Hormone-Induced Protection against Mammary Tumorigenesis Is Conserved in Multiple Rat Strains and Identifies a Core Gene Expression Signature Induced by Pregnancy

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Abstract

Women who have their first child early in life have a substantially lower lifetime risk of breast cancer. The mechanism for this is unknown. Similar to humans, rats exhibit parity-induced protection against mammary tumorigenesis. To explore the basis for this phenomenon, we identified persistent pregnancy-induced changes in mammary gene expression that are tightly associated with protection against tumorigenesis in multiple inbred rat strains. Four inbred rat strains that exhibit marked differences in their intrinsic susceptibilities to carcinogen-induced mammary tumorigenesis were each shown to display significant protection against methylnitrosourea-induced mammary tumorigenesis following treatment with pregnancy levels of estradiol and progesterone. Microarray expression profiling of parous and nulliparous mammary tissue from these four strains yielded a common 70-gene signature. Examination of the genes constituting this signature implicated alterations in transforming growth factor-β signaling, the extracellular matrix, amphiregulin expression, and the growth hormone/insulin-like growth factor I axis in pregnancy-induced alterations in breast cancer risk. Notably, related molecular changes have been associated with decreased mammographic density, which itself is strongly associated with decreased breast cancer risk. Our findings show that hormone-induced protection against mammary tumorigenesis is widely conserved among divergent rat strains and define a gene expression signature that is tightly correlated with reduced mammary tumor susceptibility as a consequence of a normal developmental event. Given the conservation of this signature, these pathways may contribute to pregnancy-induced protection against breast cancer. (Cancer Res 2006; 66(12): 6421-31)

Introduction

Epidemiologic studies clearly show that a woman's risk of developing breast cancer is influenced by reproductive endocrine events (1). For example, early age at first full-term pregnancy, as well as increasing parity and duration of lactation, have each been shown to reduce breast cancer risk (2, 3). In particular, women who have their first child before the age of 20 have up to a 50% reduction in lifetime breast cancer risk compared with their nulliparous counterparts (2). Notably, the protective effects of an early full-term pregnancy have been observed in multiple ethnic groups and geographic locations, suggesting that parity-induced protection results from intrinsic biological changes in the breast rather than specific socioeconomic or environmental factors. At present, however, the biological mechanisms underlying this phenomenon are unknown.

Several models to explain the protective effects of parity have been proposed. For instance, parity has been hypothesized to induce the terminal differentiation of a subpopulation of mammary epithelial cells, thereby decreasing their susceptibility to oncogenesis (4). Related to this, parity has been suggested to induce changes in cell fate within the mammary gland, resulting in a population of mammary epithelial cells that are more resistant to oncogenic stimuli by virtue of decreased local growth factor expression and/or increased transforming growth factor (Tgf)-β3 and p53 activity (5, 6). Others have suggested that the process of involution that follows pregnancy and lactation acts to eliminate premalignant cells or cells that are particularly susceptible to oncogenic transformation (5). Conversely, parity-induced decreases in breast cancer susceptibility could also be due to persistent changes in circulating hormones or growth factors rather than local effects on the mammary gland (7). At present, however, only limited cellular or molecular evidence exists to support any of these models.

Similar to humans, both rats and mice exhibit parity-induced protection against mammary tumorigenesis. Administration of the chemical carcinogens, 7,12-dimethylbenzanthracene or methylnitrosourea, to nulliparous rats results in the development of hormone-dependent mammary adenocarcinomas that are histologically similar to human breast cancers (8). In outbred Sprague-Dawley, and inbred Lewis and Wistar-Furth rats, a full-term pregnancy either shortly before or after carcinogen exposure results in a high degree of protection against mammary carcinogenesis (7, 9, 10). Similarly, treatment of rats with pregnancy-related hormones, such as 17-β-estradiol (E) and progesterone (P), can mimic the protective effects of pregnancy in rat mammary carcinogenesis models (11, 12). This suggests that the mechanisms of parity-induced protection and estradiol and progesterone–induced protection may be similar. Using analogous approaches, Medina and colleagues have shown parity-induced as well as hormone-induced protection against 7,12-dimethylbenzanthracene–initiated carcinogenesis in mice (13, 14). As such, rodent models recapitulate the ability of reproductive endocrine events to modulate breast cancer risk as observed in humans. This, in turn, permits the mechanisms of parity-induced protection to be studied within defined genetic and reproductive contexts.

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Previously, analyses of gene expression changes that occur in rodent models in response to parity, or hormonal treatments that mimic parity, have been used to suggest potential cellular and molecular mechanisms for pregnancy-induced protection against breast cancer (6, 15). Rosen and colleagues used subtractive hybridization analysis to identify genes in the mammary glands of Wistar-Furth rats that were persistently up-regulated 4 weeks posttreatment with estradiol and progesterone (15). Estradiol and progesterone treatment was found to increase the mRNA expression of a wide range of genes, including those involved in differentiation, cell growth, and chromatin remodeling. Similarly, we used microarray expression profiling to assess global gene expression changes induced by parity in the mammary glands of FVB mice (6). This analysis revealed parity-induced increases in epithelial differentiation markers, *Tgfb3* and its downstream targets, and cellular markers reflecting the influx of macrophages and lymphocytes into the parous gland. We also found that parity resulted in persistent decreases in the expression of a number of growth factor–encoding genes, including amphiregulin (*Areg*) and insulin-like growth factor (*Igf-I*). Together, these studies provided initial insights into cellular and molecular mechanisms that could contribute to parity-induced protection.

Notably, early first full-term pregnancy in humans primarily decreases the incidence of estrogen receptor (ER)-positive breast cancers (16). Because rats are more similar to humans than are mice with respect to the incidence of ER-positive mammary tumors (17), in the present study we used microarray expression profiling to identify persistent gene expression changes in the mammary glands of this rodent species to explore potential mechanisms of parity-induced protection. To date, a comprehensive analysis of parity-induced up-regulated and down-regulated gene expression changes in the rat has not been performed.

A major challenge posed by global gene expression surveys is the large number of differentially expressed genes that are typically identified, only a few of which may contribute causally to the phenomenon under study. Consequently, we considered approaches to identifying parity-induced changes in the rat mammary gland that would permit the resulting list of expressed genes to be narrowed to those most robustly associated with parity-induced protection against mammary tumorigenesis. Given the marked genetic and biological heterogeneity between different inbred rat strains, we reasoned that identifying expression changes that are conserved across multiple strains exhibiting hormone-induced protection against mammary tumorigenesis would facilitate the identification of a core set of genes associated with parity-induced protection against breast cancer.

To achieve this goal, we focused on gene expression changes that are conserved among different strains of rats that exhibit hormone-induced protection against mammary tumorigenesis. We first identified four genetically distinct inbred rat strains that exhibit hormone-induced protection against methylnitrosourea-induced mammary tumorigenesis independent of their inherent susceptibility to this carcinogen. We then used oligonucleotide microarrays to identify a core 70-gene expression signature that closely reflects parity-induced changes in the mammary gland that were conserved among each of these strains. The results of this analysis extend prior observations with respect to parity-induced changes in the growth hormone/Igf-I axis, identify novel parity-induced changes associated with the extracellular matrix (ECM), and implicate a core set of pathways in pregnancy-induced protection against breast cancer.

Materials and Methods

Animals and tissues. Lewis, Wistar-Furth, Fischer 344, and Copenhagen rats (Harlan, Indianapolis, IN) were housed under 12-hour light/12-hour dark cycles with access to food and water ad libitum. Animal care was performed according to institutional guidelines. To generate parous (G1P1) rats, 9-week-old females were mated and allowed to lactate for 21 days after parturition. After 28 days of postlactational involution, rats were sacrificed by carbon dioxide asphyxiation and the abdominal mammary glands were harvested and snap-frozen following lymph node removal, or whole-mounted and fixed in 4% paraformaldehyde. Whole-mounted glands were stained with carmine alum as previously described (6). For histologic analysis of whole mammary glands and tumors, paraffin-embedded tissues were sectioned and stained with H&E or Mason's trichrome as previously described (6). Tissues were harvested from age-matched nulliparous (G0P0) animals in an identical manner.

Carcinogen and hormone treatments. Twenty-five to 30 nulliparous female Lewis, Fischer 344, Wistar-Furth, and Copenhagen rats were weighed and treated at 7 weeks of age with methylnitrosourea (Sigma-Aldrich, St. Louis, MO) at a dose of 50 mg/kg by a single i.p. injection. At 9 weeks of age, animals from each strain were assigned to one of two groups and treated with hormone pellets (Innovative Research, Sarasota, FL) by s.c. implantation. Group 1 received pellets containing 35 mg of 17-β-estradiol + 35 mg of progesterone, whereas group 2 received pellets containing placebo. Pellets were removed after 21 days of treatment. No signs of toxicity were observed. The development of mammary tumors was assessed by weekly palpation. Animals were sacrificed at a predetermined tumor burden, or at 60 weeks postmethylnitrosourea. At sacrifice, all mammary glands were assessed for tumors, which were fixed in 4% paraformaldehyde and embedded in paraffin. Tumor samples from each strain were confirmed as carcinomas by histologic evaluation. Statistical differences in tumor-free survival between experimental groups were determined by log rank tests and by the generation of hazard ratios (HR) based on the slope of the survival curves using GraphPad Prism 4.0 software.

Microarray analysis. RNA was isolated from snap-frozen abdominal mammary glands by the guanidine thiocyanate/cesium chloride method as previously described (6). Ten micrograms of total RNA from individual Wistar-Furth (six G0P0 and five G1P1), Fischer 344 (eight G0P0 and six G1P1), and Copenhagen (six G0P0 and five G1P1) rats was used to generate cDNA and biotinylated cRNA as previously described (6). For Lewis rats, three G0P0 and three G1P1 samples were analyzed, each of which was comprised of 10 μg of pooled RNA from three animals. To permit the identification of epithelial as well as stromal gene expression changes, intact mammary glands (with lymph nodes removed) were used. Samples were hybridized to high-density oligonucleotide microarrays (RGU34A) containing ~8,800 probe sets representing ~4,700 genes and expressed sequence tags. Affymetrix comparative algorithms (MAS 5.0) and Chipstat were used to identify genes that were differentially expressed between nulliparous and parous samples (18). Robust Multichip Average signal values were generated using Bioconductor (19).

Genes were selected for further analysis whose expression changed significantly by the above analysis in three out of four strains. Significance was assessed by randomly generating eight lists equal in size to the up-regulated and down-regulated lists for each strain from the population of nonredundant genes called present on the chip in at least one sample (2,428 genes). One million random draw trials were performed to calculate a nominal *P* value for combined list length and to estimate the false discovery rate (FDR) using the median list size occurring by chance.

Hierarchical clustering was done using R statistical software[1] and as described (20). Mouse genes were identified using the Homologene database (National Center for Biotechnology Information).

Quantitative real-time PCR. Five micrograms of DNase-treated RNA were used to generate cDNA by standard methods. *Csn2, Mmp12, Tgfb3,*

[1] http://www.R-project.org.

Igfbp5, Areg, Igf-I, Ghr, Serpinh1, and *Sparc* were selected for confirmation by quantitative real-time PCR (QRT-PCR) using TaqMan assays (Applied Biosystems, Foster City, CA). *B2m* was used as a control (21, 22). Reactions were performed in duplicate in 384-well microtiter plates in an ABI Prism Sequence Detection System according to standard methods (Applied Biosystems). One-tailed *t* tests were performed to determine statistical significance using Prism 4.0 software.

Results

Hormone-induced protection in inbred rat strains. To determine whether hormone-induced protection against mammary tumorigenesis is a feature unique to carcinogen-sensitive strains, we compared the extent of protection induced by hormones in four different rat strains: Lewis, Wistar-Furth, Fischer 344, and Copenhagen. Two of these strains (Lewis and Wistar-Furth) have been reported to exhibit hormone-induced protection (9, 12). However, it has not been determined whether carcinogen-resistant strains of rats, such as Copenhagen (23), also exhibit protection. Female rats from each strain were treated with a single dose of methylnitrosourea at 7 weeks of age, followed by s.c. implantation of either placebo or hormone pellets (35 mg of estradiol + 35 mg of progesterone) at 9 weeks of age. Among the placebo-treated groups, Lewis rats exhibited the highest susceptibility to methylnitrosourea-induced mammary tumorigenesis with 100% penetrance and a median tumor latency of 13 weeks (Fig. 1*A*). Fischer 344 and Wistar-Furth rats displayed intermediate carcinogen sensitivity with latencies of 24 and 36 weeks, respectively. In contrast, Copenhagen rats exhibited a high degree of resistance to methylnitrosourea-induced mammary tumorigenesis with only 5 of 12 animals developing mammary tumors, with an average latency of 51 weeks.

Surprisingly, despite the wide variance in carcinogen sensitivity of nulliparous rats from these four strains, estradiol and progesterone treatment induced a significant ($P < 0.05$) degree of protection against mammary tumorigenesis in each strain (Fig. 1*B*). For example, whereas Lewis and Copenhagen strains differed markedly in their sensitivity to methylnitrosourea, they exhibited strikingly similar degrees of hormone-induced protection with HRs of 0.19 [95% confidence interval (CI), 0.05-0.40] and 0.16 (95% CI, 0.02-0.63), respectively. The Wistar-Furth (HR, 0.31; 95% CI, 0.09-0.90) and Fischer 344 (HR, 0.38; 95% CI, 0.10-0.71) strains exhibited lesser, but significant degrees of protection. These experiments show that hormone treatments that mimic pregnancy confer protection against mammary tumorigenesis in each strain irrespective of the intrinsic carcinogen susceptibility of nulliparous animals from that strain.

Morphologic changes induced by parity in the rat mammary gland. Parity-induced changes in breast cancer susceptibility have been reported to be accompanied by persistent changes in the structure of the mammary gland in humans, as well as in rats and mice (4, 6). Consistent with this, carmine-stained whole-mount analysis of nulliparous and parous mammary glands from each of the four rat strains revealed that the architecture of the parous mammary epithelial tree was more complex than that of age-matched nulliparous animals, with a higher degree of ductal side-branching (Fig. 1*C*). These effects were observed in each of the four strains analyzed, suggesting that changes in the structural and cellular composition of the mammary gland may occur as a consequence of parity.

Microarray analysis of parity-induced changes in the rat mammary gland. The similar morphologic changes induced by parity suggested that the hormone-induced protection against mammary tumorigenesis that we observed in different rat strains might be accompanied by common molecular alterations. To identify these changes, we first performed oligonucleotide microarray expression profiling on pooled samples from nulliparous and parous Lewis rats. Genes whose expression changes were considered to be statistically significant using established algorithms, and whose expression changed by at least 1.2-fold as a result of parity, were selected for further analysis (18). This combined analytic approach has previously been shown to be capable of identifying differentially expressed genes with high sensitivity and specificity (18). Gene expression analysis performed in this manner identified 75 up-regulated and 148 down-regulated genes in parous compared with nulliparous mammary glands. Examination of this list of differentially expressed genes confirmed our previous findings in mice that parity results in the persistent up-regulation of *Tgfb3*, as well as differentiation and immune markers, as well as the persistent down-regulation of growth factor encoding genes, such as *Areg* and *Igf-I* (ref. 6; data not shown).

To narrow the list of candidate genes whose regulation might contribute to the protected state associated with parity, we attempted to identify parity-induced gene expression changes that were conserved across multiple rat strains. To this end, total RNA was isolated from the mammary glands of nulliparous and parous Wistar-Furth, Fischer 344, and Copenhagen rats, and analyzed on RGU34A arrays in a manner analogous to that employed for Lewis rats. This led to the identification of 68, 64, and 92 parity up-regulated genes and 132, 209, and 149 parity down-regulated genes in Wistar-Furth, Fischer 344, and Copenhagen rats, respectively.

Unsupervised hierarchical clustering performed using the expression profiles of 1,954 globally varying genes across the nulliparous and parous data sets representing the four rat strains revealed that samples clustered primarily based on strain without regard to parity status (Fig. 2*A*). This suggested that the principal source of global variation in gene expression across these data sets was due to genetic differences between strains rather than reproductive history. This observation suggested that determining which parity-induced gene expression changes were conserved among these highly divergent rat strains could represent a powerful approach to defining a parity-related gene expression signature correlated with hormone-induced protection against mammary tumorigenesis.

To identify parity-induced gene expression changes that were conserved across strains, we selected genes that exhibited $\geq$1.2-fold change in at least three of the four strains analyzed. This led to the identification of 24 up-regulated (Table 1) and 46 down-regulated genes (Table 2). Based on the number of parity-induced gene expression changes observed for each strain, an overlap of this size is highly unlikely by chance (up-regulated: $P < 1 \times 10^{-6}$, FDR < 1%; down-regulated: $P < 1 \times 10^{-6}$, FDR = 4%). As such, this approach led to the identification of 70 genes whose expression is persistently altered by parity across multiple strains of rats that exhibit hormone-induced protection against mammary tumorigenesis.

A gene expression signature distinguishes parous and nulliparous rats and mice. To confirm the validity of the 70-gene parity-related expression signature derived from the above studies, we performed oligonucleotide microarray analysis on samples from nulliparous and parous Lewis rats that were generated independently from those used to derive this signature. Hierarchical clustering analysis of these independent samples using the 70-gene signature revealed that the expression profiles of these genes were sufficient to accurately distinguish parous from nulliparous Lewis rat samples in a blinded manner (Fig. 2*B*).

To determine whether this parity-related signature could distinguish between nulliparous and parous mammary glands from multiple strains of rats, Lewis, Wistar-Furth, Fischer 344, and Copenhagen microarray data sets were clustered in an unsupervised manner based solely on the expression of the 70 genes comprising the parity signature (Fig. 2*C*). In each of the four rat strains examined, the 70-gene signature was sufficient to distinguish parous from nulliparous rats (Fig. 2*C*). Thus, this signature reflects parity-induced gene expression changes that are highly conserved among four genetically divergent rat strains.

Early full-term pregnancy has been reported to result in protection against mammary tumorigenesis in mice, as it does in humans and rats (13). Accordingly, we mapped the 70 genes

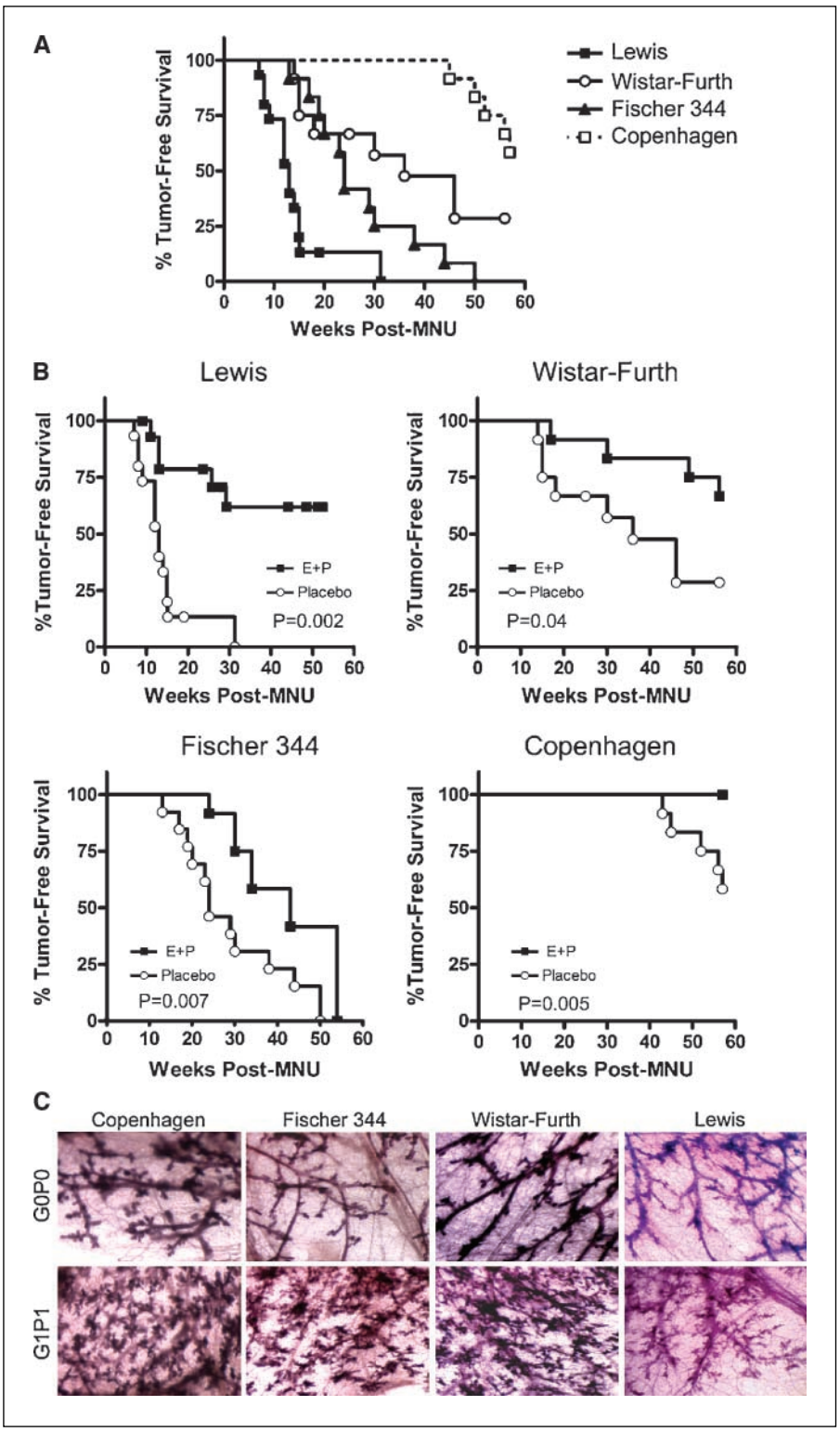

Figure 1. Hormone-induced protection against mammary tumorigenesis is conserved among multiple rat strains. *A,* Kaplan-Meier curves plotting the time to the formation of a first mammary tumor in placebo-treated groups for Lewis (n = 15), Wistar-Furth (n = 12), Fischer 344 (n = 13), and Copenhagen (n = 12) rats treated with methylnitrosourea (*MNU*) at 7 weeks of age. Significant differences in tumor incidence were identified between Lewis and Wistar-Furth (P = 0.0003), Lewis and Fischer 344 (P = 0.0005), Lewis and Copenhagen (P = 0.0001), Wistar-Furth and Copenhagen (P = 0.024), and Fischer 344 and Copenhagen (P = 0.0001) as determined by a log rank test. Wistar-Furth and Fischer 344 were not significantly different (P = 0.14). *B,* mammary tumor incidence for placebo and estradiol and progesterone–treated rats is plotted for each strain. Cohort sizes for estradiol and progesterone–treated animals were: Lewis (n = 16), Wistar-Furth (n = 12), Fischer 344 (n = 12), and Copenhagen (n = 12). Each strain exhibited significantly decreased tumor incidence in estradiol and progesterone–treated compared with placebo-treated cohorts. *C,* carmine-stained whole mounts of abdominal mammary glands from nulliparous (G0P0) and parous (G1P1) rats from each strain (original magnification, ×50). Samples are representative of three animals per group.

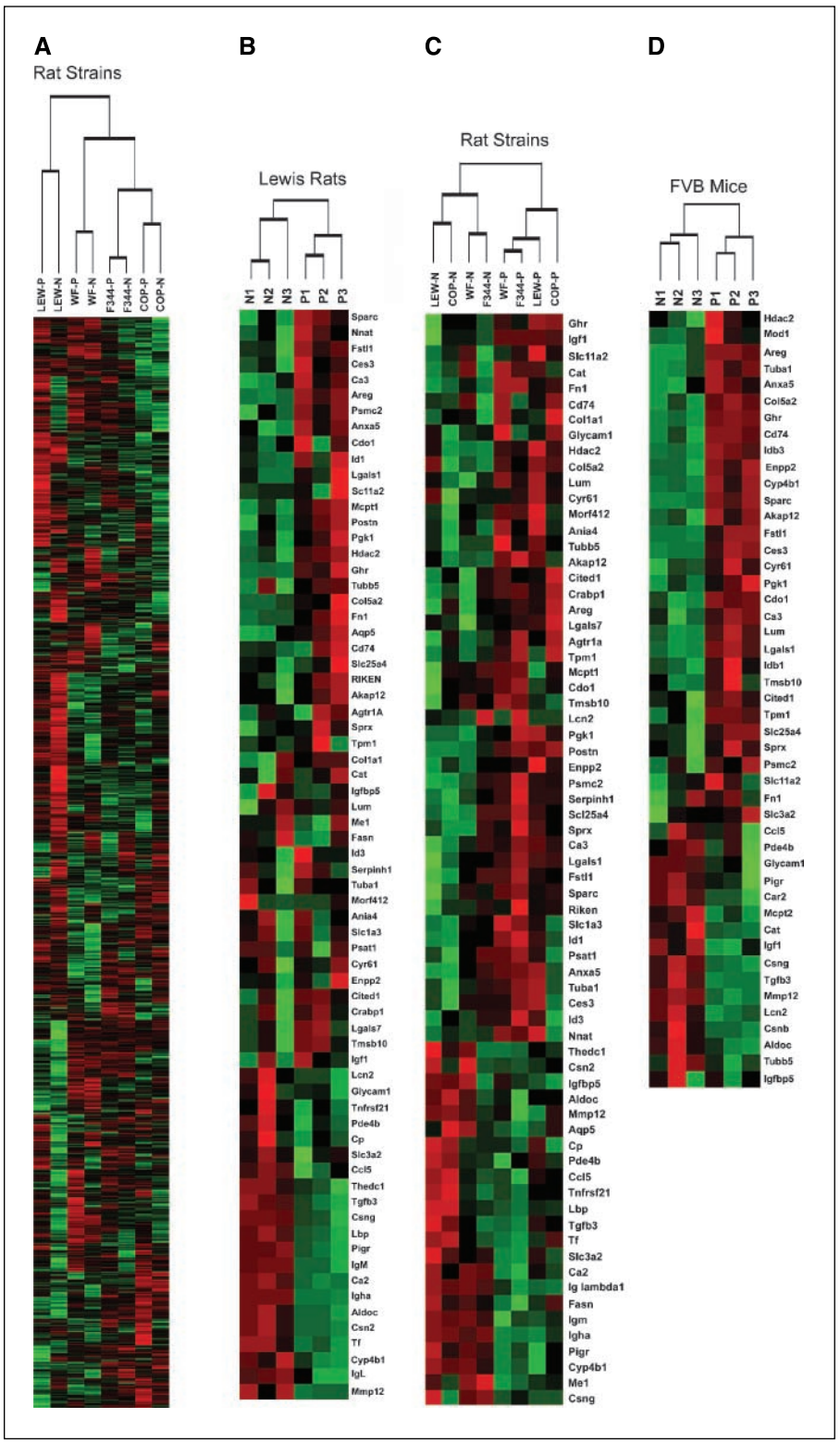

Figure 2. A parity-related gene expression signature distinguishes between nulliparous and parous rats and mice. Unsupervised hierarchical clustering analysis. Nulliparous (*N*), parous (*P*), Lewis (*LEW*), Fischer 344 (*F344*), Wistar-Furth (*WF*), and Copenhagen (*COP*). *A,* nulliparous and parous samples from each strain were clustered based on the median expression values of ~1,900 genes exhibiting global variation in gene expression across the data sets. *B,* six independent Lewis samples [three nulliparous (N1-N3) and three parous (P1-P3)] were clustered based solely on the expression of genes in the 70-gene parity signature. *C,* clustering analysis based solely on the expression of the 70-gene parity signature was performed on nulliparous and parous samples from Lewis, Wistar-Furth, Fischer, and Copenhagen rats. *D,* the 70-gene rat parity signature was mapped to the mouse genome using Homologene, yielding 47 mouse genes. Six FVB mouse samples [three nulliparous (N1-N3) and three parous (P1-P3)] were clustered based on the expression profiles of these 47 genes.

constituting the rat parity signature to the mouse genome, and assessed their expression profiles in nulliparous and parous FVB mouse mammary samples. Of the 70 genes that were mapped, 47 were represented on Affymetrix MGU74Av2 microarrays. These 47 genes were sufficient to distinguish nulliparous from parous samples in a blinded manner (Fig. 2*D*). Thus, a parity-related gene expression signature generated in the rat is able to predict reproductive history in the mouse, suggesting that the persistent molecular alterations that occur in response to parity are conserved across rodent species.

Among the 70 genes that we identified as being consistently regulated by parity, at least five categories were evident.

Table 1. Genes up-regulated in parous rats

Gene name	Symbol	Gene ID	Function	Category	Fold-change G1P1 versus G0P0				
					Lewis	WF	F344	Cop	Median
Immunoglobulin heavy chain	*Igha*	314487	Immunoglobulin	Immune	39.4	25.4	4.5	6.9	25.4
Casein β	*Csn2*	29173	Milk protein	Differentiation	8.0	5.2	1.9	1.5	5.2
IgM light chain		287965	Immunoglobulin	Immune	2.5	3.8	1.8	1.6	2.5
Matrix metalloproteinase 12	*Mmp12*	117033	Proteolysis	ECM/Immune	2.6	1.4	2.0	1.3	2.0
Casein γ	*Csng*	114595	Milk protein	Differentiation	3.1	1.9	1.2	0.9	1.9
Fatty acid synthase	*Fasn*	50671	Fatty acid biosynthesis	Metabolism/ differentiation	2.0	1.6	1.7	0.9	1.7
Cytochrome P450, family 4, subfamily b,1	*Cyp4b1*	24307	Monooxygenase activity	Metabolism	1.6	1.5	1.2	1.2	1.5
Carbonic anhydrase 2	*Ca2*	54231	Carbon dioxide hydration	Metabolism	1.5	1.5	1.4	1.1	1.5
Ig lambda-1 chain C region		363828	Immunoglobulin	Immune	1.5	1.4	1.4	1.3	1.4
Malic enzyme 1	*Me1*	24552	Pyruvate synthesis	Metabolism	1.3	1.4	1.4	1.1	1.4
Insulin-like growth factor binding protein 5	*Igfbp5*	25285	Igf-I-binding	Growth factor/ ECM	2.4	1.4	0.9	2.7	1.4
Lipopolysaccharide binding protein	*Lbp*	29469	Antibacterial	Immune	2.1	1.3	1.4	2.0	1.4
Polymeric immunoglobulin receptor	*Pigr*	25046	Trancytosis	Immune	1.7	1.4	1.2	1.1	1.4
Transforming growth factor, β3	*Tgfb3*	25717	Cell growth/ proliferation	Tgf-β	1.5	1.3	1.2	1.4	1.3
Aquaporin 5	*Aqp5*	25241	Water transport	Transporter	1.3	1.7	1.2	1.5	1.3
Phosphodiesterase 4B	*Pde4b*	24626	Cyclic AMP phoshodiesterase	Signal transduction	1.3	1.4	1.0	1.4	1.3
Thioesterase domain containing 1	*Thedc1*	64669	Fatty acid biosynthesis	Metabolism/ differentiation	1.9	1.2	1.3	1.5	1.3
Transferrin	*Tf*	24825	Iron transport	Transport/ differentiation	1.4	1.2	1.3	1.5	1.3
Ceruloplasmin	*Cp*	24268	Copper transport	Transport/ differentiation	1.3	1.0	1.2	2.2	1.2
Similar to death receptor 6	*Tnfrsf21*	316256	Apoptosis	Signal transduction	1.3	1.0	1.2	1.3	1.2
Aldolase C, fructose-biphosphate	*Aldoc*	24191	Fructose metabolism	Metabolism	1.2	1.2	1.1	1.3	1.2
Lipocalin 2	*Lcn2*	170496	Iron binding/antibacterial	Immune	1.3	1.1	1.2	1.4	1.2
Solute carrier family 3, member 2	*Slc3a2*	50567	Amino acid transporter	Transporter	1.2	1.1	1.2	1.3	1.2

NOTE: Genes identified as up-regulated by at least 1.2-fold in three out of four rat strains as a result of parity are reported from the highest to lowest median fold change. Gene names and symbols are reported based on the Rat Genome Database, and Gene ID according to Entrez Gene. Gene functions and categories are based on Gene Ontology.
Abbreviations: WF, Wistar-Furth; F344, Fischer 344; Cop, Copenhagen.

These included the previously identified differentiation, immune, Tgf-β, and growth factor categories (6), as well as an additional category of genes that are involved in ECM structure and function (Tables 1 and 2). We previously showed that clustering based on genes in each of the first four categories was sufficient to distinguish between nulliparous and parous rats (6). In an analogous manner, we tested whether unsupervised clustering based solely on ECM-related genes would be sufficient to differentiate between nulliparous and parous rat or mouse samples. In each case, ECM-related gene expression patterns alone were sufficient to distinguish between nulliparous and parous mammary samples from the four different rat strains (Fig. 3*A*), from independent mammary samples derived from nulliparous and parous Lewis rats (Fig. 3*B*), and from mammary samples derived from FVB mice (Fig. 3*C*). This indicates that differential expression of a subset of genes involved in ECM structure and function represents a conserved feature of parity-induced changes in the rodent mammary gland.

Parity up-regulates *Tgfb3* and expression of differentiation and immune markers. Our previous analysis of parity-induced gene expression changes in FVB mice was consistent with the parity-induced up-regulation of Tgf-β3 activity. Similarly, in the current study, we found that *Tgfb3* expression was up-regulated by parity in each of the four rat strains examined (Table 1). This finding was confirmed by QRT-PCR

Table 2. Genes down-regulated in parous rats

Gene name	Symbol	Gene ID	Function	Category	Fold-change G1P1 versus G0P0				
					Lewis	WF	F344	Cop	Median
Periostin	*Postn*	361945	Transcription factor	Differentiation	1.9	2.1	1.8	2.2	2.0
Amphiregulin	*Areg*	29183	Epidermal growth factor receptor ligand	Growth factor	3.5	2.1	1.7	1.9	2.0
Cellular retinoic acid binding protein I	*Crabp1*	25061	Retinoic acid receptor signaling	Signal transduction	1.8	2.1	1.3	1.5	1.7
Insulin-like growth factor 1	*Igf-I*	24482	Cell proliferation/ survival	Growth factor	1.7	1.2	1.5	1.5	1.5
Fibronectin 1	*Fn1*	25661	Integrin signaling	ECM	1.4	1.3	1.5	1.6	1.5
A kinase (PRKA) anchor protein (gravin) 12	*Akap12*	83425	Scaffolding protein	Signal transduction	1.2	1.6	1.6	1.3	1.4
Neuronatin	*Nnat*	94270	Protein transport		2.0	1.4	1.5	0.9	1.4
Glycosylation dependent cell adhesion molecule 1	*Glycam1*	25258	Selectin ligand	Differentiation	0.5	2.2	1.2	1.7	1.4
Secreted acidic cysteine rich glycoprotein	*Sparc*	24791	ECM Formation	ECM	1.6	1.1	1.4	1.4	1.4
Ectonucleotide pyrophosphatase/ phosphodiesterase 2	*Enpp2*	84050	Lysophospholipase	Cell motility	2.1	1.4	1.4	1.0	1.4
Lectin, galactose binding, soluble 1	*Lgals1*	56646	Integrin signaling	ECM	1.5	1.2	1.4	1.4	1.4
Inhibitor of DNA binding 1, helix-loop-helix protein	*Id1*	25261	Transcriptional repression	Tgf-β	1.4	1.4	1.4	1.1	1.4
Follistatin-like 1	*Fstl1*	79210			1.5	1.7	1.2	1.2	1.4
Phosphoserine aminotransferase 1	*Psat1*	293820	Serine biosynthesis	Metabolism	1.4	1.2	1.5	1.3	1.4
Lumican	*Lum*	81682	Proteoglycan	ECM	1.3	1.5	1.1	1.4	1.3
Melanocyte-specific gene 1 protein	*Cited1*	64466	Transcription factor	Signal transduction	1.4	1.9	1.2	1.3	1.3
Serine proteinase inhibitor, clade H, member 1	*Serpinh1*	29345	Procollagen binding	ECM	1.4	1.3	1.3	1.4	1.3
Sushi-repeat-containing protein	*Sprx*	64316			1.3	1.3	1.3	1.5	1.3
Carboxylesterase 3	*Ces3*	113902	Fatty acid metabolism	Metabolism	1.8	1.1	1.3	1.4	1.3
Cysteine rich protein 61	*Cyr61*	83476	Integrin signaling	ECM	1.1	1.3	1.3	1.6	1.3
Solute carrier family 1, member 3	*Slc1a3*	29483	Amino acid transporter	Transporter	1.4	1.3	1.3	1.1	1.3
Similar to RIKEN cDNA 6330406I15	*RDG1307396*	360757			1.6	1.2	1.3	1.3	1.3
Catalase	*Cat*	24248	Hydrogen peroxide reductase	ROS	1.7	1.0	1.4	1.2	1.3
Tropomyosin 1, α	*Tpm1*	24851	Actin binding		1.1	1.3	1.3	1.3	1.3
Activity and neurotransmitter-induced early gene protein 4	*Ania4*	360341	CAM kinase	Kinase	1.5	1.2	1.2	1.3	1.3
Solute carrier family 11, member 2	*Slc11a2*	25715	Divalent metal ion transporter	Transporter	1.4	1.0	1.3	1.2	1.3
Inhibitor of DNA binding 3, helix-loop-helix protein	*Id3*	25585	Transcriptional repression	Tgf-β	1.5	1.2	1.3	0.9	1.3
Solute carrier family 25 member 4	*Slc25a4*	85333	Nucleotide translocator	Transporter	1.3	1.3	1.2	1.3	1.3
Growth hormone receptor	*Ghr*	25235	Growth hormone signaling	Growth factor	2.1	1.1	1.2	1.3	1.3
Phosphoglycerate kinase 1	*Pgk1*	24644	Phosphoprotein glycolysis	Metabolism	1.6	1.2	1.3	1.2	1.3

(*Continued on the following page*)

Table 2. Genes down-regulated in parous rats (Cont'd)

Gene name	Symbol	Gene ID	Function	Category	Fold-change G1P1 versus G0P0				
					Lewis	WF	F344	Cop	Median
Cytosolic cysteine dioxygenase 1	*Cdo1*	81718	Cysteine metabolism	Metabolism	1.5	1.2	1.2	1.3	1.3
Mast cell protease 1	*Mcpt1*	29265	Proteolysis	ECM	1.6	1.3	1.2	1.2	1.2
Collagen, type V, α2	*Col5a2*	85250	ECM structural protein	ECM	1.0	1.2	1.3	1.5	1.2
Carbonic anhydrase 3	*Ca3*	54232	Carbon metabolism	Metabolism	1.8	1.2	1.1	1.3	1.2
Tubulin, α1	*Tuba1*	64158	Microtubule component	Cell structure	1.5	1.2	1.2	1.2	1.2
Angiotensin II receptor, type 1	*Agtr1A*	24180	Angiotensin receptor	Signal transduction	1.3	1.2	1.2	1.3	1.2
Collagen, type 1, α 1	*Col1a1*	29393	ECM structural protein	ECM	1.1	1.2	1.2	1.8	1.2
Annexin A5	*Anxa5*	25673	Calcium ion binding		1.6	1.2	1.2	1.2	1.2
Thymosin, β10	*Tmsb10*	50665	Actin binding		1.3	1.2	1.2	1.0	1.2
Tubulin, β5	*Tubb5*	29214	Microtubule component	Cell structure	1.1	1.3	1.2	1.2	1.2
Histone deacetylase 2	*Hdac2*	84577	Chromatin rearrangement		1.2	1.2	1.3	1.1	1.2
Lectin, galactose binding, soluble 7	*Lgals7*	29518	Galactose binding		1.1	1.8	1.2	1.2	1.2
CD74 antigen	*Cd74*	25599		Immune	1.2	1.2	1.0	1.3	1.2
Proteasome 26S subunit, ATPase 2	*Psmc2*	25581	Protein degradation		1.3	1.1	1.2	1.1	1.2
MORF-related gene X	*Morf412*	317413			1.4	1.2	1.1	1.2	1.2

NOTE: Genes identified as down-regulated by at least 1.2-fold in three out of four rat strains as a result of parity are reported from the highest to lowest median fold change. Gene names and symbols are reported based on the Rat Genome Database, and Gene ID according to Entrez Gene. Gene functions and categories are based on Gene Ontology.
Abbreviations: WF, Wistar-Furth; F344, Fischer 344; Cop, Copenhagen.

analysis of independent parous and nulliparous Lewis rat samples (Fig. 4*A*).

Also consistent with our prior observations, parity resulted in a persistent increase in the expression of genes involved in mammary differentiation, including the milk proteins β-casein and γ-casein, and the metal ion transporters ceruloplasmin and tranferrin (ref. 6; Table 1; Fig. 4*A*).

As we have previously shown in the mouse, the 70-gene rat parity-related gene expression signature reflected the increased presence of immune cells in the parous mammary gland. In particular, increased expression of multiple immunoglobulin heavy and light chain genes in the parous gland suggested an increase in the population of plasma cells, whereas up-regulation of *Mmp12* and *Tnfrsf21* was consistent with increased numbers of macrophages and T cells (Table 1; Fig. 4*A*). Similarly, increased antibacterial and antiviral activity was suggested by the up-regulation of *Lbp, Lcn2*, and *Ccl5* (refs. 24–26; Table 1).

Parity results in down-regulation of amphiregulin and the growth hormone/Igf-I axis. Previous gene expression profiling of mouse mammary development revealed that parity results in a persistent decrease in the expression of several growth factor–encoding genes, including *Areg* and *Igf-I* (6). The present study confirmed that decreased expression of *Areg* and *Igf-I* are consistent features of the parous state in rats (Table 2; Fig. 4*B*). Additional evidence supporting parity-induced down-regulation of the growth hormone/Igf-I axis in the mammary glands of multiple rat strains was suggested by a decrease in growth hormone receptor (*Ghr*) expression (Table 2; Fig. 4*B*) as well as an increase in *Igfbp5* expression (Table 1; Fig. 4*A*), which functions to sequester local Igf-I in the ECM (27).

Parity regulates ECM gene expression. Mammary epithelial-ECM interactions play an important role in both normal mammary gland development and tumorigenesis (28). Moreover, persistent changes in the structure and function of the ECM have been shown in the mammary glands of parous rats (29). In the present study, microarray expression profiling suggested that a principal effect of parity in the rodent mammary gland is alteration of ECM gene expression. Thirteen of the 70 genes constituting the parity signature encode ECM structural components or proteins that regulate ECM formation or signaling (Tables 1 and 2). Notably, the majority of ECM-related gene expression changes induced by parity represented decreases in expression, including the ECM structural components, fibronectin 1, lumican, and collagen type I and collagen type V (Table 2). Parity-induced decreases in the expression of genes that regulate ECM formation or cellular interactions were also observed, including, *Sparc, Lgals1, Lgals7, Serpinh1, Cyr61*, and *Mcpt1* (Table 2; Fig. 4*B*).

To determine whether these parity-induced ECM-related gene expression changes were accompanied by differences in ECM structure, we stained histologic sections with Mason's trichrome to evaluate total collagen content. Although proximal epithelial structures seemed similar with respect to periductal trichrome staining (data not shown), a significant decrease in the extent of trichrome staining surrounding distal ducts was observed in the parous gland (Fig. 4*C*). These results provide further evidence that parity results in structural changes in the ECM.

Discussion

Women who have their first child early in life have a substantially reduced lifetime risk of breast cancer, an effect that is largely restricted to ER-positive tumors. Similar to humans, rats frequently develop ER-positive breast cancers and exhibit parity-induced protection against mammary tumorigenesis. In the current study, we set out to identify persistent parity-induced changes in gene expression that are conserved among multiple rat strains that exhibit hormone-induced protection against mammary tumorigenesis. We found that four genetically diverse inbred rat strains exhibit hormone-induced protection against mammary tumorigenesis and share a 70-gene pregnancy-induced expression signature. Our findings constitute the first global survey of parity-induced changes in gene expression in the rat—which represents the principal model for studying this phenomenon—as well as the first study to show conservation of parity-induced gene expression changes in multiple inbred rat strains that exhibit hormone-induced protection. Beyond suggesting that parity-induced protection is as robust and widely conserved a phenomenon in rats as it is in humans, our findings provide new insights into potential mechanisms by which early first-full term pregnancy decreases breast cancer risk.

These current studies extend our previous observations that parity results in persistently increased mammary expression of *Tgfb3* to include multiple additional strains of rats. Notably, loss of Tgf-β signaling in stromal fibroblasts promotes the growth and invasion of mammary carcinomas (30). Tgf-β may also have direct effects on mammary epithelial cells, resulting in the inhibition of mammary tumorigenesis (31). The sum of these effects is predicted to decrease the susceptibility of the parous gland to oncogenic transformation.

One of the most consistent and robust parity-induced changes in gene expression that we have observed in the rodent mammary gland is down-regulation of the epidermal growth factor receptor ligand, *Areg*. AREG is overexpressed in a high proportion of human breast cancers and correlates with large tumor size and nodal involvement (32). Studies in genetically engineered mice and mammary epithelial cell lines suggest an important role for AREG in driving mammary epithelial proliferation, whereas recent evidence indicates that this growth factor may alter the ECM by the regulation of protease expression and secretion, including matrix metalloproteinase-2, matrix metalloproteinase-9, urokinase-type plasminogen activator, and plasminogen activator inhibitor-1 (33). Thus, parity-mediated down-regulation of *Areg* may not only inhibit epithelial proliferation, but may also hinder the invasive abilities of transformed cells in the mammary gland.

In addition to the down-regulation of *Areg*, we have confirmed that parity also results in the persistent down-regulation of *Igf-I*. Notably, a strong positive correlation exists between serum IGF-I levels and breast cancer risk in premenopausal women (34). Local and serum levels of IGF-I are regulated by growth hormone through its interaction with growth hormone receptor (35). Additional findings indicate that parity results in a persistent decrease in circulating growth hormone levels in rats (7); moreover, treatment of parous rats with Igf-I results in an increase in carcinogen-induced mammary tumorigenesis to levels similar to those observed in nulliparous controls (36). Consistent with this, spontaneous dwarf rats, which lack functional growth hormone, are highly resistant to carcinogen-induced mammary tumorigenesis (37).

Additional evidence for down-regulation of the growth hormone/Igf-I axis within the parous mammary gland was suggested in the present study by increases in *Igfbp5* expression and decreases in *Ghr* expression. As such, our findings suggest that—in addition to reducing circulating levels of growth hormone—parity may modulate local expression and activity of Igf-I within the mammary gland. Whereas Igf-I acts directly on mammary epithelial cells to promote proliferation and inhibit apoptosis (38), Igf-I in the mammary gland is likely produced in the stromal compartment in response to Ghr signaling (39). Local regulation of Igf-I activity also occurs through interactions with Igf-I binding proteins, such as Igfbp5, which binds and sequesters Igf-I in the ECM (40). As such, parity-induced down-regulation of Ghr and Igf-I expression in the mammary gland, coupled with up-regulation of Igfbp5 expression, would be predicted to result in decreased Igf-I activity. This represents a

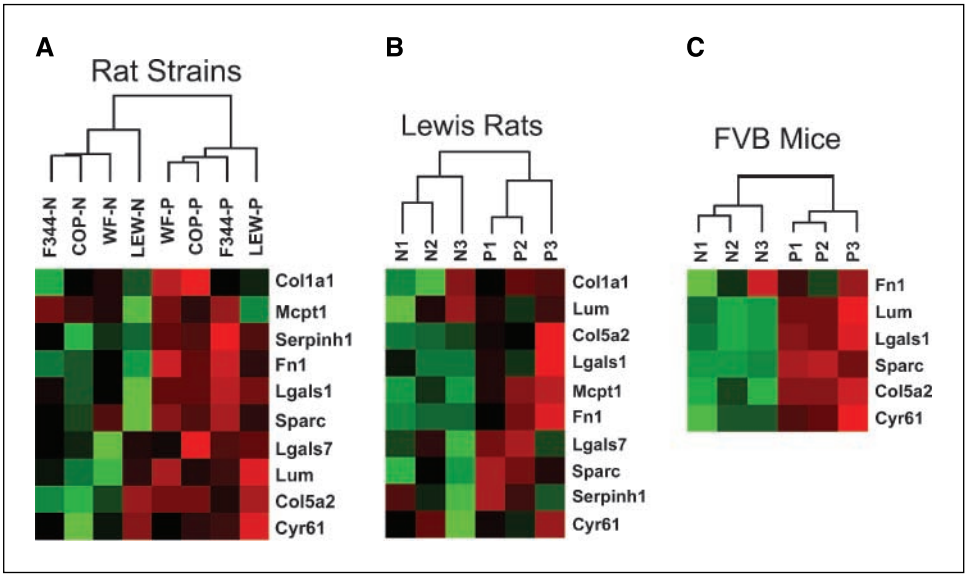

Figure 3. ECM gene expression distinguishes between nulliparous and parous rats and mice. Unsupervised hierarchical clustering analysis. *A,* a subset of parity-regulated genes involved in ECM structure and regulation was used to cluster nulliparous and parous mammary samples from Lewis (*LEW*), Wistar-Furth (*WF*), Fischer (*F344*), and Copenhagen (*COP*) rats. *B,* six independent Lewis samples [three nulliparous (N1-N3) and three parous (P1-P3) samples] were clustered based on the expression of ECM-related genes. *C,* six FVB mouse samples [three nulliparous (N1-N3) and three parous (P1-P3)] were clustered based on the expression of ECM-related genes identified in the rat parity signature that were mapped to the mouse genome.

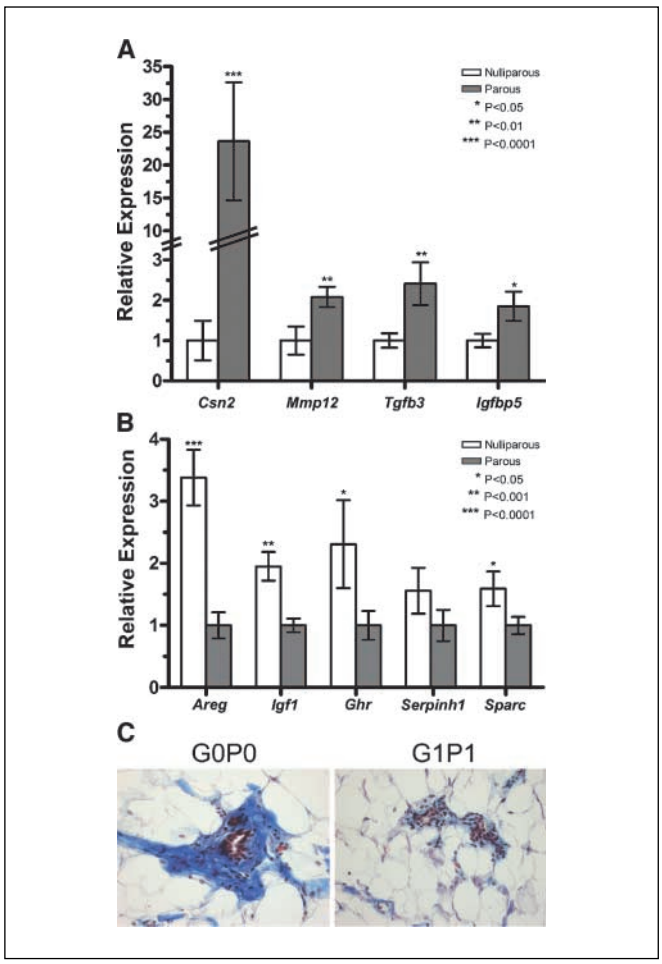

Figure 4. Confirmation of gene expression changes. *A* and *B,* TaqMan QRT-PCR was performed on cDNAs generated from 21 nulliparous and 21 parous Lewis rat mammary samples. Each reaction was performed in duplicate. Expression values for each gene were normalized to *B2m*. *A,* relative expression of parity up-regulated genes. *White columns*, mean expression in nulliparous samples normalized to 1.0 for each gene; *gray columns*, mean expression of each gene in parous relative to nulliparous samples; *bars*, ±SE. *B,* relative expression of parity down-regulated genes. *White columns*, mean expression of each gene in nulliparous relative to parous samples; *gray columns,* mean expression in parous samples normalized to 1.0 for each gene; *bars*, ±SE. *P* values were generated using a one-tailed, unpaired Student's *t* test. *C,* Mason's trichrome staining. Abdominal mammary glands from nulliparous and parous Lewis rats were stained with Mason's trichrome to assess total collagen present in the ECM surrounding epithelial structures. Images are representative of distal structures in the mammary glands of three nulliparous and three parous Lewis rats (original magnification, ×200).

plausible mechanism by which parity may confer protection against breast cancer.

The functional unit of the mammary gland consists of a complex stroma that surrounds the epithelial compartment. Stromal-epithelial interactions play a prominent role, not only in mammary development, but also in tumorigenesis (28). Fibroblasts represent the most prominent cell type of the periductal stroma and, in addition to secreting growth factors that activate epithelial receptors, they are the primary synthesizers of ECM constituents such as fibronectin, collagen, and proteoglycans. Accumulating evidence indicates that stromal constituents, including fibroblasts and ECM structural components, could have differential effects on epithelial cells depending on the source of the tissue from which they are isolated (41). Consistent with this, Schedin et al. have shown that the ability of mammary epithelial cells to form ductal structures in culture is markedly influenced by the developmental context of the ECM in which they are cultured (29). Further support for the role of ECM regulation in parity-induced protection against breast cancer comes from our observation that parous mammary glands exhibit decreased trichrome staining as well as persistent down-regulation of ECM structural and regulatory genes. Because cross-talk between epithelial and stroma cells occurs through local growth factors and their receptors (42), it is possible that parity-induced down-regulation of *Areg* and *Igf-I* in combination with up-regulation of *Tgfb3* may alter stromal-epithelial interactions in such a way as to decrease susceptibility to mammary carcinogenesis.

Finally, it is interesting to speculate that parity-induced changes in the ECM may be related to measures of breast cancer risk associated with mammographic breast density. Increased mammographic density has been consistently shown to correlate with high breast cancer risk (43). Mammographic density has also been reported to be negatively correlated with parity (44). Although breast density was initially believed to reflect the epithelial content of the breast, current evidence suggests that ECM composition—in particular collagen and proteoglycans such as lumican—may be the primary determinant of mammographic density (44, 45). Intriguingly, recent studies have implicated the ratio of serum IGF-I to IGFBP3 as a major determinant of mammographic density (46). Consistent with this, Guo et al. found increased IGF-I tissue staining in samples from women with increased breast density (45). Our findings support the hypothesis that parity decreases Igf-I expression and activity and diminishes the expression of selected ECM structural components. Together, these changes may lead to decreases in both mammographic breast density and breast cancer risk. Validation of this hypothesis will require confirmation that parity alters local IGF-I levels and mammographic breast density in women, and that modulation of Igf-I in rodent models will alter breast density as well as pregnancy-induced protection against breast cancer.

In summary, the results presented in this study extend previous observations that parity results in local changes in growth factor gene expression in the mammary gland. We hypothesize that the evolutionarily conserved parity-induced alterations in gene expression identified in this study result in the modification of the extracellular environment and changes in stromal-epithelial interactions. We hypothesize that the ultimate effect of these changes is to create a tumor suppressive state, thereby providing a potential mechanism to explain parity-induced protection against mammary tumorigenesis. Whether analogous parity-induced changes occur in the human breast remains an important yet unresolved question.

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Hormone-Induced Protection against Mammary Tumorigenesis Is Conserved in Multiple Rat Strains and Identifies a Core Gene Expression Signature Induced by Pregnancy

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