis. Briefly, cDNA was reverse transcribed from 50 μg/mL of total input RNA using Taqman Reverse Transcription Reagents (Applied Biosystems). The reverse transcription reaction was carried out in a Taqman master mix under the following conditions: 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes. Next, PCR products were generated from the cDNA samples using the Taqman Universal PCR Master Mix (Applied Biosystems) and Assays-on-Demand (Applied Biosystems) for the appropriate target gene (*Vegfa*, Pleiotrophin, *Mapk 9*, and *Eif4e*). The 18S Assay-on-Demand (Applied Biosystems) was used as an endogenous control in all assays.

Table 1. Effects of exposure to leptin or estradiol on rat dams' pregnancy weight gain

Treatment	Baseline (g) (mean ± SEM)	Third week of gestation (g) (mean ± SEM)	Net weight gain (g) (mean ± SEM)
Control	223.98 ± 2.15	267.92 ± 3.04	43.20 ± 4.16
Leptin	224.39 ± 2.52	266.44 ± 2.82	42.59 ± 1.53
Estradiol	224.00 ± 1.94	264.76 ± 2.13	40.90 ± 1.83

Rats were administered DMBA at 50 days of age and mated 2 weeks later. Pregnant dams were exposed to leptin, estradiol, or vehicle control between days 8 of and 19 of gestation. Body weight values (in grams) collected at baseline and on the last week of gestation are expressed as the mean ± SEM. There were no significant differences in pregnancy weight gain among the groups.

All assays were run on 384-well plates so that each cDNA sample was run in triplicate for the target gene and the endogenous control. qRT-PCR was performed on an ABI Prism 7900 Sequence Detection System and the results assessed by relative quantification of gene expression using the $\Delta\Delta C_T$ method.

Statistical analysis

Data for pregnancy hormone levels and gene expression were analyzed by ANOVA (only assessed in parous rats). Some mammary tumor endpoints (latency and multiplicity) were analyzed by two-way ANOVA, using nulliparous or parous, and treatments as independent variables. Cell proliferation and apoptosis were only assessed in parous rats, and because the estrous cycle may influence mammary cell proliferation and apoptosis in rats (low proliferation: pro-estrus, estrus, and the second part of diestrus; and high proliferation: metestrus and the first part of diestrus), the proliferation and apoptosis indices data were analyzed by two-way ANOVA, using the stage of estrous cycle and E2/leptin exposure as independent variables. Where appropriate, comparisons between groups were done using Holm–Sidak method. Kaplan–Meier curves were used to compare differences in tumor incidence, followed by the log-rank test. Tumor incidence was also analyzed just for post-DMBA weeks 13 and 22, and in this analysis nulliparous control rats were compared to parous control rats and either parous rats exposed to leptin or E2 were included in the analysis. Final tumor incidence was determined using χ^2 test. All tests were performed using the SPSS SigmaStat software, and differences were considered significant if the *P*-value was less than 0.05. All probabilities were two-tailed.

Results

Effects on weight gain and pregnancy hormone levels

Neither E2 nor leptin affected weight gain during pregnancy (Table 1). Birth weights of the pups also were similar, as were the numbers of pups born per litter (Table 1). The concentrations of circulating E2 and leptin, measured in serum samples collected on day 19 of pregnancy, are shown in Fig. 1. Leptin levels were significantly higher in the leptin-exposed dams when compared to either the E2 or control dams ($P < 0.001$). Circulating E2 levels were significantly higher in the E2-treated group, when compared to the control or leptin-treated dams ($P = 0.004$).

Effects on mammary tumorigenesis

Because pregnancy has a transient and long-term effect on breast cancer risk, we considered tumors that developed between weeks 1 and 12 after DMBA as early appearing tumors, and those developing on week 13 or after as long term. Twelve weeks post-DMBA treatment coincided with completion of mammary gland involution in parous rats, as the rats became pregnant 2 weeks after DMBA, gave birth 5 weeks after DMBA and started undergoing involution 8 weeks after DMBA. It then takes 4 weeks for the rat

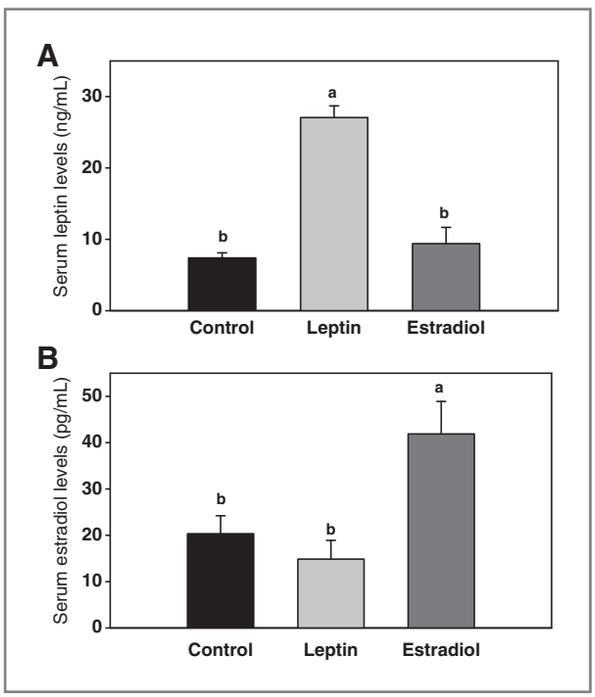

Figure 1. Serum (A) leptin and (B) estradiol levels on day 19 of pregnancy in rat dams exposed to 15 μg of leptin or 10 mg of estradiol between days 8 and 19 of gestation. All values are expressed as the mean ± SEM of 5 to 7 rats/group. Means with a different letter are significantly different from each other: $P < 0.05$.

Table 2. Effects of exposure to leptin or estradiol on later mammary carcinogenesis in nulliparous and parous rats

Treatment	Tumor incidence (%) Weeks after treatment 0–22	0–12	13–22	Tumor multiplicity (mean ± SEM)	Tumor latency (weeks) (mean ± SEM)
Nulliparous rats					
Control	17/29	7/17	10/17[a]	1.88 ± 0.40	13.29 ± 1.09
	59%	41%	59%		
Leptin	14/28	11/14	3/14[b]	1.43 ± 0.14	11.14 ± 1.22
	50%	79%	21%		
Estradiol	22/41	5/22	17/22[c]	1.73 ± 0.24	14.9 ± 0.84
	54%	23%	77%		
Parous rats					
Control	14/43[b]	13/14	1/14[a]	1.93 ± 0.29	7.07 ± 0.76[a]
	32%	93%	7%		
Leptin	26/40[a]	14/26	12/26[b]	1.33 ± 0.12	12.27 ± 1.11[b]
	65%	54%	46%		
Estradiol	19/42[a,b]	9/19	10/19[b]	1.37 ± 0.18	12.31 ± 1.38[b]
	45%	47%	53%		

Nulliparous rats were administered DMBA at 50 days of age and 3 weeks later treated with vehicle, leptin, or estradiol for 2 weeks. Parous rats were also administered DMBA at 50 days of age and mated 2 weeks later. Pregnant dams were exposed to leptin or estradiol between days 8 and 19 of gestation. Values for tumor latency and multiplicity are expressed as the mean ± SEM. Total tumor incidence values are expressed as the number of rats with mammary tumors per group, and between weeks 0–12 or 13–22 after DMBA as number and percentage of all tumors per group. Values marked with a different letter are significantly different from each other: $P < 0.05$.

mammary gland to return to a prepregnancy stage (13, 35); that is, this occurred on week 12 in our study.

Effect of E2 and leptin exposures in nulliparous rats. We first determined whether a 2-week exposure of nulliparous rats to E2 or leptin alters mammary tumorigenesis. Table 2 indicates that the mean mammary tumor latency in nulliparous control rats is about 13 weeks. Tumor latency did not differ among the nulliparous control-, E2-, or leptin-exposed rats. In the vehicle-treated nulliparous rats, 41% of the tumors become palpable during weeks 1 and 12, and 59% during weeks 13 to 22. The majority of the tumors in the leptin group (79%) were detected before week 13, whereas in the E2 group 23% of the tumors were detected early and 77% were detected after week 12 ($P < 0.004$). Final mammary tumor incidence and multiplicity were similar in the 3 groups of nulliparous rats exposed to vehicle, leptin, or E2. These results are shown in Table 2 and Fig. 2A.

Effect of parity. Next, we compared mammary tumorigenesis in the vehicle (control)-treated nulliparous and parous rats. Latency of mammary tumor appearance was shorter in the parous than nulliparous rats ($P < 0.005$). In the parous control rats, 93% of the tumors appeared during weeks 1 and 12, compared to 41% in the nulliparous group ($P < 0.001$). The final tumor incidence during weeks 1 and 22 ($P < 0.001$) and during weeks 13 and 22 ($P < 0.001$) in the nulliparous controls was higher than in the parous rats, but tumor multiplicity was similar (Table 2). Thus, similar to women, we found that after a transient increase in mammary cancer risk, pregnancy provided protection against breast cancer in rats.

Effect of E2 and leptin exposures during pregnancy. In the parous control group, all but one (7%) of 14 tumors became palpable within 12 weeks of DMBA exposure, whereas 12 (46%) of the 26 tumors in the leptin group and 10 (53%) of the 19 tumors in the E2 group appeared after week 12 of pregnancy ($P < 0.018$; Table 2). This is similar to what was seen in nulliparous control rats in which 59% developed mammary tumors after week 12. Thus, although the mean tumor latency period was longer in both the leptin ($P < 0.001$) and E2-treated parous rats ($P < 0.002$) than in the vehicle-treated parous rats, it did not differ between the parous hormone-treated rats and nulliparous control rats; that is, the treatments did not delay tumor development.

To determine whether an exposure to leptin or E2 during pregnancy affected mammary tumorigenesis, differences were assessed between weeks 13 and 22. Both the leptin ($P < 0.001$) and E2 groups ($P < 0.0037$) exhibited significantly higher mammary tumor incidence than the parous control rats (Fig. 2), but neither group differed from nulliparous control rats. At the end of the monitoring period, final tumor incidence was higher in the parous rats exposed during pregnancy to either leptin (65%) or E2 (45%), when compared to the controls (33%) (Table 2), but this difference reached statistical significance in the leptin group only ($P < 0.039$). However, tumor multiplicity among the groups was not statistically different (Table 2).

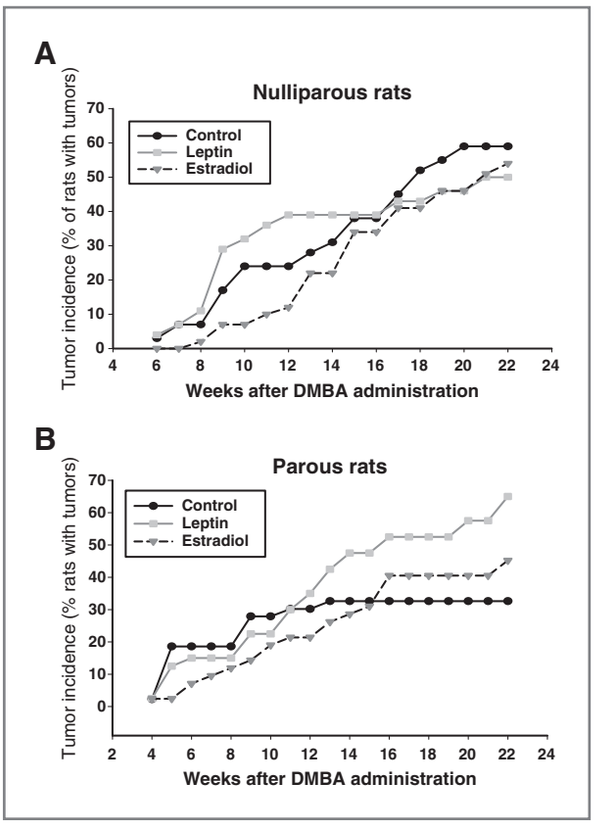

Figure 2. Effects of an exposure to leptin (15 μg) or estradiol (10 mg) on mammary tumorigenesis in (A) in nulliparous rats (received hormonal treatments for two weeks) and (B) in parous rats (received hormonal treatments between gestation days 8 and 19). Tumor incidence values are expressed as percentage of animals with mammary tumors in each group. No statistically significant differences were seen among the nulliparous rats, but parous rats exposed to leptin during pregnancy exhibited significantly higher mammary tumor incidence than vehicle treated parous controls ($P < 0.039$).

Effects on mammary cell proliferation and apoptosis

Cell proliferation and apoptosis were determined in mammary glands obtained from rats sacrificed 22 weeks after exposure to DMBA. Figure 3 shows that the proliferation index, determined by PCNA staining, was significantly higher in the mammary glands of E2-treated parous rats compared to those of vehicle-treated parous control rats ($P < 0.001$). The number of apoptotic cells present in the mammary glands of rats in the 2 treatment groups and controls were determined using the ISOL assay. There were no significant differences among these 2 treatment groups, when compared to the controls ($P = 0.17$; Fig. 4).

Gene microarray analysis

To explore the long-term effects on gene expression in the mammary glands of rats exposed to E2 or leptin during pregnancy, microarray experiments were performed using RNA extracted from mammary glands collected 22 weeks after DMBA exposure. In the comparison between the control and leptin groups, 352 genes were found to be differentially expressed (criteria for differential expression was 2-fold difference and $P < 0.05$). The comparison between the control and E2 groups revealed 252 differentially expressed genes. We then compared the E2 and leptin groups, and found only 11 genes to be differentially expressed between these 2 groups. For this reason, these 2 groups were combined into 1 high-risk group and compared to controls. In this analysis, we identified 143 genes associated with changes in tumorigenesis between the control and high-risk groups. Of those, 62 genes were downregulated (Table 3) and 80 genes upregulated (Table 4) in the high-risk group compared to controls.

Confirmation of changes in gene expression by qRT-PCR. Several of the genes that were differentially expressed in the mammary glands of parous rats exposed to either leptin or E2 during pregnancy, compared to controls, are involved in cell growth, survival, and angiogenesis. These genes included *Mapk9* (mitogen-activated protein kinase 9), *Nras* (neuroblastoma ras oncogene), *Ptn* (pleiotrophin), *Vegfa* (vascular endothelial growth factor), and *Eif4e* (eukaryotic initiation translation factor 4e), which were upregulated in the mammary gland of rats exposed to leptin or E2 during pregnancy when compared to vehicle treated controls (Table 4). We also found that the expression of genes inducing mammary epithelial differentiation, such as α-lactalbumin and α-casein, were downregulated in the leptin- or E2-exposed dams (Table 3).

Differential expression of these genes was confirmed by real-time PCR. As illustrated in Fig. 5, transcripts for *Vegfa* and *Ptn* were more abundant in the rats treated with either leptin or E2 during pregnancy than in the controls ($P < 0.001$ and $P < 0.001$, respectively). *Vegfa* levels were 3.8- and 6.8-fold higher in mammary glands of leptin and E2-treated dams than in the controls, respectively (Fig. 5A). *Ptn* mRNA levels were 3.3-fold higher in leptin-treated and 21-fold higher in E2-treated dams than in the controls (Fig. 5B).

Real-time PCR data indicated that the levels of *Mapk9* mRNA were 1.3-fold higher in the leptin-treated parous rats than in the vehicle- or E2-treated dams (Fig. 5C; $P = 0.008$). Transcription levels of *Eif4e* were 1.2-fold higher in mammary glands of leptin-treated animals compared to the controls ($P = 0.003$; Fig. 5D). Differential expression of *Nras* in the microarray was not confirmed by real-time PCR.

Comparison to data obtained in previous studies assessing effect of parity on gene expression. Several earlier studies have outlined a gene expression signature characterizing the effect of parity on the mammary gland. We investigated whether there were any similarities between these signatures and changes in gene expression induced by an exposure to excess leptin or E2 during pregnancy. For that purpose, we used the tables of differentially expressed genes between parous and nulliparous rat and mouse strains generated in studies by D'Cruz and colleagues (13) and Blakely and colleagues (14), and humans by Asztalos and colleagues (36).

Several common genes in the parous rats exposed to E2 or leptin versus vehicle, and parous versus nulliparous animals

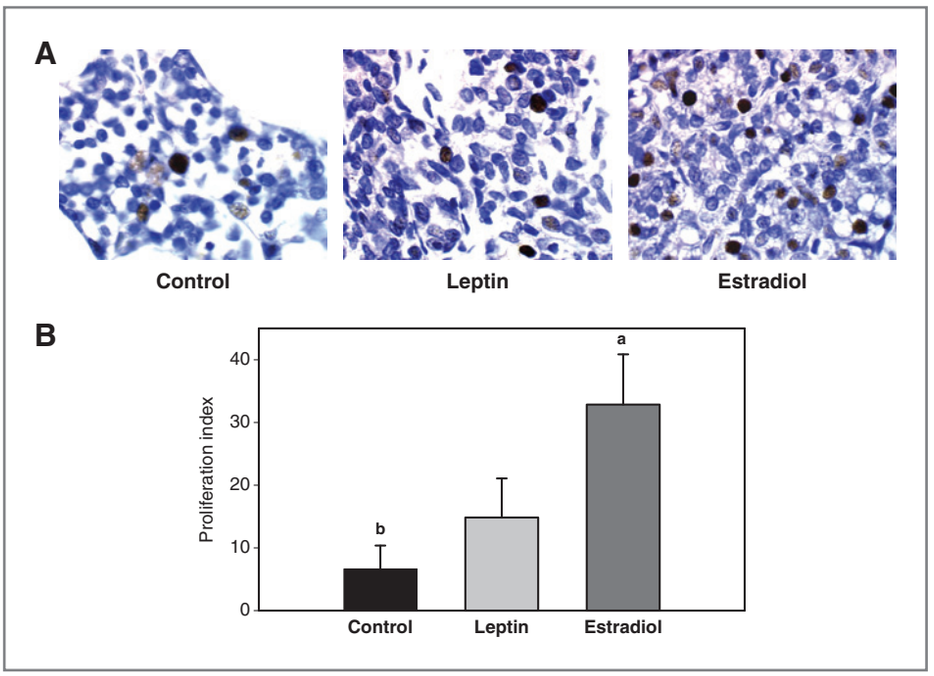

Figure 3. Effects of an exposure to leptin or estradiol during pregnancy on mammary gland cell proliferation, determined 17 weeks after pregnancy. A, PCNA staining (dark nuclei) in representative mammary gland sections (400× magnification), and B, proliferation index (percentage of PCNA positive cells/1,000 cells). All values are expressed as the mean ± SEM, $n = 6$ rats/group. Means with a different letter are significantly different from each other: $P < 0.05$.

and women were identified. The genes identified in this comparison are shown in Tables 5 and 6. Importantly, genes that were upregulated (or downregulated) in parous rats, compared to nulliparous rats, were also upregulated (or downregulated) in vehicle-treated parous rats, compared to parous rats treated with E2 or leptin during pregnancy, suggesting that these hormonal exposures prevented parous-induced signaling changes in the mammary glands. For example, TGF-β3 has been reported to be upregulated in parous animals and humans, compared to nulliparous controls, and we found that is was also upregulated in parous control rats, compared to parous rats treated with leptin or E2 during pregnancy. The downregulated genes are those that induce differentiation (*Csn1*, *Cp*, and *Lalba*) or regulate immune functions (*Lcn2* and *Lbp*), whereas the upregulated genes are those that promote growth (*Ghr* and *Ptn*), angiogenesis (*Vegfa*) and induce epithelial-to-mesenchymal transition (*Col1a1*).

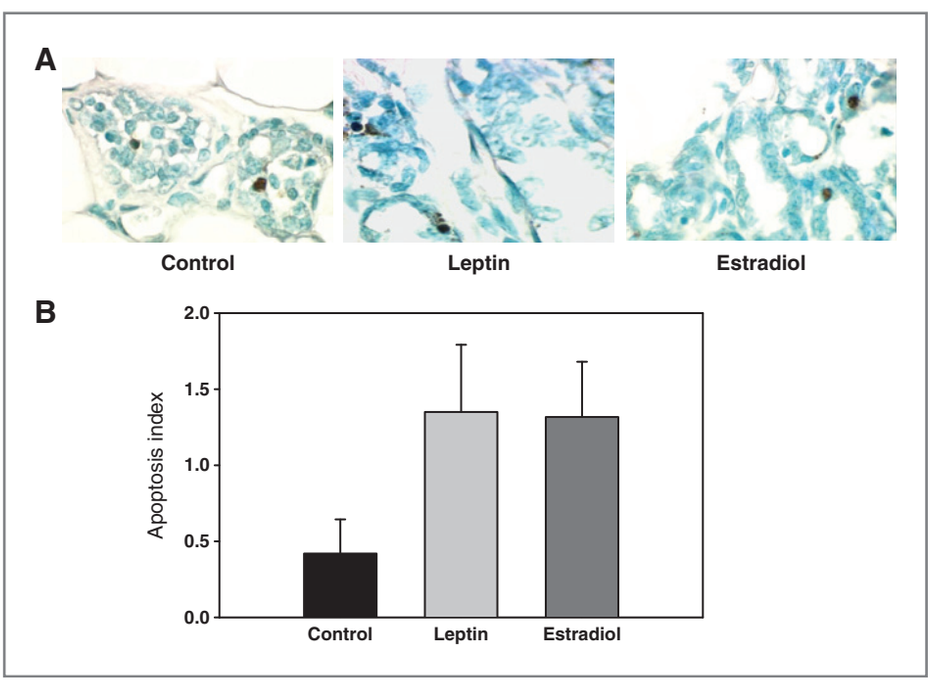

Figure 4. Effects of an exposure to leptin or estradiol during pregnancy on mammary gland apoptosis, determined 17 weeks after pregnancy. A, ISOL staining in representative mammary gland sections (400× magnification), and B, apoptosis index (percentage of ISOL positive cells/1,000 cells). All values are expressed as the mean ± SEM of 6 rats/group.

Table 3. Genes downregulated in parous rats treated with E2 or leptin during pregnancy, compared to vehicle-exposed parous rats

Gene name	Symbol	Accession No.	Function	Category	Fold
Cytochrome c oxidase, subunit Va	*Cox5a*	rc_AI104513_at	Electron transport	Metabolism	0.41
Glucosamine (UDP-N-acetyl)-2-epimerase/ N-acetylmannosamine kinase	*Gne*	rc_AI145931_at	Amino sugar biosynthesis	Metabolism	0.49
Parathymosin	*Ptms*	M33025_s_at	Regulation of glycolysis	Metabolism	0.34
Acidic nuclear phosphoprotein 32 family, member A	*Anp32a*	rc_AI070967_at	Nucleocytoplasmic transport	Transporter/ differentiation	0.42
Apolipoprotein E	*Apoe*	X04979_at	Lipid transport	Transporter	0.42
Aquaporin 5	*Aqp5*	U16245_g_at	Carbon dioxide and water transport	Transporter	0.42
Calcium channel, voltage-dependent, L type, α 1D subunit	*Cacna1d*	D38101_s_at	Calcium ion transport	Transporter	0.47
Epsin 2	*Epn2*	AF096269_at	Endocytosis/Notch signal transduction	Transporter/signal transduction/ embryonic development	0.4
Ferritin light polypeptide	*Ftl1*	rc_AI231807_g_at	Iron transport	Transporter	0.44
Lectin, galactoside-binding, soluble, 9	*Lgals9*	U72741_at	Ion transport/NFκB signaling	Transporter/Signal Transduction	0.41
Low-density lipoprotein receptor-related protein 3	*Lrp3*	AB009463_g_at	Lipoprotein receptor/ endocytosis	Transporter	0.43
PDZK1 interacting protein 1	*Pdzk1ip1*	rc_AA892264_at	Glucose transport	Transporter	0.37
Rab38, member RAS oncogene family	*Rab38*	rc_AI136175_at	Vesicle mediated transport	Transporter	0.37
Secretogranin V (7B2 protein)	*Scg5*	M63901_g_at	Regulation of hormone secretion	Transporter	0.34
Sodium channel, nonvoltage-gated 1 gamma	*Scnn1g*	X77933_at	Sodium ion transport	Transporter	0.47
Integrin β4	*Itgb4*	U60096_at	Integrin signaling	ECM/cell motility	0.49
Syndecan 4	*Sdc4*	S61868_g_at	Focal adhesion assembly	ECM	0.5
TGF-β3	*Tgfb3*	U03491_g_at	Growth Factor	ECM/EMT	0.34
Tropomyosin 3, γ	*Tpm3*	L24776_at	Actin binding	Structural	0.11
Troponin T type 2 cardiac	*Tnnt2*	M80829_at	Actin binding	Muscle contraction	0.35
α-2-Macroglobulin	*A2m*	X13983mRNA_at	Inflammatory response	Immune	0.33
Granzyme F	*Gzmf*	U57063_at	Protease	Immune	0.45
Kininogen 1	*Kng1*	K02814_g_at	Inflammatory response	immune	0.47
Lipopolysaccharide binding protein	*Lbp*	L32132_at	Inflammatory response	Immune	0.43
Lipocalin 2	*Lcn2*	rc_AA946503_at	Protease and iron binding/ transporter	Immune/Apoptosis	0.47
Myxovirus (influenza virus) resistance 2	*Mx2*	X52713_at	Antiviral	Immune	0.36

(Continued on the following page)

Table 3. Genes downregulated in parous rats treated with E2 or leptin during pregnancy, compared to vehicle-exposed parous rats (Cont'd)

Gene name	Symbol	Accession No.	Function	Category	Fold
Protein tyrosine phosphatase, nonreceptor type 1	*Ptpn1*	M33962_at	Endocytosis/Insulin receptor signaling	UPR/signal transduction	0.5
Heat shock protein 1A	*Hspa1a*	Z27118cds_s_at	Stress-inducible chaperone	UPR/antiproliferative	0.35
B-cell CLL/lymphoma 2	*Bcl2*	L14680_g_at	Intrinsic apoptotic pathway	Antiapoptosis	0.5
Cystatin C	*Cst3*	rc_AI231292_g_at	Cystein protease inhibitor	Apoptotsis	0.49
Guanine nucleotide binding protein (G protein), β polypeptide 1	*Gnb1*	U88324_at	GTPase	Apoptosis/ proliferation/signal transduction	0.33
G protein–coupled receptor kinase 1	*Grk1*	U63971_at	G protein coupled receptor signaling	Apoptosis/signal transduction	0.36
Nitric oxide synthase 3, endothelial cell	*Nos3*	U02534_at	NO synthesis	Apoptosis/ angiogenesis	0.42
SMAD family member 3	*Smad3*	U66479_at	Transcription factor/TGF-β signaling	Apoptosis/signal transduction	0.48
Adrenergic receptor, α2b	*Adra2b*	M32061_at	G-protein coupled receptor	Signal transduction/ angiogenesis	0.43
ArfGAP with dual PH domains 1	*Adap1*	U51013_at	Inositol phosphate-mediated signaling	Signal transduction	0.48
Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	*Cited1*	AF104399_g_at	Transcription factor/ Estrogen receptor-α and TGF-β signaling	Signal transduction	0.48
GNAS complex locus	*Gnas*	L10326_at	G-protein α subunit	Signal transduction/ differentiation	0.48
G protein–coupled estrogen receptor 1	*Gper*	U92802_at	Estrogen receptor activity	Signal transduction	0.43
Guanylate cyclase 1, soluble, β2	*Gucy1b2*	M57507_at	Guanylate cyclase	Signal transduction	0.31
High mobility group box 1	*Hmgb1*	rc_AI029805_at	Transcription Factor/ cytokine	Signal transduction/ immune	0.38
Mitogen-activated protein kinase kinase 1	*Map2k1*	rc_AI178835_at	MAPK signaling	Signal Transduction/ differentiation	0.45
Prepronociceptin	*Pnoc*	S79730_s_at	Opiod-like orphan receptor ligand	Signal transduction	0.43
Ret proto-oncogene	*Ret*	AF042830_at	Proto-oncogene/receptor tyrosine kinase	Signal transduction/ embryonic development	0.45
Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, epsilon polypeptide	*Ywhae*	rc_AA965154_at	Calcium/calmodulin-dependent signal transduction	Signal transduction	0.48
Brain abundant, membrane attached signal protein 1	*Basp1*	D14441_at	Transcriptional corepressor	Differentiation	0.46
Ceruloplasmin (ferroxidase)	*Cp*	Y12178_at	Copper transport	Differentiation	0.44
Casein αs1	*Csn1s1*	J00710_at	Milk protein	Differentiation	0.46

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Table 3. Genes downregulated in parous rats treated with E2 or leptin during pregnancy, compared to vehicle-exposed parous rats (Cont'd)

Gene name	Symbol	Accession No.	Function	Category	Fold
D4, zinc, and double PHD fingers family 1	*Dpf1*	X66022mRNA#3_i_at	Transcription factor	Differentiation	0.41
Jun-B proto-oncogene	*Junb*	X54686cds_at	Transcription factor/ proto-oncogene	Differentiation/ response to hormone stimulus	0.34
Keratin 19	*Krtt19*	X81449cds_at	Keratin	Differentiation/ response to estrogen stimulus	0.36
Lactalbumin α	*Lalba*	X00461_at	Lactose biosynthesis	Differentiation	0.49
Growth hormone releasing hormone	*Ghrh*	U41183_at	Growth hormone receptor binding	Growth factor	0.31
H3 histone, family 3B	*H3f3b*	rc_AA875069_at	Nucleosome assembly	Response to hormone stimulus/gene expression	0.25
Prolactin family 8, subfamily a, member 4	*Prl8a4*	AB009889_f_at	Hormone activity	Hormone	0.32
FEV (ETS oncogene family)	*FEV*	U91679_at	Transcription factor	Gene expression	0.48
Forkhead box O1A	*Foxo1a*	rc_AA893671_at	Transcription factor	Gene expression/ proliferation/ antiapoptosis	0.46
Proline-rich nuclear receptor coactivator-1	*Pnrc1*	rc_AI235492_at	Nuclear receptor coactivator	Gene expression	0.45
Ribosomal protein L10	*Rpl10*	rc_AA945611_at	Translation	Gene expression	0.48
Ribosomal protein L30	*Rpl30*	D84480_s_at	Translation	Gene expression	0.47
Ribosomal protein S14	*Rps14*	rc_AA945806_at	Translation	Gene expression/ differentiation	0.49
Calpain 8	*Capn8*	D14478_s_at	Proteolysis	Protein regulation	0.39

Only one gene, Cited 1, was found to be altered in a similar manner both in the parous animals (compared to nulliparous animals) and in the leptin- or E2-exposed parous rats (compared to control parous rats) in our study. Cited 1 is a transcriptional coregulator of ER-α and affects estrogen sensitivity in a gene-specific manner (37). Therefore, pregnancy suppresses ER-α signaling, with increasing suppression the higher the hormone levels were during pregnancy. However, we did not observe any changes in the expression of ER-α between the parous rats which received E2 or leptin during pregnancy and parous control rats. Instead, G protein–coupled estrogen receptor 1 (*Gper*) that localizes to the endoplasmic reticulum and binds estrogen was downregulated in the parous E2- and leptin-treated rats. This protein is involved in the rapid nongenomic signaling events observed with estrogen.

Discussion

Results obtained in our study indicate that parous control rats had a lower mammary tumor incidence than nulliparous rats which is consistent with the protective effect of pregnancy against breast cancer in women who have their first child before age 20 (5) and previous reports in rats (27, 38). Importantly, the majority of mammary tumors in parous rats in our study appeared before mammary gland involution had been completed. These findings are in accordance with the transient increase in breast cancer risk caused by pregnancy in women (1–4). An exposure to E2 or leptin during pregnancy increased mammary cancer risk in parous rats. Specifically, E2- or leptin-treated parous rats continued to develop mammary tumors also after the initial transient increase in risk. Thus, the pattern of mammary tumor development in the rats treated with E2 or leptin during pregnancy mimicked that of nulliparous rats, suggesting that the hormonal exposures prevented the protective effects of parity on mammary cancer risk.

The increase in mammary cancer risk in rats exposed to E2 or leptin during pregnancy is consistent with findings reported in humans. Women who took the synthetic estrogen DES during pregnancy are at an increased risk of developing breast cancer (18, 19). Furthermore, women who exhibit the highest pregnancy estrogen levels, either in the first (16) or third (17) trimesters of gestation are at elevated breast cancer risk later in life. We are not aware of

Table 4. Genes upregulated in parous rats treated with E2 or leptin during pregnancy, compared with vehicle treated parous rats

Gene name	Symbol	Accession No.	Function	Category	Fold
Acyl-CoA dehydrogenase, short/branched chain	*Acadsb*	U64451_at	Fatty acid metabolism	Metabolism	3.3
Aminolevulinate, delta-, synthase 1	*Alas1*	J03190_g_at	Heme biosynthesis/Amino acid metabolism	Metabolism	2
Antizyme inhibitor 1	*Azin1*	D89983_at	Polyamine biosynthesis	Metabolism	2.2
Biotinidase	*Btd*	rc_AI236721_r_at	Nitrogen compound metabolism	Metabolism	2.9
Fumarate hydratase	*Fh*	rc_AI171734_s_at	Tricarboxylic acid cycle	Metabolism	2.4
Glutamate cysteine ligase, modifier subunit	*Gclm*	rc_AI233261_i_at	Glutathione metabolism	Metabolism	2.2
Glutathione S-transferase, mu 5	*Gstm5*	U86635_g_at	Glutathione metabolism	Metabolism/response to estrogen stimulus	2.2
Hypoxanthine phosphoribosyltransferase 1	*Hprt1*	M63983_s_at	Purine metabolism	Metabolism	2.2
Methionine adenosyltransferase II, α (Mat2a)	*Mat2a*	J05571_s_at	Methionine metabolism	Metabolism	2.1
Nicotinamide nucleotide transhydrogenase	*Nnt*	rc_AA891872_at	Proton transport	Metabolism	2
Ornithine aminotransferase	*Oat*	rc_AA893325_at	Amino acid metabolism	Metabolism	2
Phosphoribosyl pyrophosphate synthetase 1	*Prps1*	X16554_at	Purine biosynethesis	Metabolism	2.1
Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase 1	*Pcmt1*	M26686_g_at	Amino acid metabolism	Metabolism	2.3
Pyruvate dehydrogenase E1 α1	*Pdha1*	Z12158cds_at	Glycolysis	Metabolism	2.1
Blocked early in transport 1 homolog	*Bet1*	U42755_at	Vesicular membrane trafficking	Transporter	2.3
Exocyst complex component	*Exoc5*	U79417_at	Exocytosis	Transporter	2.6
Nucleoporin 155	*Nup155*	Z21780_at	Nucleocytoplasmic transport	Transporter	2.1
Solute carrier family 11 member 2	*Slc11a2*	AF008439_at	Iron transport	Transporter	2.5
Solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	*Slc16a1*	rc_AI145680_s_at	Organic anion transport	Transporter	2.1
Synaptosomal-associated protein 23	*Snap23*	rc_AA892759_at	Exocytosis/protein transport	Transporter	2
Syntaxin 12	*Stx12*	AF035632_s_at	Vesicle-mediated transport	Transporter	2.2
Trans-golgi network protein 1	*Tgoln1*	X53565_at	Endosome transport	Transporter	3.1
Transmembrane emp24 domain trafficking protein 2	*Tmed2*	X92097_at	Protein transport	Transporter	2.2

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Table 4. Genes upregulated in parous rats treated with E2 or leptin during pregnancy, compared with vehicle treated parous rats (Cont'd)

Gene name	Symbol	Accession No.	Function	Category	Fold
Uncoupling protein 3	*Ucp3*	AF035943_at	Hydrogen ion transmembrane transporter/oxidative phoshorylation uncoupling	Transporter/ Response to hormone stimulus	2.6
Annexin A4	*Anxa4*	rc_AI171167_at	Exocytosis	ECM	2.1
CD36 molecule (thrombospondin receptor)	*Cd36*	AB005743_g_at	Thrombospondin receptor	ECM	4.5
Collagen type 1 α1	*Col1a1*	Z78279_at	ECM structural protein	ECM/EMT	2.5
Discs, large homolog 1	*Dlgh1*	U14950_at	Cell adhesion	ECM	2.1
Fat tumor suppressor homolog 1 (*Drosophila*)	*Fat1*	L41684mRNA_at	Cell–cell adhesion	ECM	2.3
Prolyl 4-hydroxylase α polypeptide 1	*P4ha1*	X78949_at	Collagen fibril organization	ECM	2
Caldesmon 1	*Cald1*	rc_AI180288_s_at	Actin binding	Structural	2
LIM motif-containing protein kinase 1	*Limk1*	D31873_g_at	Kinase involved in actin dynamics	Structural	2.1
Cathepsin C	*Ctsc*	D90404_g_at	Aminopeptidase	Immune	2.2
Proteasome (prosome, macropain) subunit, α type 2	*Psma2*	E03358cds_at	Antiviral response	Immune	2.3
Protein phosphatase, Mg^{2+}/Mn^{2+}-dependent 1B	*Ppm1b*	S90449_at	Serine/Threonine phosphatase/Antiviral	Immune	3.1
Calnexin	*Canx*	L18889_at	Protein folding	UPR	2
Eukaryotic translation initiation factor 2-α kinase 2	*Eif2ak2*	rc_AI013987_s_at	Interferon-inducible RNA-dependent protein kinase/antiviral response	UPR/immune	2.6
Nuclear factor, erythroid-derived 2, like 2	*Nfe2l2*	rc_AI177161_g_at	Transcription factor	UPR/antioxidant response	2.1
Stress-associated endoplasmic reticulum protein 1	*Serp1*	AF100470_g_at	Protein glycosylation	UPR	2.4
Cytochrome c, somatic	*Cycs*	rc_AI008815_s_at	Electron transport chain	Apoptosis/ metabolism	2.9
Dynamin 1-like	*Dnm1l*	AF019043_at	Membrane fission	Apoptosis	2
Mitogen-activated protein kinase 9	*Mapk9*	rc_AI231354_at	Cysteine-type endopeptidase/MAPK signaling	Apoptosis/Signal Transduction	2.5
Glutamate cysteine ligase, modifier subunit	*Gclm*	rc_AI233261_i_at	Glutathione metabolism	Antiapoptosis	2.2
Serpine1 mRNA binding protein 1	*Serbp1*	rc_AA893338_at	mRNA 3′-UTR binding	Antiapoptotic	2.2
Heme oxygenase (decycling) 1	*Hmox1*	J02722cds_at	Heme catabolism	Angiogenesis/ Antiapoptosis	2.1
VEGF A human	*Vegfa*	L20913_s_at	Growth factor	Angiogenesis	3.2
v-Crk sarcoma virus CT10 oncogene homolog (avian)	*Crk*	D44481_at	Oncoprotein/adaptor protein/actin cytoskeleton organization	Signal transduction/ structural	2.4

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Table 4. Genes upregulated in parous rats treated with E2 or leptin during pregnancy, compared with vehicle treated parous rats (Cont'd)

Gene name	Symbol	Accession No.	Function	Category	Fold
Guanylate cyclase 2G	*Gucy2g*	U33847_at	cGMP biosynthesis	Signal transduction	3.3
Multiple inositol-polyphosphate phosphatase 1	*Minpp1*	rc_AI111401_s_at	Phosphatase	Signal transduction	2
Neuroblastoma RAS viral (v-ras) oncogene homolog	*Nras*	rc_AA943331_s_at	GTPase/actin cytoskeleton organization	Signal transduction/ structural	2.3
O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine: polypeptide-N-acetylglucosaminyl transferase)	*Ogt*	U76557_at	O-GlcNAcylation of proteins	Signal transduction/ Insulin receptor signaling	2
Protein kinase, camp-dependent regulatory, type II β	*Prkar2b*	M12492mRNA#1_g_at	PKA signaling/fatty acid metabolism	Signal transduction/ metabolism	2.2
RAB28, member RAS oncogene family	*Rab28*	X78606_at	GTPase	Signal transduction	2.1
Arginine-glutamic acid dipeptide repeats	*Rere*	U44091_at	Chromatin remodeling/ transcription factor	Signal transduction	2.2
Calpastatin	*Cast*	Y13591_s_at	Endopeptidase inhibitor	Cell cycle	4.3
Eukaryotic translation initiation factor 4E	*Eif4*	X83399_at	Translation initiation	Cell cycle	2.2
Cyclin G1	*Ccng1*	X70871_at	Cyclin	Cell growth/mitosis	2.9
Dynein, cytoplasmic 1 light intermediate chain 2	*Dync1li2*	AB008521_s_at	Centrosome localization/ retrograde organelle transport	Mitosis/transport	2.3
Microtubule-associated protein, RP/EB family, member 1	*Mapre1*	U75920_at	Microtubule binding	Mitosis	2.4
Growth hormone receptor	*Ghr*	Z83757mRNA_g_at	Growth hormone signaling	Growth-promoting	2.9
Hydroxysteroid (17-β) dehydrogenase 12	*Hsd17b12*	U81186_at	Estrogen biosynethesis/ extracellular matrix organization	Growth-promoting/ ECM	2.6
Pleiotrophin	*Ptn*	rc_AI102795_at	Growth factor	Growth promoting	2.3
Peroxisomal biogenesis factor 2	*Pex2*	E03344cds_s_at	Peroxisome biogenesis	Antiproliferative	2.1
Heterogeneous nuclear ribonucleoprotein K	*Hnrpk*	D17711cds_s_at	mRNA processing	Gene expression	2.1
Iron responsive element binding protein 2	*Ireb2*	U20181_at	Transcriptional regulation	Gene expression/Iron homeostasis	2.3
Pleiomorphic adenoma gene-like 1	*Plagl1*	rc_AA900750_s_at	DNA binding/ transcriptional regulation	Gene expression	2.5
Ring finger protein 4	*Rnf4*	AF022081_at	Nuclear receptor coregulator	Gene expression	2.5
Splicing factor proline/ glutamine-rich	*Sfpq*	AF036335_g_at	mRNA splicing	Gene expression	2.5

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Table 4. Genes upregulated in parous rats treated with E2 or leptin during pregnancy, compared with vehicle treated parous rats (Cont'd)

Gene name	Symbol	Accession No.	Function	Category	Fold
Transformer 2β homolog (Drosophila)	*Tra2b*	rc_AA851749_s_at	mRNA splicing	Gene expression/ response to ROS	2.3
Aspartyl-tRNA synthetase	*Dars*	rc_AI009682_s_at	Protein biosynethesis	Protein regulation	3.1
Phosducin-like	*Pdcl*	L15354_s_at	Protein folding	Protein regulation	2.6
Praja ring finger 2	*Pja2*	rc_AA894089_s_at	Regulation of protein kinase A signaling/protein ubiquitination	Protein regulation	2.3
Prostaglandin F2 receptor negative regulator	*Ptgfrn*	rc_AI145502_s_at	Prostaglandin inhibitor/ negative regulation of translation	Protein regulation	2
Tripeptidyl peptidase II	*Tpp2*	rc_AI071507_s_at	Proteolysis	Protein regulation	2.3
Ubiquitin-conjugating enzyme E2D 2	*Ube2d2*	U13176_at	Protein degradation	Protein regulation	2
Ubiquitin-conjugating enzyme E2G 1	*Ubc7*	AF099093_at	Protein degradation	Protein regulation	2.1
Delta-like 1, *Drosophila*	*Dll1*	U78889_at	Notch receptor ligand	Development/ Differentiation/cell signaling	2.9
Cytochrome c oxidase subunit Vb	*Cox5b*	D10952_i_at	Cytochrome-c oxidase activity	Response to hormone stimulus	2.2
Oxidation resistance 1	*Oxr1*	rc_H33461_at	Cell wall macromolecule catabolic process	Response to oxidative stress	2.6
Protein phospatase 3, regulatory subunit B, α isoform, type 1	*Ppp3r1*	D14568_at	Protein phosphatase	EMT	2.7

any studies that have investigated whether leptin levels during pregnancy affect later breast cancer risk among mothers, but indirect parameters of high leptin levels, such as obesity or weight gain (23–25) indicate that parous women who had the highest leptin levels during pregnancy also are at an increased risk of developing breast cancer. Excessive weight gain during pregnancy is common: close to 50% of pregnant women gain more than recommended by the IOM (26, 39). Because these women are at an increased risk of developing breast cancer after menopause (26), the results obtained in our animal model suggest that high leptin levels during pregnancy are responsible, at least partly, for this finding.

The mechanisms responsible for the association between elevated E2 or leptin levels during pregnancy and increased breast cancer risk remain to be elucidated. We performed microarray analysis to identify differentially expressed genes in the mammary glands between the parous control rats and parous rats exposed to E2 or leptin during pregnancy. Intriguingly, only 11 (0.05%) of >7,000 genes were differentially expressed between the rats that were exposed to E2 or leptin during pregnancy, although both groups exhibited a number of differentially expressed genes compared to controls. The similarity of gene expression in the 2 hormone-treated groups may reflect the close association between leptin and estrogen signaling in the mammary gland (28–31, 33). We therefore focused on the 142 differentially expressed genes, shown in Table 4, between the mammary glands of rats exposed to vehicle or E2/leptin during pregnancy.

The differentially expressed genes included *Eif4e*, *Mapk9*, *Nras*, *Ptn*, and *Vegfa*. All these genes have been linked to breast cancer. Deregulation of protein synthesis is a hallmark of many cancers, and overexpression of eukaryotic translation factor *Eif4e* contributes to the deregulation. It is overexpressed in breast cancers and high expression is linked to an elevated risk of recurrence (40). When overexpressed, *Eif4e* may enable the translation of a select pool of mRNAs encoding for proteins involved in malignant growth, such as those for cyclin D1, c-MYC, VEGF, and matrix metalloprotease-9 (MMP-9; ref. 41). *Mapk9* regulates cell proliferation and apoptosis (42) and inhibition of its activity reduces cell proliferation in breast cancer cells (43). *Ptn* is overexpressed in at least 60% of human breast cancers (43), and this overexpression is linked to high risk of metastasis (44). *Vegfa* is often upregulated in breast tumors, especially in those expressing HER-2/neu (45) or mutant p53 (46). Furthermore, both leptin and estrogens activate *Vegfa* (47, 48). Leptin itself can induce angiogenesis *in vitro* and *in vivo* (49), and a neutralizing anti-leptin receptor monoclonal antibody suppresses leukemia cell growth by inhibiting angiogenesis in rats (50). Thus, we

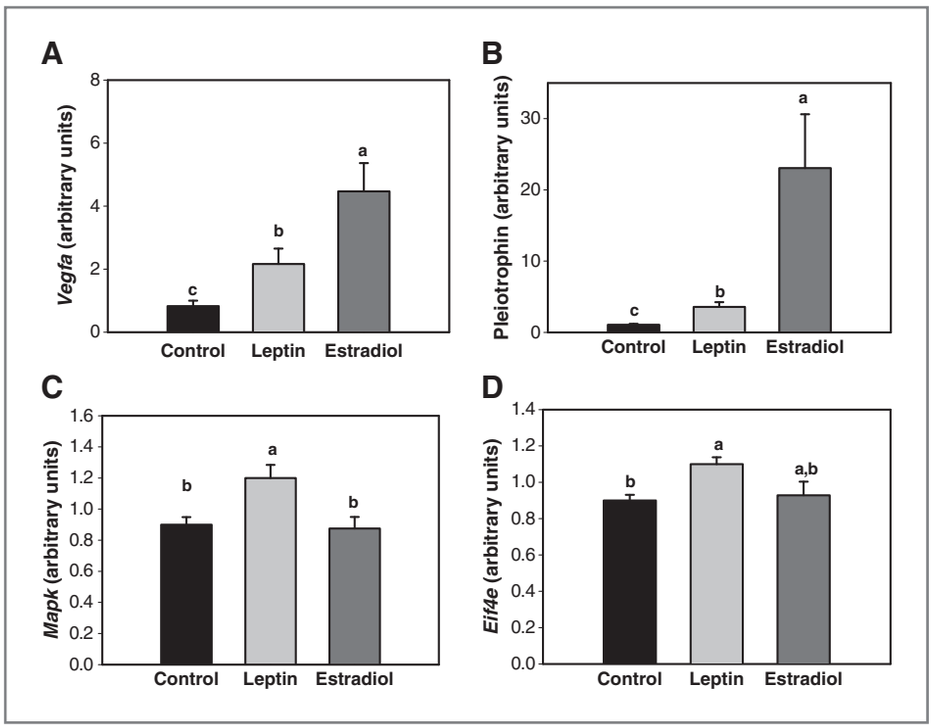

Figure 5. Effects of an exposure to leptin or estradiol during pregnancy on mammary gland mRNA levels of (A) *Vegfa*, (B) *Ptn*, (C) *Mapk 9*, and (D) *Eif4e*, determined 17 weeks after pregnancy. All values are expressed as the mean ± SEM of 6 to 8 rats/group. Means with a different letter are significantly different from each other: $P < 0.05$.

were able to confirm upregulation of *Eif4e*, *Mapk9*, *Ptn*, and *Vegfa* in the mammary glands of parous rats exposed to leptin or E2 during pregnancy, compared to parous control rats, and these changes may be associated with their increased mammary tumorigenesis. Increase in *Nras* expression in the microarray analysis was not confirmed by qRT-PCR.

In addition to these genes, several others were differentially expressed between control and E2/leptin-exposed parous rats. We were particularly interested in those genes that have been suggested to explain the protective effect(s) of pregnancy in rodents (13, 14) and humans (36). Thirteen of them were identified as differentially expressed between the parous control and E2/leptin-exposed rats. Importantly, genes that have been reported to be upregulated in the parous women/rodents, compared with nulliparous women/rodents, were upregulated in the parous control rats in our study, compared to parous rats treated with E2 or leptin during pregnancy. Thus, gene expression patterns in the E2/leptin-treated parous rats resembled those in the nulliparous rats. The same applied to downregulated genes: those that are found to be downregulated in parous versus nulliparous women/rodents were downregulated in parous control rats, compared to parous rats treated with E2 or leptin during pregnancy. Most of the downregulated genes (that are upregulated by parity) in the mammary glands of parous rats treated with E2 or leptin during pregnancy were those that induce differentiation (*Csn1*, *Cp*, and *Lalba*), inhibit growth (*Tgfβ3*), or regulate immune functions (*Lcn2*, and *Lbp*). Among the upregulated genes in the parous E2/leptin rats (and downregulated in parous women and rodents) were *Vegfa*, *Ghr* and *Ptn* that promote growth, and *Col1a1* that induces cancer progression by stimulating epithelial mesenchymal transition. Our findings suggest

Table 5. Genes upregulated in parous human breast and/or rodent mammary gland, but downregulated in parous mammary gland of rats treated with E2 or leptin during pregnancy

Gene name	Symbol	Reference	Category	Fold-change ($P < 0.05$)
Aquaporin 5	*Aqp5*	(14)	Transporter	0.42
Casein αs1	*Csn1*	(13)	Differentiation	0.46
Ceruloplasmin	*Cp*	(14)	Differentiation	0.44
Lactalbumin	*Lalba*	(13)	Differentiation	0.49
Lipocalin 2	*Lcn2*	(14)	Immune	0.47
Lipopolysaccharide binding protein	*Lbp*	(14, 36)	Immune	0.43
TGF-β3	*Tgfb3*	(13, 14, 36)	Growth inhibition	0.34

Table 6. Genes downregulated in parous human breast and/or rodent mammary gland, but upregulated in parous mammary gland of rats treated with E2 or leptin during pregnancy

Gene name	Symbol	Reference	Category	Fold-change ($P < 0.05$)
Collagen type 1, α1	*Col1a1*	(14, 36)	ECM, cancer progression	2.5
Growth hormone receptor	*Ghr*	(14)	Growth factor	2.9
Pleiotrophin	*Ptn*	(13)	Growth promoter, angiogenesis inducer	2.3
Solute carrier family 11 member 2	*Slc11a2*	(14)	Transporter	2.5
VEGF A	*VegfA*	(36)	Angiogenesis	3.2

that high levels of E2 and leptin during pregnancy may prevent parity-induced reduction in breast cancer risk by preventing protective signaling changes in mammary gland.

The parity-induced protective signaling patterns are likely to induce or reflect functional changes that result in reduced breast cancer risk. During pregnancy, the mammary gland undergoes substantial morphological changes, but after the gland has involuted, it returns to a stage resembling that seen in nulliparous animals (51, 52) or humans (12, 53). Findings in mice suggest that pregnancy promotes functional differentiation at a cellular level, and causes a reduction in the proportion of mammary epithelial stem/progenitor cells and an increase in differentiated luminal and myoepithelial cells (54, 55). Because breast cancer is thought to be initiated in epithelial stem/progenitor cells or differentiated cells that acquire stem cell like properties (56), reduction in stem/progenitor cell population may explain why early pregnancy reduces later breast cancer risk. In our study, parous rats exposed to E2 or leptin during pregnancy exhibited a sustained increase in cell proliferation, compared with parous control rats. Proliferating cells represent a progenitor cell population (57), and thus it is possible that a high hormonal environment during pregnancy prevents a pregnancy-induced reduction in stem cells. Although we did not determine whether there were less differentiated cells in the mammary glands of rats treated with E2 or leptin during pregnancy than in the controls, microarray analysis indicated that several genes that induce differentiation were downregulated, and those increasing cell proliferation were upregulated (Tables 5 and 6). In addition to the ones already discussed earlier, these included downregulation of Keratin 19, which is a marker of differentiated luminal cells (58).

Conclusion

In our study, parous rats treated with E2 or leptin during pregnancy exhibited higher mammary cancer risk than parous control rats, consistent with the findings in humans showing that women exposed to DES (19), having the highest pregnancy E2 levels (16, 17), or gaining more weight during pregnancy than recommended by the IOM (26), are at an increased risk of developing breast cancer. Parous control rats exhibited a transient increase in mammary cancer risk that lasted until their mammary gland had completed involution. After this transient period, the risk of developing mammary cancer was very low. However, in the parous rats treated with E2 or leptin during pregnancy the risk of developing mammary tumors remained elevated past the transient increase. Thus, the pattern of mammary tumor appearance in the parous E2- and leptin-exposed rats was similar to that seen in nulliparous rats, suggesting that parity does not protect against breast cancer if the levels of E2 or leptin during pregnancy are excessive. This conclusion is supported by comparing the pregnancy-induced protective mRNA signature obtained in earlier microarray analyses in rodents and humans (13, 14, 36) to the signature in parous rats treated with E2 or leptin during pregnancy. Gene expression in the mammary glands of E2- or leptin-treated parous rats was similar to that of nulliparous individuals. Thus, an exposure to excess E2 or leptin during pregnancy prevents the protective effects of pregnancy on the mammary gland and increases subsequent breast cancer risk. These findings suggest that pregnant women should avoid being exposed to the highest levels of E2 and leptin during pregnancy, caused for example by gaining excessive amounts of weight during pregnancy, because it may not only put them at risk of developing gestational diabetes and hypertension (59), but also increase later breast cancer risk.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L.A. Hilakivi-Clarke
Development of methodology: L.A. Hilakivi-Clarke
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. de Assis, L.A. Hilakivi-Clarke
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. de Assis, M. Wang, L. Jin
Writing, review, and/or revision of the manuscript: S. de Assis, L. Jin, K. Bouker, L.A. Hilakivi-Clarke
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. de Assis, K. Bouker, L.A. Hilakivi-Clarke
Study supervision: L.A. Hilakivi-Clarke

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