Induction of a novel histone deacetylase 1/c-Myc/Mnt/Max complex formation is implicated in parity-induced refractoriness to mammary carcinogenesis

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Refractoriness to carcinogen-induced increases in epithelial cell proliferation is a very important characteristic of parous mammary glands. We found that *N*-methyl-*N*-nitrosourea (MNU)-induced proliferative burst in the mammary ductal epithelium was blocked in parous glands but not in age-matched virgin (AMV) glands. The inhibition of the proliferative burst in MNU-treated parous mammary glands coincided with the upregulation of Mnt, a Myc-suppressor, and the formation of histone deacetylase 1/Mnt/Max complexes that unexpectedly contained c-Myc. These complexes formed on the promoters of Myc targets, such as ornithine decarboxylase, cyclin D2, and transforming growth factor β1 genes, in quiescent fibroblasts, and were disassembled in serum-stimulated cells. These results suggest that the complexes also function as transcription repressors of the growth-related Myc targets in MNU-treated parous mammary glands. Using the chemical mammary carcinogenesis model of human *c-Ha-ras* transgenic (Tg) rats, we confirmed that parity protected the mammary glands at the postinitiation phase of tumorigenesis. Although the incidence of 7,12-dimethylbenz[α]anthracene-induced palpable tumors was reduced from 61.5% in the AMV Tg rats to 28.5% in the parous animals, the incidence of early neoplastic lesions in the parous rats was the same as that in the AMV rats. Restriction fragment length polymorphism analysis detected mutations in the human *c-Ha-ras* gene in most of the normal-appearing parous Tg glands, as well as in the virgin glands. We propose that accelerated formation of HDAC1/c-Myc/Mnt/Max complexes in response to carcinogen exposure results in down-regulation of growth-related genes, leading to the refractoriness of parous mammary glands at the postinitiation phase of carcinogenesis. (*Cancer Sci* 2008; 99: 309–315)

Strong epidemiological evidence indicates that women who experience a full-term pregnancy early in their lives have a significantly reduced risk of developing breast cancer.[1,2] In both rat and mouse models, full-term pregnancy confers resistance to chemical carcinogen-induced mammary tumorigenesis[3,4] and this effect can be mimicked by treatment with estrogen,[5] estrogen and progesterone,[4] or human chorionic gonadotropin.[6] However, the actual mechanism involved in parity protection or hormone-induced protection against breast cancer has not yet been clearly defined. The most widely accepted explanation for pregnancy protection against mammary cancer is that the pregnancy-induced differentiation of terminal end buds and terminal ducts reduces the number of target cells for carcinogenesis.[7] It has also been reported that parous rats have decreased levels of mammogenic hormones, and this decrease has been attributed to the decreased incidence of mammary cancers in these animals.[8,9] However, complete differentiation of the mammary gland does not appear to be an obligatory prerequisite for protection against mammary carcinogenesis.[4,10,11] We have shown that pregnancy following carcinogen exposure as well as pregnancy prior to carcinogen exposure reduces mammary carcinogenesis in rats.[12] This result indicates that pregnancy has the potential to affect the carcinogen-initiated cells that are static at the promotion phase, thus providing further support to the conclusion mentioned above.

The proto-oncogene *myc* has long been known to stimulate cellular proliferation. Aberrant expression of Myc in the mammary glands of transgenic mice results in the development of invasive mammary adenocarcinomas.[13,14] Myc proteins are basic helix–loop–helix leucine-zipper (bHLH-Zip) transcription factors whose function relies on heterodimerization with Max through their related bHLH-Zip domain.[15] The Myc–Max heterodimer binds to E-box DNA sequences (CAYGTG) and can activate transcription through a tethered complex of proteins that contains histone acetyltransferase and other activities that remodel chromatin.[16] Max also heterodimerizes with several other proteins that contain Myc-like bHLH-Zip domains, including the Mad family proteins Mxd 1–4, the Mad-related protein Mnt, and Mga.[17,18] These are all transcription repressors that can block Myc-dependent cell transformation.[19,20] The transcription repression activity of Mnt and Mxd 1–4 is dependent on the interaction with Sin 3A and Sin 3B co-repressors.[16] Sin 3A and Sin 3B interact with a number of different proteins, including histone deacetylase (HDAC)1 and HDAC2.[21,22] A current working model proposes that E-boxes are occupied by Mnt–Max in quiescent cells, whereas Mnt–Max complexes are displaced by Myc–Max complexes in growth-stimulated cells.[23,24] Furthermore, Mnt deletion provokes many responses triggered by Myc, even in cells lacking *c-myc*.[18,25] Therefore, Mnt has been proposed to be a tumor suppressor behaving as a master regulator of the Max network.[26,27] Strikingly, conditional inactivation of Mnt in mammary epithelium leads to elevation of cell proliferation and development of adenocarcinomas.[25,28]

In the present study, we found a close correlation between accelerated formation of novel HDAC1/c–Myc/Mnt/Max complexes and suppression of the proliferative burst in *N*-methyl-*N*-nitrosourea (MNU)-treated parous mammary glands. These quaternary complexes were assembled on the promoters of Myc-target genes in quiescent fibroblasts and were disassembled after proliferative stimulation of the cells. In addition, parity effectively protected the mammary glands from carcinogenesis mainly at the postinitiation phase.

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Materials and Methods

Animals and treatment. Nine-week-old female Lewis rats (Charles River Laboratories Japan, Kanagawa, Japan) were divided into two groups. Animals in the first group ($n = 25$) were mated and allowed to complete their pregnancy and nurse their litters (at least eight pups for each mother) for 21 days; the second group consisted of age-matched virgin (AMV) rats ($n = 25$). Each group was maintained for 4 additional weeks prior to receiving an i.p. injection of 50 mg/kg body weight of MNU (Wako Pure Chemical, Osaka, Japan) or vehicle only (1 mM HCl) at 19 weeks of age. Five animals in each group were killed before the injection and at 1, 3, 7, and 35 days after the injection. One side of the abdominal–inguinal mammary glands was removed, quick-frozen in liquid nitrogen, homogenized, and kept at –80°C until use. The other side of the glands was fixed overnight in 10% neutral-buffered formalin and embedded in paraffin.

In a separate experiment, female transgenic (Tg) rats with Sprague–Dawley background were used to assess the protective effect of pregnancy on mammary carcinogenesis. Briefly, the animals were separated into the AMV and parous groups as described above. The latter group of rats was mated at 7 weeks of age and allowed to complete their pregnancy and lactation. Each group then received an intragastric intubation of 50 mg/kg body weight of dimethylbenz(a)anthracene (DMBA; Tokyo Chemical Industries, Osaka, Japan) or the vehicle only (corn oil) at 15 weeks of age. The animals were maintained for 20 weeks then killed. DMBA instead of MNU was used as the mammary carcinogen as MNU causes malignancies of multiple organs in carcinogen-sensitive Tg rats, resulting in unexpected deaths of the animals during experiments. The tumor incidence was assessed by palpation during the period prior to euthanasia. 'Early neoplastic lesions' included atypical ductal hyperplasia and small adenocarcinomas identified by microscopic examination of the abdominal–inguinal mammary glands that did not have palpable tumors. All animal experiments were conducted according to the Guidelines for Animal Experimentation of Kansai Medical University (Osaka, Japan).

Quantitation of cell proliferation in mammary ductal epithelium. To analyze cell proliferation, the rats were injected, i.p., with 100 mg/kg body weight of bromodeoxyuridine (BrdU; Wako Pure Chemical) 1 h before they were killed. Tissue sections were deparaffinized and rehydrated according to standard protocols, incubated in 1 N HCl for 20 min, and in 0.01% actinase E (Kaken Pharmaceutical Co., Tokyo, Japan) in phosphate-buffered saline (PBS) for 5 min at 37°C, then blocked with 1% skim milk in PBS for 1 h. Mouse monoclonal anti-BrdU antibody (1:200 dilution) (Becton Dickinson, Franklin Lakes, NJ) was applied overnight at 4°C, and the immunocomplexes were visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Labeling indices were calculated for >300 ductal epithelial cells.

TdT-mediated dUTP-biotin nick-end labeling. Apoptotic cells in the mammary ducts were detected by using a TdT-mediated dUTP-biotin nick-end labeling-based detection kit according to the manufacturer's protocol (Chemicon International, Temecula, CA). At least three microscopic fields viewed with a 40× objective were counted in each rat, representing a total of >1000 cells.

Cell culture. 3Y1 rat fibroblasts were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. For serum starvation, 3Y1 cells were grown to subconfluency then rendered quiescent by incubation in Dulbecco's modified Eagle's medium containing 0.1% serum. In some experiments, after serum starvation for 3 days, the cells were incubated with 10% serum for 3 h.

Reverse transcription–polymerase chain reaction (RT-PCR). First-strand cDNA synthesis from total RNA and PCR amplifications using the following primers were carried out as described elsewhere:[29,30] *ODC* forward, 5′-ATGGGCAGCTTTACTAAGGAAGAG-3′, reverse, 5′-CTGAGCCGACAAACTGCTTTGGAAT-3′; *Ccnd2* forward, 5′-CCGCAACCTGCTGGAAGACC-3′, reverse, 5′-TCACAGGT-CAACATCCCGCAC-3′; *Tgfb1* forward, 5′-AGACCATCGA CATG-GAGCTG GTGAA-3′, reverse, 5′-CAAAAGACAG CCACTCAGGC GTATC-3′; *gapdh* forward, 5′-TTCAACGGCACAGTCAAGG-3′, reverse, 5′-CATGGACTGTGGTCATGAG-3′. The expression of *ODC* was expressed as the value of *ODC*/*gapdh* assessed by densitometric scans of agarose gels.

Immunoprecipitation. Extracts from the homogenized mammary glands (25 mg) and 3Y1 cells were prepared in immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% IGEPAL-CA630, 1 mM dithiothreitol, 1 mM Na_3VO4, 1 μM okadaic acid, 1 μM phenylmethylsulphonyl fluoride, and 10 μg/mL leupeptin). In the case of mammary glands, five individual lysates at each time point were combined and subjected to the following procedure. For preclearing, 1 mL of the extract was mixed with 50 μL of protein G magnetic bead suspension (New England Biolabs, Beverly, MA), rotated for 1 h at 4°C, and applied to a magnetic field for 5 min at 4°C. Extracts were then incubated with the indicated antibodies for 2 h at 4°C, and the antigen–antibody complexes were collected with the protein G beads for 1 h at 4°C. Protein complexes were washed twice with the low-salt chromatin immunoprecipitation (ChIP) wash buffer as described below and washed once with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Then proteins were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gels.

Immunoblot. Western blot analysis was carried out as described previously.[31] The following antibodies were used: goat polyclonal anti-ODC antibody (sc-21515; 0.8 μg/mL); rabbit polyclonal anti-HDAC1 (sc-7872; 0.8 μg/mL); antic-Myc (sc-764; 0.8 μg/mL); anti-Mnt (sc-769; 0.8 μg/mL); anti-Mad1 (sc-222; 0.8 μg/mL); and anti-Max (sc-765; 0.8 μg/mL) antibodies, all from Santa Cruz Biotechnology (Santa Cruz, CA); and mouse monoclonal antiβ-actin antibody (1:500 dilution; Sigma, St. Louis, MO). The primary antibodies were detected using horseradish peroxidase-conjugated goat antirabbit (Cell Signaling Technology, Beverly, MA) and rabbit antigoat immunoglobulin (Ig)G antibodies (Invitrogen, Carlsbad, CA) and enhanced chemiluminescence plus Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

Restriction fragment length polymorphism (RFLP) analyses. Three to five regions of ducts or neoplastic lesions from abdominal mammary glands were carefully scraped out of paraffin sections with scalpels, and DNA was extracted using Takara DEXPAT (Takara Biomedicals, Ohtsu, Japan). RFLP analyses of codon 12 of the human *c-Ha-ras* transgene were carried out as described previously.[32]

ChIP assay. Sub-confluent (~80%) cultures of randomly growing and 3-day serum-starved 3Y1 cells on ϕ10-cm dishes were cross-linked with 1% (w/v) formaldehyde in PBS at room temperature (20°C) for 10 min. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M. The cells were pelleted and lyzed in ChIP lysis buffer (50 mM Tris/HCl, pH 8.1, 10 mM EDTA, and 1% SDS) plus protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin). Lysates were sonicated until the DNA fragments were 600–1000 bp long then diluted 10-fold with immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, and 167 mM NaCl). Samples for total chromatin (input) were collected at this point to use as positive controls in the PCR. For preclearing, 300 mL of the diluted sample per antibody was mixed with 6 mg bovine serum albumin, 0.2 mg normal rabbit IgG, and 20 mL salmon sperm DNA/Protein G–agarose suspension (Upstate Biotechnology, Lake Placid, NY), rotated for 60 min at 4°C, and

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spun at 13 000*g* for 1 min at 4°C. The diluted samples (300 μL) were mixed with 1 μg each of normal rabbit IgG, anti-HDAC1 (sc-7872X), antic-Myc (sc-764X), anti-Mnt (sc-769X), or anti-MAX (sc-765X) antibody purchased from Santa Cruz Biotechnology and rotated overnight at 4°C. The samples were then mixed with 20 μL of salmon sperm DNA/Protein G–agarose suspension (Upstate Biotechnology) and rotated for 60 min at 4°C. Immune complexes were collected by centrifugation, washed with 1 mL each of low-salt wash buffer (20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), high-salt wash buffer (20 mM Tris-HCl, pH 8.1, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), LiCl buffer (10 mM Tris-HCl, pH 8.1, 250 mM LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid), then with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) twice. Immune complexes were eluted twice in 100 μL of elution buffer (100 mM $NaHCO_3$ and 1% SDS) at room temperature for 15 min. DNA–protein cross-links were reversed by incubation with 200 mM NaCl at 65°C for 4 h for all samples, including the total input sample. The DNA was incubated sequentially with 50 μg/mL of RNase A at 37°C for 30 min and 50 μg/mL of proteinase K at 55°C for 1 h then purified with the GenElute Mammalian Genomic DNA Purification Kit (Sigma) according to the manufacturer's instructions. PCR amplification of the intronic E-box-containing region in the rat *ODC* promoter was carried out with the following set of primers: forward, 5′-TGCGGCGGGCTCGACGAGGCGGCTGA-3′; reverse, 5′-TCCCC-TGCCGGCGACCGCAGTC-3′.

Statistics. All statistical evaluations were carried out with multiple comparison by Sheffe's *F*-test in Statcel 2 for Excel (OMS Publishing Inc, Tokorozawa, Japan).

Results

Proliferative burst triggered by MNU exposure was blocked in parous mammary glands. As a differential regulation of cell proliferation and cell death could explain the difference in sensitivity to carcinogenesis for nulliparous and parous mammary glands, we analyzed cell proliferation in each of the glands after MNU challenge (Fig. 1). At 19 weeks of age, before the carcinogen inoculation, the proliferation was low in the AMV (2.4%) as well as parous (2.3%) mammary glands. The proliferation remained low in both AMV and parous glands 3 days after the challenge (2.3% and 1.8% for virgin and parous, respectively). However, 7 days after MNU inoculation, the BrdU labeling index in the parous glands (1.3%) was significantly lower than that in the AMV glands (3.6%, $P < 0.05$). At day 35 after the treatment, the proliferation was lower in both types of glands, but was still relatively higher in the virgin (1.7%) glands than in the parous glands (1.3%) (Fig. 1). There was no significant difference in apoptotic cell deaths between the virgin and parous ductal epithelia (virgin, 1.07 ± 0.46%, parous, 0.93 ± 0.09%) at 7 days after MNU injection.

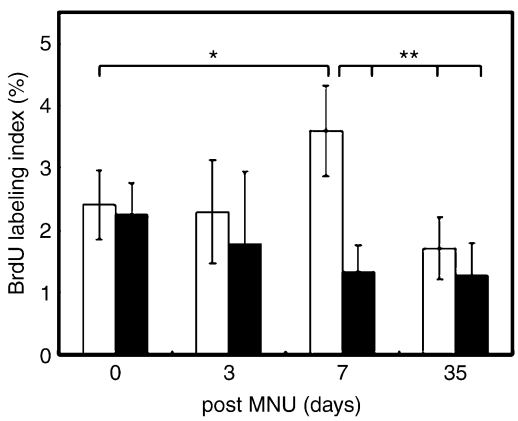
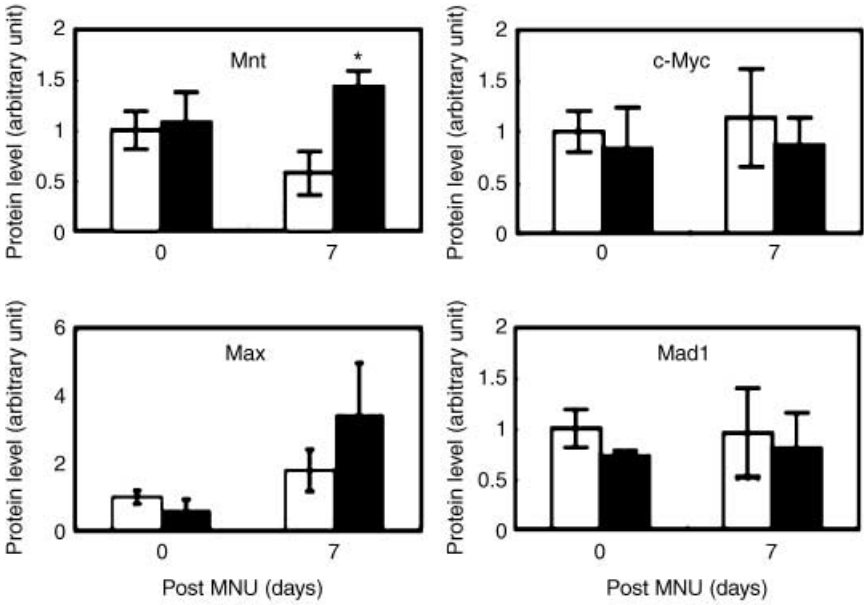


Fig. 1. Comparison of cell proliferation between age-matched virgin (AMV; open columns) and parous (solid columns) rat mammary glands after *N*-methyl-*N*-nitrosourea (MNU) inoculation. Bromodeoxyuridine-labeling indices in the mammary ductal epithelium of AMV and parous rats at 0, 3, 7, and 35 days after MNU injection. Each value represents mean ± SD calculated from five glands at each time point. Differences between groups were tested for statistical significance by using Sheffe's *F*-test. *$P < 0.05$; **$P < 0.01$.

Upregulation of Myc-suppressor Mnt and accelerated formation of novel HDAC1/c-Myc/Mn/Maxt complexes in MNU-treated parous mammary glands. We then examined the mechanism underlying parity-induced suppression of proliferative burst after carcinogen treatment. As an initial approach, the levels of c-Myc, Mnt, Mad1, and Max were compared between virgin and parous mammary glands at 0 and 7 days after MNU injection, because each of these proteins forms functionally different protein complexes with Max to regulate the expression of the Myc-target genes. The most significant difference in the two types of glands was observed for the level of Mnt at 7 days after MNU exposure. Mnt was significantly higher in the parous glands compared to the AMV glands. Actually, Mnt tended to increase in the parous glands, whereas Mnt significantly decreased in the AMV glands at this time point after the exposure (Fig. 2). It is noteworthy that the level of Max at 7 days after the exposure in the parous but not in the AMV glands was significantly ($P < 0.05$) higher than the levels before MNU exposure in the respective glands. There were no significant differences in any of these protein levels in the AMV and parous glands before the treatment, and the levels of c-Myc and Mad1 were stable (Fig. 2).

Immunoprecipitation experiments were carried out to determine if more HDAC1 was recruited into Mnt/Max transcription-repressor complexes in the MNU-treated parous mammary glands because of the increased level of Mnt. Indeed, higher amounts of HDAC1 co-immunoprecipitated with Mnt/Max complexes from the lysate of parous glands as compared to that of virgin glands after MNU treatment (Fig. 3a). Surprisingly, a large amount of c-Myc was associated with the HDAC1/Mnt/Max complexes in the MNU-treated parous glands (Fig. 3a). Other experiments using anti-HDAC1, antic-Myc, and anti-Max antibodies confirmed that c-Myc was trapped in the ternary complexes in the MNU-exposed parous glands (Fig. 3b and data not shown). HDAC1/c-Myc/Mnt/Max complexes were also detected in the virgin and parous glands before MNU exposure. However, c-Myc appeared to be released from the quaternary complexes in the virgin glands after the treatment (Fig. 3). It is also noteworthy that a higher amount of Mad1 was associated with Mnt–Max complexes in the parous glands compared to the virgin glands before MNU challenge (Fig. 3a). Thus, it is possible that the HDAC1/c-Myc/Mnt/Max complexes preferentially form in place of the HDAC1/Mad1/Mnt/Max complexes after the carcinogenic stimuli in the parous glands.

HDAC1/c-Myc/Mnt/Max complexes are potential transcription repressors. We then examined whether the formation of HDAC1/c-Myc/Mnt/Max complexes has a causal relationship to the downregulation of cell proliferation and gene expression. When 3Y1 rat fibroblasts were starved for serum, protein levels of c-Myc and Mnt in the nucleus gradually increased (data not shown), whereas expression levels of the *ODC*, *Ccnd2*, and *Tgfb1* genes, which are established targets of c-Myc, were reciprocally reduced (Fig. 4a). In the quiescent cells, c-Myc was trapped in anti-Mnt immunoprecipitates that also contained HDAC1, and these complexes were disassembled by the growth stimuli (Fig. 4b). ChIP assays clearly indicated the assembly and disassembly of the quaternary complexes on the promoter (presumably on the E-boxes) of *ODC* in the quiescent and proliferating cells, respectively (Fig. 4c). The same results were obtained for the promoters of *Ccnd2* and *Tgfb1* (data not shown). Therefore, we



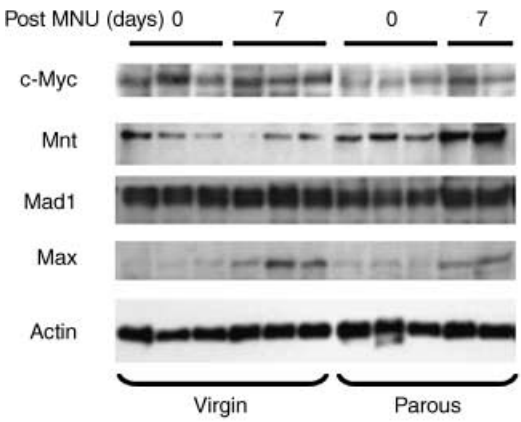


Fig. 2. Immunoblot analyses of levels of the c-Myc regulators in age-matched virgin (AMV; open columns) and parous (solid columns) rat mammary glands after *N*-methyl-*N*-nitrosourea exposure. Changes in the protein levels of the E-box-interacting proteins were monitored with immunoblots at 0 and 7 days after the carcinogen inoculation. Densitometric quantification data ($n = 5$ at each time point) were obtained from three independent blots. The mean value of the AMV glands at day 0 is expressed as 1, and each value represents mean ± SD. $*P < 0.05$ *versus* AMV at the indicated time point. The blots are representative of three independent experiments. Actin was used as a loading control.

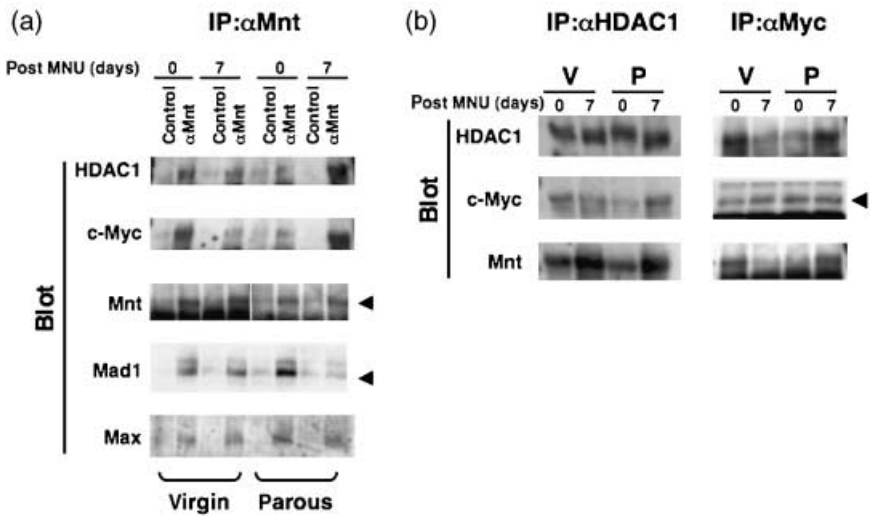


Fig. 3. Induction of HDAC1/c-Myc/Mnt/Max complex formation in *N*-methyl-*N*-nitrosourea (MNU)-treated parous rat mammary glands. (a) Western blot data from immunoprecipitation with anti-Mnt antibody from each combined lysate of age-matched virgin (V) and parous (P) mammary glands ($n = 5$ each) at 0 and 7 days after MNU treatment. Normal rabbit immunoglobulin G was used as the control. (b) Immunoblot data from immunoprecipitations with anti-HDAC1 (αHDAC1) and anti-c-Myc (αMyc) antibodies from the corresponding samples in panel (a). Note the induction of HDAC1/c-Myc/Mnt association in the parous sample after MNU treatment. The immunoprecipitations were carried out simultaneously in three independent experiments. Arrows indicate the corresponding protein bands. IP, immunoprecipitation.

concluded that the formation of novel HDAC1/c-Myc/Mnt/Max quaternary complexes contributes to downregulation of the Myc-target genes and cell proliferation in parous mammary glands after carcinogen exposure.

Parity protects mammary carcinogenesis at the postinitiation phase. The carcinogenesis model of human *c-Ha-ras* Tg rats was used to directly test our hypothesis that there is a major postinitiation effect of parity in protection from mammary tumorigenesis.

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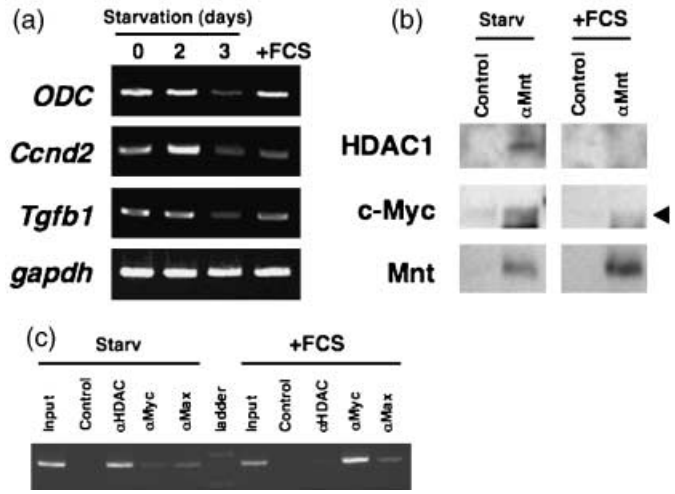

Fig. 4. Assembly and disassembly of HDAC1/c-Myc/Mnt/Max complexes on the promoter of Myc-target gene in fibroblasts. (a) Reverse transcription–polymerase chain reaction analysis of mRNA expression of Myc-target genes during serum starvation. The suppression of target gene expressions was relieved 3 h after restimulation by serum of the quiescent fibroblasts (+FCS). (b) Western blot data from immunoprecipitation with anti-Mnt antibody from the quiescent (Starv) and serum-stimulated (+FCS) fibroblast lysates. Normal rabbit immunoglobulin (Ig)G was used as the control. (c) Chromatin immunoprecipitation assays of the quiescent (Starv) and serum-stimulated (+FCS) fibroblasts were carried out using antibodies against HDAC1, c-Myc, or Max. Normal rabbit IgG was used as the control.

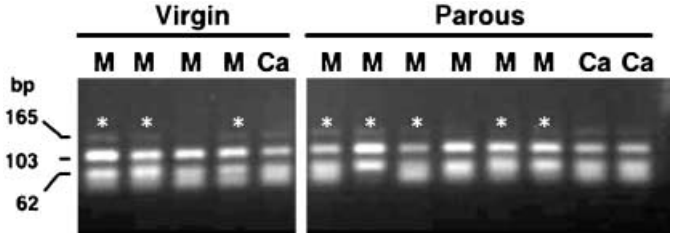

Fig. 5. Mutations in the human *c-Ha-ras* genes in the mammary glands of transgenic (Tg) age-matched virgin (AMV) and parous rats after dimethylbenz(a)anthracene treatment. Representative restriction fragment length polymorphism analysis data for codon 12 of the human *c-Ha-ras* gene. DNA samples were collected from several regions of normal mammary ducts in AMV and parous rats. *Presence of 165 bp bands corresponding to the mutant human *c-Ha-ras*. Ca, DNA from three individual microscopic carcinomas in virgin and parous Tg rats; M, DNA from mammary ductal epithelium with normal morphology.

Mammary tumors became palpable in 1 of 13 AMV Tg rats at 6 weeks after DMBA inoculation, and the tumor incidence gradually increased to 61.5% at 20 weeks. In contrast, the first tumor became palpable in a parous Tg rat at 12 weeks, and only 28.5% (4/14) of the animals developed lesions by 20 weeks (Table 1). Although there was not a significant difference in the mean weight of the mammary tumors in the two groups at the time of autopsy, significantly fewer tumors per animal were observed in the parous rats than in the AMV animals (Table 1). Thus, parity was protective against carcinogen-induced tumorigenesis even in this highly tumorigenic rat strain. However, microscopic surveys of mammary glands that lacked palpable tumors from all rats revealed that the incidence of early neoplastic lesions (atypical hyperplasia and small adenocarcinomas) did not differ between the AMV (53.8%) and parous (64.3%) groups at 20 weeks.

As the mutation rate of the human *c-Ha-ras* transgene is markedly higher than that of the endogenous rat gene, the transgene was used as a probe to monitor ras mutations, thought to occur during the initiation step of carcinogenesis. RFLP analysis detected mutations in codon 12 of the human Ha-ras transgene in most of the normal epithelial samples, regardless of their parous status (Fig. 5). Therefore, the initiation steps occurred with equal frequency in the virgin and parous mammary glands of Tg rats. In conclusion, parity effectively protects the mammary glands from cancer development by a reduction in cell proliferation mediated by the formation of HDAC1/c-Myc/Mnt/Max complexes even at the promotion and/or progression phases of carcinogenesis.

Discussion

An early full-term pregnancy induces a refractory state of the mammary gland against carcinogenesis in both humans[1,2] and rodents.[3,12] Despite extensive efforts to reveal the mechanisms of parity-induced protection against mammary cancer, the cellular and molecular mechanisms are still largely unresolved. The most widely accepted explanation for pregnancy protection against mammary cancer is that the protective effect is attributable to the pregnancy-induced differentiation of the target structures, terminal end buds and terminal ducts.[33] A corollary to this hypothesis is that differences in susceptibility to carcinogen-induced tumorigenesis between parous and nulliparous glands could be explained by the differences in proliferation indices, alterations of the properties associated with carcinogen uptake, binding, and metabolism, and an enhanced capacity for DNA repair.[7] However, studies in which differentiation of the mammary glands was achieved by hormonal stimulation have indicated that the protective effect is independent of the level of differentiation.[4,5,10,11] Furthermore, other studies have shown no consistent differences in cellular kinetics between parous and nulliparous animals.[10]

Estrogen alone or estrogen plus progesterone treatment can induce protection from mammary carcinogenesis in rats and mice.[5,34] Using this model, Sivaraman *et al*.[35] showed that an important molecular alteration that occurs in the hormone-treated gland is the induction, sustained expression, activation, and nuclear sequestration of p53 tumor suppressor protein. This change is persistent and present at the time of carcinogen treatment. Furthermore, the absence of the p53 gene abrogates

Table 1. Incidences and numbers of gross mammary tumors in transgenic rats at 20 weeks after *N*-methyl-*N*-nitrosourea injection

	No. of rats	Atypical hyperplasia		Adenocarcinoma		Total		Weight of tumors/rat (g) (mean ± SD)
		Incidence (%)	No./rat (mean ± SD)	Incidence (%)	No./rat (mean ± SD)	Incidence (%)	No./rat (mean ± SD)	
Virgin	13	1 (7.7)	0.08 ± 0.277	8 (61.5)	1.92 ± 2.99	8 (61.5)	2.00 ± 2.97	5.37 ± 14.04
Parous	14	1 (7.7)	0.07 ± 0.267	4 (28.5)	0.43 ± 0.85*	4 (28.5)	0.50 ± 0.94*	2.20 ± 7.94

*$P < 0.05$, as compared to Virgin.

the protective effect of hormones against carcinogen-induced mammary carcinogenesis in mice.[34] Therefore, the authors developed a cell-fate hypothesis that proposes that at a critical period in adolescence the hormonal milieu of pregnancy affects the developmental fate of a subset of mammary epithelial cells. This hypothesis is attractive and might explain parity-induced protection against mammary cancer at the initiation phase of carcinogenesis when DNA damage is thought to occur.

However, several laboratories have reported that the short-term inoculation of ovarian steroids (estrogens and progesterone) or human chorionic gonadotropins not only before but also after carcinogen treatment decreases the incidence of mammary carcinomas in rodents.[4,11,36] Moreover, we have shown that pregnancy following the carcinogen exposure also reduces mammary carcinogenesis in rats.[12] Human *c-Ha-ras* proto-oncogene Tg rats have an increased susceptibility to chemical carcinogens that target the mammary gland.[37,38] All of the rats developed preneoplastic mammary lesions within 20 days of the injection of MNU[39] and mammary carcinomas appeared within 8 weeks of treatment with a variety of chemical carcinogens.[29,40] Interestingly, activating mutations in the human transgene are readily detectable in preneoplastic lesions that developed after carcinogen treatment or spontaneously.[29] Therefore, mammary carcinogenesis in Tg rats is an excellent model to study multistep development of cancers. In the present study, we found that parity effectively protected the Tg mammary glands as well as the wild-type glands from carcinogenesis preferentially at the postinitiation phase (Table 1 and Fig. 5). Therefore, both pregnancy and the hormones might have potential to protect mammary glands from carcinogenesis at the postinitiation phase.

The most striking finding in the present study was the discovery of HDAC1/c-Myc/Mnt/Max complexes in the MNU-treated parous mammary glands and the quiescent rat fibroblasts. We have also found assembly and disassembly of HDAC1/c-Myc/Mnt/Max complexes in the quiescent and growth-stimulated fibroblasts, respectively (Fig. 4). Thus, based on our findings, c-Myc/Mnt/Max interactions probably are more complex in some types of cells as compared to the current model, in which Mnt–Max and Myc–Max complexes are mutually exclusively formed depending on the growth states of cells.[23,24] Induction of *c-myc* mRNA and protein expression in quiescent fibroblasts and transformed epithelial cells has previously been shown, although the precise role of c-Myc in these cells is still unclear.[41,42] One laboratory has suggested a role for c-Myc 1, the non-AUG-initiated form of the c-Myc protein, in growth inhibition of cells.[43] Interestingly, in the parous mammary glands after MNU treatment, we found the accelerated formation of HDAC1/c-Myc/Mnt/Max complexes as seen in the fibroblasts. DNA damage generated by carcinogens such as MNU and DMBA induces *ODC* expression, replicative DNA synthesis, and cell proliferation in different types of cells including mammary epithelium and fibroblasts under certain conditions ([44,45] and Matsuoka *et al*. this study and unpublished data, 2007). Therefore, it is reasonable to propose that HDAC1/c-Myc/Mnt/Max quaternary complexes function as transcription repressors of growth-related genes such as *ODC* and *Ccnd2* at the promotion phase of preneoplastic epithelial cells in parous mammary glands. Indeed, we have previously shown that the parous glands show signs of inhibition of upregulation of growth-related genes including *ODC*, *Stmn1*, *Cdc2a*, *Igf2*, and *Msln* at 21 days after MNU treatment.[46]

In summary, our results indicate that full-term pregnancy leads to the accelerated formation of HDAC1/c-Myc/Mnt/Max complexes in parous mammary glands when they are exposed to DNA-damaging agents. The quaternary complexes might function as transcription repressors to directly and/or indirectly downregulate growth-related genes; this downregulation leads to refractoriness of the parous glands, probably at the promotion phase of carcinogenesis. The differentiation theory proposed by Russo *et al*.[33] and the cell-fate theory proposed by Medina and Kittrell[34] to explain parity-induced protection mainly apply to the initiation phase of mammary carcinogenesis. Hence, we speculate that the protection provided by parity might operate with different molecular mechanisms at both the initiation and promotion phases. Because it is impossible to determine when mammary epithelial cells are initiated for tumorigenesis in humans, protection at the initiation phase of breast cancer by practical means such as hormone treatments might be incomplete. Rajkumar *et al*.[5] recently showed that short-term exposure to pregnancy levels of estrogen after carcinogen inoculation effectively induces refractoriness to mammary carcinogenesis in rats. Therefore, we believe that protection at the promotion phase is more practical than protection at the initiation phase. We are currently working to identify all constituents of the HDAC1/c-Myc/Mnt/Max complexes in mammary glands as well as in fibroblasts. This information will provide deeper insights into the underlying mechanisms of parity-induced and hormone-induced protection against mammary cancer.

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