Parous mammary glands exhibit distinct alterations in gene expression and proliferation responsiveness to carcinogenic stimuli in Lewis rats

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Abstract. Early full-term pregnancy affords lifetime protection against the development of breast cancer. Parity-induced protection can be reproduced in a carcinogen-induced rat mammary carcinoma model, but the molecular mechanisms of this protection against carcinogenic stimuli in rat mammary glands have not been fully characterized. To gain a better understanding of these molecular mechanisms, we used an oligonucleotide microarray to examine gene expression in parous and age-matched virgin (AMV) mammary glands of Lewis rats before and after carcinogen (*N*-methyl-*N*-nitrosourea; MNU) treatment. Parous mammary glands before MNU treatment showed up-regulation of multiple differentiation-related genes, such as *whey acidic protein* (Wap), *casein beta* (Csn2), *casein gamma* (Csng), *lipopolysaccharide binding protein* (Lbp), *secreted phosphoprotein 1* (Spp1) and *glycosylation-dependent cell adhesion molecule 1* (Glycam1). Also, parous mammary glands before MNU treatment exhibited down-regulation of growth-related genes such as *regenerating islet-derived 3 alpha* (Reg3a), *mesothelin* (Msln), *insulin-like growth factor 2* (Igf2) and *insulin-like growth factor binding protein 4* (Igfbp4). After MNU treatment, AMV mammary glands exhibited up-regulation of growth-related genes, such as Msln, *cell division cycle 2 homolog A* (Cdc2a), Igf2, Igfbp4, *stathmin 1* (Stmn1) and *homeobox*, *msh-like 1* (Msx1), whereas expression of these genes remained low in parous mammary glands. AMV mammary glands also exhibited marked up-regulation of Cdc2a and Stmn1 in response to MNU. After MNU treatment, the PCNA labeling index increased significantly in AMV mammary epithelial cells (13.7±1.1%), but remained low in parous mammary glands (3.6±0.4%). The response of AMV mammary glands to carcinogenic stimuli includes up-regulation of growth-related genes and increased cell proliferation. The lack of a similar response in parous mammary glands may explain parity-induced protection against mammary tumor development.

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Introduction

Reproductive history is the most consistent risk factor affecting breast cancer incidence, regardless of geographic region and ethnicity (1). There is evidence that age at menarche, menopause and first full-term pregnancy has a significant impact on individual susceptibility to breast cancer. In particular, a number of epidemiological studies have shown that early full-term pregnancy results in a significant reduction of lifetime risk of breast cancer compared with nulliparous women (2,3). Parity-induced protection against mammary cancer has also been observed in rodents. Rats (4,5) and mice (6) that have undergone a full-term pregnancy are more refractory to chemically induced mammary carcinogenesis than age-matched virgins (AMVs). Pregnancy is the best-known means of physiological protection against breast cancer, and can be mimicked by short-term treatment with estrogen and progesterone (E+P) (7,8) or with human chorionic gonadotrophin (hCG) (9,10). Parity- or hormone-induced protection has been demonstrated using several types of chemical carcinogens [including 7, 12-dimethylbenz (α) anthracene (DMBA) and *N*-methyl-*N*-nitrosourea (MNU)] and several strains of mice (C3H/Sm and BD2/fF1) and rats (Sprague-Dawley, Wistar-Furth and Lewis) (4-6,11).

Several hypotheses have been proposed to explain the mechanism(s) of parity-induced protection against rat mammary carcinogenesis, based on phenotypic features of parous and hormone-treated mammary glands. The best-known hypothesis is that protection is the result of differentiation of mammary gland target structures (terminal end buds; TEBs), which are considered to be the site of origin of mammary carcinoma (12). In addition, changes in the phenotypes of parous mammary epithelial cells are

thought to be important, e.g. decreased proliferation activity, increased capacity for DNA repair, and decreased carcinogen binding to DNA of epithelial cells (13,14). Another hypothesis is that protection is the result of an altered systemic hormonal milieu with reduced circulating levels of prolactin (PRL) and growth hormone (GH), which regulate mammary gland development (4). Parous mammary glands have also exhibited decreased expression of estrogen receptor α and epidermal growth factor receptor (4). Thus, persistent changes in mammary gland structures, mammary epithelial cell phenotype or hormonal environment may be involved in parity-induced protection against carcinogenesis in rats. Studies have shown persistent changes in gene expression in parous rodents, compared to AMVs (15,16). However, the cellular and molecular mechanisms that underlie parity-induced protection remain largely unknown. Therefore, there is a need to elucidate the molecular mechanisms responsible for parity-induced protection against mammary carcinoma in rats.

In the present study, we performed oligonucleotide microarray analyses to identify candidate genes for parity-induced protection against mammary carcinoma in rats, and used real-time PCR analyses to quantify differences in expression of these genes between parous and AMV mammary glands of inbred Lewis rats before and after MNU treatment. In addition, we compared levels of cell proliferation before and after MNU treatment between parous and AMV mammary glands.

Materials and methods

Animals. Twelve-week-old pregnant and AMV inbred Lewis rats were purchased from Charles River Japan (Hino). The animals were maintained under standard laboratory conditions (22˚C, 60±10% humidity, 12 h dark/light cycle), and fed a commercial pellet diet (CMF; Oriental Yeast, Kyoto, Japan) and water *ad libitum* throughout the experiment. The Animal Experimentation Committee at Kansai Medical University approved all procedures involving animals. Pregnant and AMV rats (10 animals each) were used for the experiments. After parturition (at 87 days of age), parous animals were allowed to nurse their offspring for 21 days. At 108 days of age, the litters were removed, and the parous animals underwent 28 days of postlactational involution. At 136 days of age, parous and AMV rats (5 animals each) were sacrificed, and the remaining animals (5 parous and 5 AMV rats) received a single intraperitoneal 50 mg/kg dose of *N*-methyl-*N*-nitrosourea (MNU; Nacalai Tesque, Kyoto, Japan). Three weeks after MNU administration, both parous and AMV rats (age, 157 days) were sacrificed. A schematic representation of the experimental protocol is shown in Fig. 1.

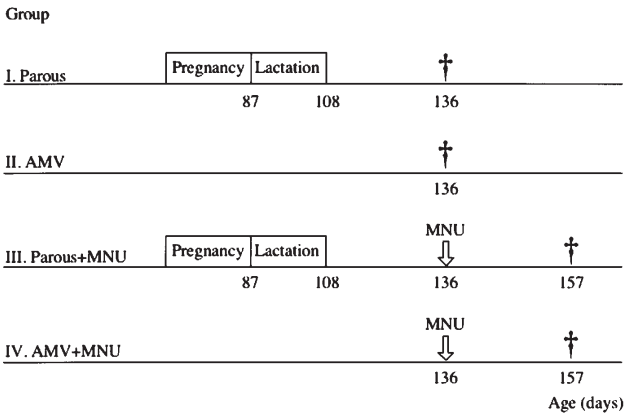

Figure 1. Schematic representation of the experimental protocol. †, Time at which rats were sacrificed. MNU, 50 mg/kg body weight MNU was injected into parous and AMV animals (groups III and IV, respectively) at 136 days of age.

Table I. List of primers used for real-time PCR analysis.

Gene name (gene symbol)	Forward primer	Reverse primer
Whey acidic protein (Wap)	GCCCAGAATGACATGTGTTG	TTGTTGCAGCATTTCATGGT
Casein beta (Csn2)	TGGGCCTATCATTTCTCCTG	GTCTGAGGAAAAGCCTGCAC
Casein gamma (Csng)	AGGGCAATGGTGTGTTCTTC	GGGCCATTGGTTCACACTAC
Lipopolysaccharide binding protein (Lbp)	AATCCAAAGTCGGCATGTTC	AGTCGAGGTCGTGGAGCTTA
Glycosylation-dependent cell adhesion molecule 1 (Glycam1)	GCAGGAAGAGACCACCTCAG	CCAAGAGGTGGTGGTCAGAT
Secreted phosphoprotein 1 (Spp1)	GAGGAGAAGGCGCATTACAG	ATGGCTTTCATTGGAGTTGC
Amphiregulin (Areg)	TGGCAGTGAACTCTCCACAG	CCTTTGCCTCCCTTTTTCTT
Regenerating islet-derived 3 alpha (Reg3a)	TCTGAAGTGGGGAGACCATC	CGGATGAAGGAAGATGGAAA
Mesothelin (Ms1n)	ACCTGGACAGTCTGGGTTTG	CAGGAGCCTTAGGAGTGGTG
Cell division cycle 2 homolog A (Cdc2a)	GGGTCCGTTGTAACCTGTTG	TCCTTCTTCCTCGCTTTCAA
Insulin-like growth factor 2 (Igf2)	GTCCTGCTCCCAACCATTTA	CAGAAGTCAGGCCAGGTAGC
Insulin-like growth factor binding protein 4 (Igfbp4)	ATGGCCAAAGTGAGAGATCG	GACACTGTTTGGGGTGGAAG
Stathmin 1 (Stmnl)	AGCAAAATGGCAGAGGAGAA	TTAGTCAGCCTCGGTCTCGT
Homeobox, msb-like 1 (Msxl)	CTACGCAAGCACAAGACCAA	TTCACCTGGGTCTCGGTAAG
Actin, beta (Actb)	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA
Cytokeratin 8 (Ck8)	CCAAGCTGGAGGATCTGAAG	TTCGTGTGGATGCTCATGTT

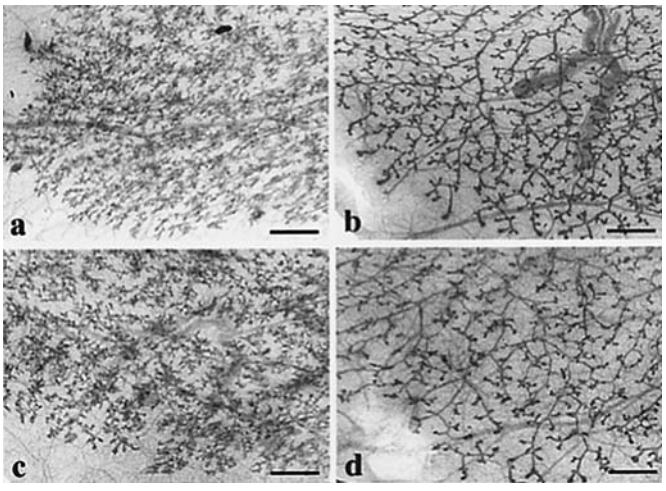

Figure 2. Whole mounts of mammary glands obtained from parous (a), AMV (b), parous plus MNU (c) and AMV plus MNU (d). Note the increased ductal branching and alveolar formation in parous mammary glands before (a, 136 days of age) and after (c, 157 days of age) MNU treatment, compared with corresponding AMV mammary glands (b and d, respectively). Scale bar, 1.0 mm.

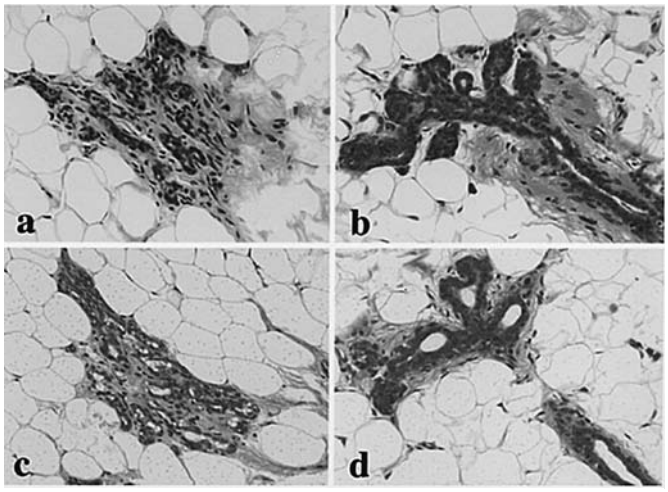

Figure 3. Photomicrographs of mammary glands. Untreated parous mammary glands (a), untreated AMV mammary glands (b), MNU-treated parous mammary glands (c), and MNU-treated AMV mammary glands (d). Atrophic cells without secretory activity were observed in untreated parous mammary glands and MNU-treated parous mammary glands (a and c, respectively). Original magnification, x200.

Tissue collection and RNA isolation. For microarray and real-time PCR analyses, 4th inguinal mammary glands were collected from 5 animals per group (group I, parous; group II, AMV; group III, parous+MNU; and group IV, AMV+MNU). Mammary tissues were snap-frozen in liquid nitrogen immediately after removal and stored at -80˚C until use. Total RNA was isolated using QIAzol reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Frozen tissues were ground to a powder in liquid nitrogen using a mortar and pestle before being added to QIAzol reagent for homogenization. RNA was further purified using the RNeasy Mini kit (Qiagen).

Microarray analysis. For microarray analyses, equal amounts of purified RNA from 5 animals per group were combined into RNA pools. The quality of each RNA pool was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). An oligonucleotide array, containing approximately 20,000 sequences corresponding to known genes, expression sequence tags (ESTs) and unknown genes, was used to compare the gene expression patterns of parous and AMV mammary glands before and after MNU treatment. Rat oligo microarray kits (G4130A) were purchased from Agilent Technologies. Labeled cRNA was synthesized from 1.0 μg of total RNA using a Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies) according to the manufacturer's instructions. The first-strand cDNA was generated by *in vitro* transcription using the Moloney murine leukemia virus (MMLV) reverse transcriptase and oligo dT-T7 promoter primer. cRNA was then generated using T7 RNA polymerase, and labeled with cyanine 3 (Cy3)-CTP (Perking-Elmer/NEN Life Sciences, Boston, MA). For the purification of labeled cRNA samples, unincorporated dye-labeled nucleotides were removed using an RNeasy Mini kit (Qiagen). The quality of each labeled cRNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies). Each array was hybridized in hybridization buffer containing fragmented Cy3-labeled cRNA for 17 h at 60˚C with constant rotation (5 rpm). After hybridization, the slides were washed and scanned using an Agilent Microarray Scanner (Agilent Technologies). Fluorescence intensities on scanned images were quantified, and the values were corrected for the background level and normalized using Agilent Feature Extraction software (Agilent Technologies). The complete dataset of the microarray used in this study has been deposited in the database of the National Center for Biotechnology Information's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE2726 [NCBI GEO]).

Quantitative analysis of gene expression. Gene expression was analyzed by performing quantitative real-time PCR using iCycler iQ Optical System Software version 3.0A (Bio-Rad, Hercules, CA). The cDNA was reverse-transcribed from 1.0 μg of RNA from each of 5 animals per group, using a first-strand cDNA Synthesis kit (Invitrogen, Grand Island, NY). Primers for the quantitative real-time PCR analysis were designed using Primer3 software available at http://www.primer3.com for genes differentially expressed in microarray analyses (Table I). The PCR for each primer set was performed in triplicate using iQ SYBR Green Supermix (Bio-Rad). The expression of each target cDNA, relative to rat ß-actin, was calculated using the comparative threshold cycle (CT) method and the Q-gene software (17). In addition, *cytokeratin 8* (Ck8) expression was compared among groups to compensate for differences in the number of epithelial cells in the samples.

Histology, immunohistochemistry and whole-mount analyses. For histological and immunohistochemical analyses, the left 2nd and 3rd thoracic mammary glands were removed, fixed in 10% neutral-buffered formalin, sectioned, then either stained with hematoxylin and eosin or used for immunohistochemistry. For whole mounts, the right 2nd and 3rd thoracic mammary glands were dehydrated in graded alcohol, defatted in methyl salicylate, stained with hematoxylin and

Table II. Differences in gene expression between parous and AMV mammary glands.

Genes	Gene symbol	Function	Fold change	GenBank accession no.
Up-regulated				
Whey acidic protein	Wap	Milk protein	5.3	NM_053751
Casein beta	Csn2	Milk protein	7.7	NM_017120
Casein gamma	Csng	Milk protein	16.3	XM_341197
Lipopolysaccharide binding protein	Lbp	Antibacterial/milk protein	2.2	NM_017208
Ig germline alpha H-chain C-region gene		Immune response	5.3	M13801
Secreted phosphoprotein 1	Spp1	Immune response	2.4	NM_012881
Matrix metalloproteinase 12	Mmpl2	Macrophage elastase activity	2.6	NM_053963
Granzyme A	Gzma	Granzyme A activity	2.3	NM_153468
Glycosylation-dependent cell adhesion molecule 1	Glycam1	Cell adhesion	5.3	NM_012794
Glycoproiein (transmembrane) nmb	Gpnmb	Cell adhesion	2.4	NM_133298
Neuropilin 2	Nrp2	Cell adhesion	2.1	NM_030869
Aquaporin 2	Aqp2	Plasma membrane	2.5	NM_012909
Leukotriene C4 synthase	Ltc4s	Membrane fraction	2.0	NM_053639
Complement component/actor h	Cfh	Lipid transporter activity	2.0	NM_130409
Carbonic anhydrase 2	Ca2	Hydratase	2.3	NM_019291
Down-regulated				
Amphiregulin	Areg	Cell proliferation	2.2	NM_017123
Regenerating islet-derived 3 alpha	Reg3a	Cell proliferation	2.9	NM_172077
Mesothelin	Ms1n	Cell adhesion	2.2	NM_031658
Latent transforming growth factor beta binding protein 2	Ltbp2	Cell adhesion	2.0	NM_021586
Fibronectin 1	Fnl	Cell adhesion	2.2	NM_019143
Myosin, light polypeptide 2	Myl2	Cytoskeleton	2.1	NM_012605
Peptidyl arginine deiminase, type II	Pdi2	Protein modification	2.3	NM_017226

mounted in balsam. The proliferative status of mammary epithelial cells in parous and AMV rats before and after MNU treatment was quantified using the proliferating cell nuclear antigen (PCNA) labeling index. PCNA immunohistochemistry was performed using the labeled streptavidin-biotin (LSAB) method with an antibody against PCNA (PC10; Novocastra, Newcastle upon Tyne, UK), using an LSAB Staining kit (Dako Cytomation, Carpinteria, CA) according to the manufacturer's instructions. The PCNA labeling index was determined as described previously (5).

Statistical analysis. Data were analyzed using the Student's t-test. Differences were considered significant at a p-value <0.05.

Results

Morphological analyses of mammary glands. Morphological changes in parous and AMV mammary glands before and after MNU treatment were confirmed by whole-mount analyses (Fig. 2). In parous mammary glands after 28 days of postlactational involution, we observed highly branched epithelial trees with small alveoli (Fig. 2a), compared with AMVs (Fig. 2b). At 21 days after MNU treatment (after 49 days of postlactational involution), parous mammary glands continued to exhibit highly branched epithelial structures, compared with AMV mammary glands (Fig. 2c and d, respectively). After MNU treatment, parous mammary glands exhibited a degree of ductal branching that was similar to the branching observed in parous glands before MNU treatment (Fig. 2a and c). Histologically, after 28 days of postlactational involution, epithelial structures of parous mammary glands consisted mainly of non-secretory atrophic cells (Fig. 3a), and similar cell features were seen after 49 days of postlactational involution (Fig. 3c). Before and after MNU treatment (Fig. 3b and d), AMV mammary glands exhibited a lesser degree of alveolar structural development than parous mammary glands (Fig. 3a and c).

Comparison of gene expression before MNU treatment between parous and AMV mammary glands by microarray analyses. A total of 57 genes, including EST genes and unknown genes, exhibited a 2-fold difference in expression between parous and AMV mammary glands with 44 up-regulated and 13 down-regulated genes in parous mammary glands, compared with AMV mammary glands (data not shown). A summary of 22 known genes is shown in Table II. Several of these genes encode mammary differentiation markers [such as *whey acidic protein* (Wap), *casein beta* (Csn2) and *casein gamma* (Csng)] that were up-regulated in

Table III. Differences in gene expression between parous and AMV mammary glands after MNU treatment.

Genes	Gene symbol	Function	Fold change	GenBank accession no.
Up-regulated				
Casein beta	Csn2	Milk protein	3.8	NM_017120
Casein gamma	Csng	Milk protein	3.3	XM_341197
Lipopolysaccharide binding protein	Lbp	Antibacterial/milk protein	2.1	NM_017208
Ig germline alpha H-chain C-region gene		Immune response	3.6	M13801
Glycosylation-dependent cell adhesion molecule 1	Glycam1	Cell adhesion	3.0	NM_012794
Corticotrophin releasing hormone receptor 2	Crhr2	Plasma membrane	2.3	NM_022714
Angiotensin 11 type-1 receptor	Agtrl	Plasma membrane	2.4	NM_031009
Putative G protein-coupled receptor snGPCR32	Edg7	Plasma membrane	2.0	NM_023969
D site albumin promoter binding protein	Dbp	DNA binding	2.8	NM_012543
Down-regulated				
Amphiregulin	Areg	Cell proliferation	2.0	NM_017123
Regenerating islet-derived 3 alpha	Reg3a	Cell proliferation	2.1	NM_172077
Mesothelin	Ms1n	Cell adhesion	2.2	NM_031658
Cell division cycle 2 homolog A	Cdc2a	Cell cycle	2.4	NM_019296
Insulin-like growth factor 2	Igf2	Growth factor	2.2	NM_031511
Insulin-like growth factor binding protein 4	Igfbp4	Growth factor binding	2.0	XM_340897
Stathmin 1	Stmnl	Cell proliferation	2.1	NM_017166
Homeobox, msb-like 1	Msxl	Development	2.1	NM_031059
Nuclear factor, interleukin 3, regulated	Nfil3	DNA binding	2.1	NM_053727
Vascular cell adhesion molecule 1	Vcami	Cell adhesion	2.4	NM_012889
Peptidyl arginine deiminase, type II	Pdi2	Protein modification	2.4	NM_017226
Stearoyl coenzyme A desaturase 2	Scd2	Fatty acid biosynthesis	2.3	NM_031841
Fatty acid elongase 2	rELO2	Fatty acid biosynthesis	2.1	NM_134383
Calcium channel, voltage-dependent, T-type, alpha IG subunit	Cacnalg	Ion channel activity	2.0	NM_031601

parous mammary glands. In addition, *lipopolysaccharide binding protein* (Lbp) (18), *secreted phosphoprotein 1* (Spp1) (19) and *glycosylation-dependent cell adhesion molecule 1* (Glycam1) (20), which are reportedly involved in mammary gland differentiation, were found to be up-regulated in parous mammary glands. In contrast, genes involved in cell growth, such as *amphiregulin* (Areg) and *regenerating islet-derived 3 alpha* (Reg3a), were found to be down-regulated in parous mammary glands. We also found that *mesothelin* (Msln), which has been shown to be involved in mammary epithelial cell proliferation (21), was down-regulated in parous mammary glands.

Comparison of gene expression after MNU treatment between parous and AMV mammary glands by microarray analyses. A total of 71 genes that included EST and unknown genes exhibited a 2-fold difference in expression with 42 up-regulated and 29 down-regulated genes in parous mammary glands, compared with AMV mammary glands (data not shown). A summary of 23 known genes is shown in Table III. Among those 23 genes, up-regulation of differentiation-related genes (Csn2, Csng, Lbp and Glycam1) and down-regulation of growth-related genes (Areg, Reg3a and Msln) were observed in parous mammary glands after MNU treatment. In addition to these alterations in gene expression, we observed down-regulation of growth-related genes, such as *cell division cycle control 2 homolog A* (Cdc2a), *insulin-like growth factor 2* (Igf2), *insulin-like growth factor binding protein 4* (Igfbp4), *stathmin 1* (Stmn1), *homeobox*, and *msh-like 1* (Msx1) in parous mammary glands after MNU treatment.

Differences in gene expression in parous and AMV mammary glands in response to MNU treatment by microarray analyses. Changes in gene expression in 157-day-old parous and AMV mammary glands (obtained 21 days after MNU treatment) were compared to those of 136-day-old (MNU-untreated) parous and AMV mammary glands. In parous mammary glands, 34 genes, including EST genes and unknown genes, exhibited a 2-fold difference in expression with 21 up-regulated and 13 down-regulated genes after MNU treatment (data not shown). A summary of 5 known genes is shown in Table IV. Of those 5 genes, *zinc finger protein 4* (Azf4) and *angiotensin II type-1 receptor* (Agtr1) were up-regulated, and *claudin-7* (Cldn7), *alpha-3 type IV collagen* (COL4A3) and Spp1 were down-regulated. In AMV mammary glands, 55 genes, including EST and unknown genes, exhibited a 2-fold

Table IV. Changes in gene expression in parous mammary glands in response to MNU treatment.

Genes	Gene symbol	Function	Fold change	GenBank accession no.
Up-regulated				
Zinc finger protein 4	Azf4	Transcription factor	2.5	U78115.1
Angiotensin II type 1 receptor	Agtrl	Plasma membrane	2.6	NM_031009
Down-regulated				
Claudin-7	Cldn7	Cell adhesion	2.8	BM386659
Alpha-3 type IV collagen	COL4A3	Cell adhesion	2.5	L47281.2
Secreted phosphoprotein 1 (*osteopontin*)	Spp1	Immune response	2.1	NM_012881

Table V. Changes in gene expression in virgin mammary glands in response to MNU treatment.

Genes	Gene symbol	Function	Fold change	GenBank accession no.
Up-regulated				
Cell division cycle 2 homolog A	Cdc2a	Cell cycle	2.4	NM_019296
Stathmin 1	Stmn1	Cell proliferation	2.3	NM_017166
High mobility group box 2	Hmgb2	DNA binding	2.3	NM_017187
Pyruvate carboxylase	Pc	Pyruvate carboxylase activity	3.1	NM_012744
Malic enzyme I, soluble	Mel	Malate dehydrogenase activity	2.7	NM_012600
Transketolase	Tkt	Transketolase activity	2.6	BI282629
ATP citrate lyase	Acly	ATP citrate synthase activity	2.2	NM_016987
Carbonyl reductase I	Cbr1	NADPH activity	2.2	NM_019170
Fatty acid elongase 2	rELO2	Fatty acid biosynthesis	3.7	NM_134383
Fatty acid synthase	Fasn	Fatty acid metabolism	3.2	NM_017332
Thyroid hormone responsive protein	Thrsp	Lipid metabolism	3.6	NM_012703
Pyruvate dehydrogenase kinase 1	Pdkl	Protein kinase activity	2.0	NM_053826
Diazepam binding inhibitor	Dbi	Acyl-CoA binding	2.1	NM_031853
Retinal-binding protein	Rbp	Retinoid binding	2.5	AA858962
Brain protein 44-like	Brp441	Unknown	2.6	NM_133561
Down-regulated				
Connective tissue growth factor	Ctgf	Plasma membrane	2.4	NM_022266
Nectin-1		Cell adhesion	2.1	AF091111.1
D site albumin promoter binding protein	Dbp	DNA binding	2.1	NM_012543
Neuronatin	Nnat	Development	2.4	NM_053601
Sodium channel,type 10, alpha polypeptide	Scn10a	Sodium ion transport	2.1	NM_017247

difference in expression with 35 up-regulated and 20 down-regulated genes after MNU treatment (data not shown). A summary of 20 known genes is shown in Table V. Among those 20 genes, growth-related genes such as Cdc2a and Stmn1 were up-regulated. In addition, we observed the up-regulation of genes involved in energy metabolism and fatty acid metabolism, such as *pyruvate carboxylase* (Pc), *malic enzyme 1* (Me1), *transketorase* (Tkt), *ATP citrate lyase* (Acyl), *carboxyl reductase 1* (Cbr1), *fatty acid elongase 2* (rELO2), *fatty acid synthase* (Fasn) and *thyroid hormone responsive protein* (Thrsp).

Confirmation of differences in expression of differentiation- and growth-related genes in parous and AMV mammary glands by real-time PCR analysis. The results of oligonucleotide microarray analyses showed the up-regulation of differentiation-related genes and down-regulation of growth-related genes in parous mammary glands before and after MNU treatment, compared to AMV mammary glands. We next used quantitative real-time PCR analysis to further analyze the expression levels of differentiation-related genes (Wap, Csn2, Csng, Lbp, Spp1 and Glycam1) and growth-related genes (Areg, Reg3a, Msln, Cdc2a, Igf2, Igfbp4, Stmn1 and

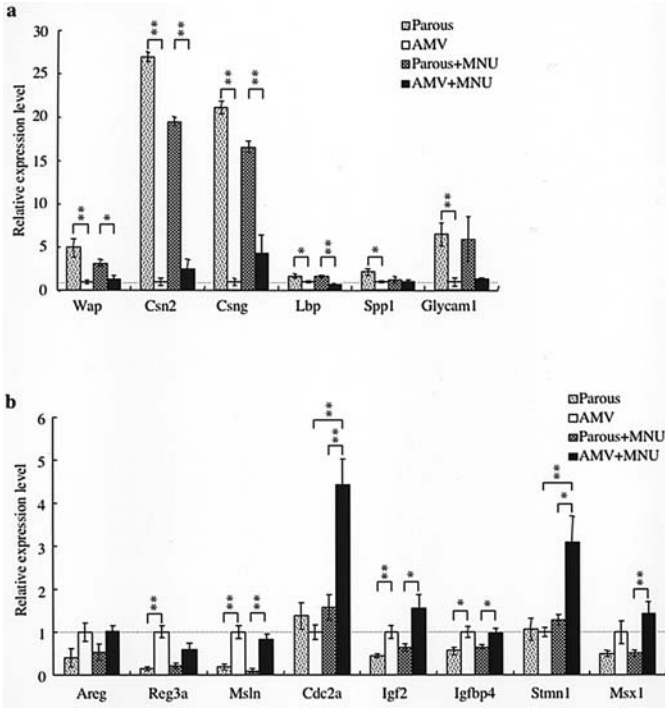

Figure 4. Analyses of gene expression in parous and AMV mammary glands before and after MNU treatment. (A) Differentiation-related and (B) growth-related genes identified by microarray analyses were further analyzed by quantitative real-time PCR. For each target gene, the expression was normalized to ß-actin, and the expression level is shown relative to the value for AMV (designated as 1) for comparison. Data represent the means ± SE of triplicate PCR assays for each of 5 mammary glands per group. $^{**}p<0.01$ and $^{*}p<0.05$.

Msx1) using each of the 5 mammary gland samples obtained from each of the 4 experimental groups. Results obtained for differentiation- and growth-related genes are shown in Fig. 4a and b, respectively. Regardless of MNU treatment, parous mammary glands exhibited persistent up-regulation of Wap, Csn2, Csng, Lbp and Glycam1. In contrast, Spp1 expression in parous mammary glands was only transiently up-regulated, and declined until it reached the same level as that of AMV mammary glands after MNU treatment (Fig. 4a). Before MNU treatment, parous mammary glands exhibited significant down-regulation of growth-related genes such as Reg3a, Msln, Igf2 and Igfbp4, compared with AMV mammary glands. After MNU treatment, AMV mammary glands showed significant up-regulation of growth-related genes such as Msln, Cdc2a, Igf2, Igfbp4, Stmn1 and Msx1 compared with parous mammary glands. In addition, we observed marked up-regulation of Cdc2a and Stmn1 in AMV mammary glands in response to MNU, whereas the expression of these genes in parous mammary glands remained at a steady level (Fig. 4b). To determine whether there were differences in the number of epithelial cells between groups, we compared levels of Ck8 expression. There were no significant differences in the Ck8 expression levels between the groups (data not shown), indicating that the changes we observed in differentiation- and growth-related gene expression were not due to differences in the number of epithelial cells.

Cell proliferation in parous and AMV mammary glands before and after MNU treatment. Before MNU treatment,

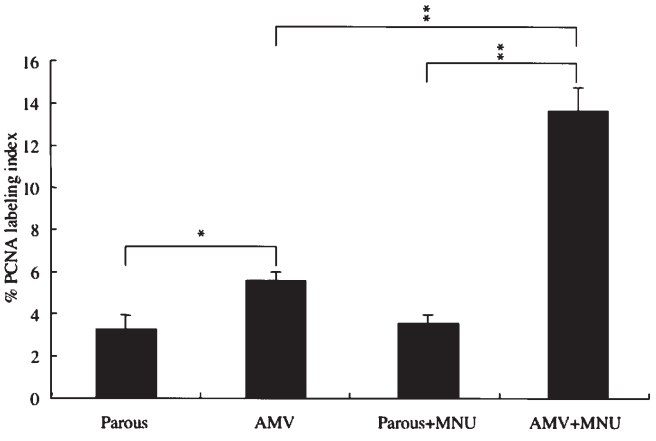

Figure 5. Analysis of cell proliferation in mammary epithelial cells of AMV and parous rats before and after MNU treatment. $^{**}p<0.01$ and $^{*}p<0.05$. Data represent the means ± SE of 5 mammary glands per group.

PCNA-positive cells comprised 3.3±0.6% and 5.6±0.4% of cells in parous and AMV mammary glands, respectively (Fig. 5). In contrast, after MNU treatment, a significant increase in PCNA labeling was observed in AMV mammary glands (13.7±1.1%), whereas PCNA labeling remained at a low level in parous mammary glands (3.6±0.4%).

Discussion

The preventive effects of early full-term pregnancy against breast cancer are well documented in humans and rodent models of chemically induced mammary carcinogenesis (2-6). However, the cellular and molecular mechanisms of parity-induced protection are still largely unknown. Using oligonucleotide array analyses, we identified known genes that were differentially expressed in parous and AMV mammary glands before (22 genes) and after (23 genes) MNU treatment. Researchers have speculated that parity-induced protection against mammary cancer may be due to structural changes in the mammary glandular tree caused by pregnancy (12). In the present study, whole-mount analyses showed that parous mammary glands exhibited highly branched epithelial trees possessing alveoli, and were more differentiated than AMV mammary glands (Fig. 2). Histological analyses revealed that parous mammary glands consisted mainly of non-secretory atrophic cells (Fig. 3) (4,5). These structural changes in parous mammary glands were accompanied by the up-regulation of genes encoding mammary epithelial differentiation markers such as Wap, Csn2 and Csng, indicating that parous mammary glands consist of mammary cells that are more differentiated than those of AMV mammary glands. Moreover, we also found that differentiation-related genes such as Lbp (18), Spp1 (19) and Glycam1 (20) were up-regulated in parous mammary glands. The highly branched structure of ductal trees observed in parous mammary glands after 28 days postlactational involution was still present 21 days after MNU treatment (after 49 days of postlactational involution) (Fig. 2). Consistent with this observation, parous mammary glands exhibited persistent up-regulation of differentiation-related genes such as Wap, Csn2, Csng, Lbp and Glycam1, but not Spp1, after MNU treatment (Fig. 4a).

Parity induces increased expression of differentiation-related genes such as Wap, Csn2, Csng, Lbp and Spp1 (also known as *osteopontin* or *early T-cell activating protein*) (16). In addition to pregnancy, short-term treatment with E+P has protective effects against mammary cancer (7,8), and it has been reported that casein genes are up-regulated in response to E+P treatment (15). Induction of differentiation of mammary glands as a consequence of pregnancy appears to be important for protection against mammary cancer. However, induction of mammary gland differentiation by the potent differentiating agent perphenazine does not confer protection against mammary carcinogenesis (22). Structural changes in mammary glands may not be the only major factor in parity-induced protection; it is possible that up-regulation of differentiation-related genes and alteration of their downstream pathways affects refractoriness to mammary carcinogenesis. In addition, parity-induced up-regulation of genes specifically expressed in hematopoietic cells has been reported (16). Consistent with this finding, our microarray data showed up-regulation of Lbp, *Ig germline alpha H-chain C-region gene*, Spp1 and *matrix metalloproteinase 12* (Mmp12), which may influence the immune system in parous mammary glands.

Parous mammary glands tend to down-regulate growth-related genes such as Areg, Reg3a and Msln, in contrast to their up-regulation of differentiation-related genes. Areg belongs to the epidermal growth factor family, and plays an important role in promoting mammary epithelial cell proliferation and development of mammary ducts (23,24). In addition, Areg overexpression in primary human breast cancers has been reported (25). Consistent with the present findings, a previous study has shown persistent up-regulation of Areg in parous mammary glands of rats and mice (16). Although there have been no reports of expression of Reg3a (a member of the Reg family) in mammary glands, Reg3a has been found to be involved in regeneration and growth of pancreatic ß-cells after DNA damage (26). Msln has been shown to be elevated in Wnt-1 transgenic mice whose mammary epithelial cells exhibit transformed phenotype (20). These findings suggest that parity-induced down-regulation of these genes is important for maintenance of the refractory state against mammary carcinogenesis.

After MNU treatment, the expression of Msln, Cdc2a, Igf2, Igfbp4, Stmn1 and Msx1 was down-regulated in parous mammary glands, compared with AMV mammary glands (Fig. 4b). Overexpression of Cdc2 (27), Igf2 (28), Igfbp4 (29) and Stmn1 (30) in human breast cancer has been reported. Cyclin D1 plays a major role in mammary gland development and carcinogenesis (31), and Areg (32), Msln (21,33) and Msx1 (34) are involved in cyclin D1 expression. The regulation of cyclin D1 via these genes in parous mammary glands may be a key factor in the protection against mammary carcinogenesis. Reduced circulating levels of PRL and GH in rats are thought to play pivotal roles in parity-induced protection against mammary carcinogenesis (4). It has been reported that Igf2 is a mediator of PRL-induced mammary gland development (35). Reduced circulating levels of PRL may result in suppression of Igf2 in parous mammary glands after MNU treatment.

The most important findings of the present study were the alterations in gene expression in AMV mammary glands after MNU treatment. Parous and AMV mammary glands exhibited quite different changes in gene expression in response to carcinogen challenge. It is interesting that AMV mammary glands exhibited marked up-regulation of Cdc2a and Stmn1, whereas expression of these genes remained unchanged in parous mammary glands after MNU treatment (Fig. 4b). Stmn 1 product (Stathmin) is an important molecule that plays a key role in cell cycle progression. In Jurkat and HeLa cells, the phosphorylation level of Stathmin peaks during the mitotic phase (36). It is interesting that Stathmin is phosphorylated by Cdc2, a major protein kinase that regulates entry into mitosis (36,37). The marked up-regulation of Stmn1 and Cdc2a we observed in AMV mammary glands after MNU treatment may be related to subsequent mammary tumor development.

PCNA immunohistochemistry revealed that the mammary epithelial cell proliferation rate was significantly higher in AMV mammary glands than in parous mammary glands after MNU treatment (Fig. 5). This increased proliferation rate of mammary epithelial cells may account for the up-regulation of growth-related genes in AMV mammary glands after MNU treatment. In contrast, after MNU treatment, the PCNA labeling index of parous mammary epithelial cells remained low, and the expression of growth-related genes remained unchanged or was suppressed. These results suggest that MNU acts on alveolar and ductal cells in AMV mammary glands, increasing their rate of proliferation. Consistent with the present findings, previous studies have shown that mammary epithelial cells of virgin mammary glands exhibit a higher proliferation rate after carcinogen challenge than mammary epithelial cells of parous or hormone-treated mammary glands (11). p53 is considered to be a potent mediator of parity- and/or hormone-induced protection against mammary carcinogenesis (38). However, no changes in p53 gene expression were observed in the present study.

After carcinogen challenge, parous mammary glands showed greater refractoriness to epithelial cell proliferation, and exhibited suppression of multiple growth-related genes, whereas AMV mammary glands exhibited greater cell proliferation and up-regulation of growth-related genes. It has previously been hypothesized that the hormonal milieu during pregnancy causes persistent changes in gene expression patterns in the mammary gland via epigenetic modifications in a specific population of cells (11,15). Alterations in gene expression patterns in parous mammary glands after MNU treatment appear to be epigenetic changes induced by parity. In the present study, we identified several genes that may be involved in parity-induced protection against breast cancer. Further investigation of the function and transcriptional regulation of these genes is needed to gain a better understanding of the parity-induced protection mechanisms.

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