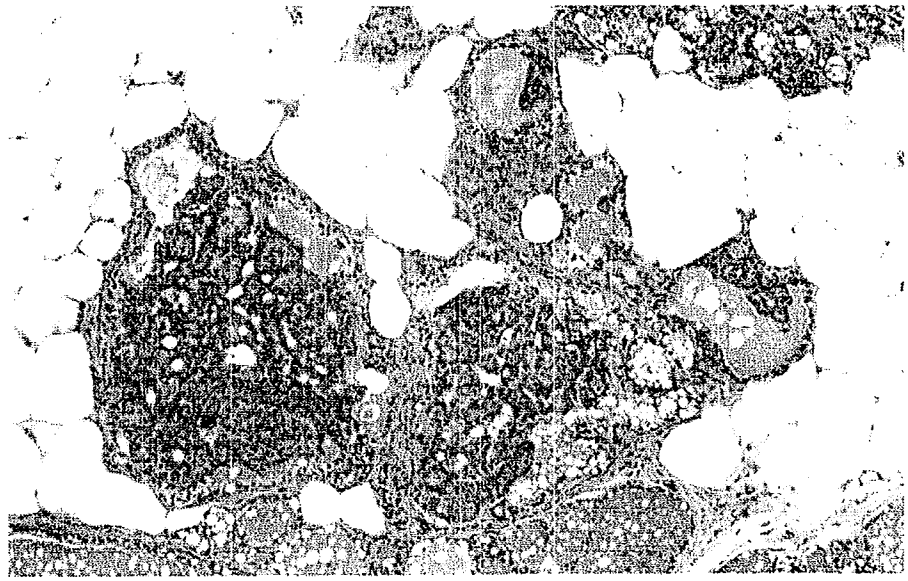

Figure 5. Alveolar hyperplasia with cellular atypia in c-Ha-ras proto-oncogene transgenic rat A marked increase in the number of mammary terminal branches and alveoli in 35-week-old transgenic virgin rats is described in Hamaguchi *et al.* [39]. Occasionally, the epithelial cells of these pre-neoplastic lesions displayed atypia.

Table 2. Genetic alterations in chemically induced rat mammary carcinomas.

	DMBA	NMU	PhIP
Ha-ras	23%[121, 122]	29-80%[41, 121-123]	18-42%[31, 124]
Ki- ras	—	18%[123]	0%[31]
p53	3% [122]	0%[122]	0-10%[31, 125, 126]
brca 1	—	—	0%[127]

detected (Table 2). In contrast, approximately 30% of sporadic human breast cancers have mutations in TP53 (human p53 gene) [58-60], but few human cancers feature Ha-ras or Ki-ras alterations [61, 62]. Amplification and/or overexpression of Ha-, Ki-, and N-ras, cyclin D1, and neu/erbB-2/HER-2 are common in breast malignancies in humans [62, 63]. Two laboratories have demonstrated that overexpression of Ha-ras is sufficient to induce mammary carcinomas in rats [64, Ueda and Tsuda, unpublished data]. Thus, aberrant expression of the ras, cyclin D1, or erbB-2 genes can initiate mammary carcinogenesis in rats as well as in humans.

In both rats and humans there is strong evidence of genetic predisposition. Hereditary breast cancer is characterized by early age at onset (an average of 5 to 15 years earlier than sporadic cases), bilaterality, vertical transmission through both maternal and paternal lines, and familial association with tumors of other organs, particularly the ovary and prostate gland. The search for genes associated with hereditary susceptibility to breast cancer has led to the identification of several susceptibility genes, including BRCA1, BRCA2, TP53, and PTEN/MMAC1. Mutations in the BRCA1 or BRCA2 genes confer a lifetime risk of breast cancer of between 60% and 85% [65, 66]. However, mutations in these genes account for only 2% to 3% of all human breast cancers [67, 68], and susceptibility alleles in TP53 and PTEN/MMAC1 are even less common causes of human breast cancer [69]. The 1100delC mutation

in the cell-cycle-checkpoint kinase gene (CHEK2 or CHK2) has been discovered as an additional gene variant conferring susceptibility to breast cancer [70]. CHEK2 protein is implicated in DNA repair processes involving BRCA1 and p53 [71-73]. While the 1100delC mutation, a truncating variant that abrogates the kinase activity, has a frequency of 1.1% in healthy individuals this variant is present in 5.1% of individuals with breast cancer from 718 families that do not carry mutations in BRCA1 or BRCA2. This mutation doubles the risk of breast cancer among women and increases the risk among men by a factor of 10 [70]. The CHEK2 protein is activated after phosphorylation by the checkpoint gene product ATM and in turn activates BRCA1. The role of ATM mutations in the predisposition to the early onset of breast cancer remains controversial, but some missense mutations appear to increase susceptibility to breast cancer in humans [74] and mice [75]. There is also convincing evidence of the existence of additional high-penetrance genes that increase susceptibility to breast cancer [69].

In human lesions, ductal carcinoma in situ shows multiple losses involving loci on chromosomes 2q, 13q, 16q, 17p, and 17q, which also happen to be sites where important tumor suppressor genes are located [76]. A few studies have been conducted in lobular neoplasia and show losses at 11q, 16q, and 17q in these lesions [47]. Specific genomic alterations have been described in rat mammary carcinomas induced by DMBA, NMU, and PhIP [77, 78]. However, the tumorigenic potential of each of these alterations mostly remains to be tested in rat models.

Risk assessment using the rat carcinogenesis model

Reproductive history is the strongest and most consistent risk factor outside of genetic background and age [79, 80]. Especially, early age of first full-term pregnancy ($\leqq$20 years old) is a strong protective factor. On the other hand, a history of induced abortion appears to have little influence on the breast cancer risk [81, 82]. A collaborative re-analysis of data shows that the relative risk of breast cancer decreases by 4.3% for each year that women breastfeed [83]. Protection by parity from mammary carcinogenesis is also observed in rats [84-87], and short-term exposure to pregnancy levels of estrogen is sufficient to this effect and equally protective for even nulliparous rats [88]. Whereas pregnancy alone has been as effective as pregnancy and lactation in most experiments, Yang *et al.* [87] have reported that pregnancy followed by lactation has an additive effect in protection when rats are exposed to NMU prior to pregnancy. Interrupted pregnancy appears to be protective with lower efficiency compared to full-term pregnancy, although the interruption experiments have yielded contradictory results [86, 89, 90].

The age-adjusted death rates from breast cancer are 2 to 8-fold less in Asian countries than in the United States and Western Europe. The smaller death rate appears to be related to the 20- to 50-times greater consumption of soybean products [91]. A case-control study in Shanghai suggested that regular soy consumption reduced the risk of hormone-receptor-positive tumors [92]. Studies using the rat model as well as carcinoma cell lines have indicated that genistein, one of the isoflavones in soybean, may be responsible for tumor suppressive effects [43, 93-96]. A diet rich in folate and carotenoids might also be protective [97-99]. The possibility of a protective role for folate is somewhat controversial in the rat models since moderate folate deficiency inhibits, whereas dietary folate supplementation does not significantly promote, the progression of NMU-induced mammary neoplastic foci [100, 101]. There are relatively few studies of the effects of carotenoids in the rat models. The majority, but not all, of these studies indicate a protective effect of lycopene-rich tomato carotenoid oleoresin, whereas β-carotene shows no protection against the development of mammary cancer [102-104]. A Canadian case-control study found an association with dioxin-like polychlorinated biphenyls, suggesting that exposure to these substances might increase the risk [105]. In rats, however, inconsistent results have been obtained: 3,3',4,4'-tetrachlorobiphenyl significantly inhibits the tumor growth [106] whereas 2,3,7,8-tetrachlorodibenzo-p-dioxin slightly increases the tumor incidence when neonatal rats are initiated with NMU [107].

Greater consumption of dietary fat (especially n-6 polyunsaturated fatty acids) enhances breast cancer

risk [108, 109]. In the rat model, high levels of dietary fat increase the incidence of chemically-induced mammary carcinomas, and affect the promotion phase but not the initiation phase of the carcinogenesis [5]. Both the quantity and the constituents of fat should be considered as the risk factors. An n-6 polyunsaturated fatty acid, linoleic acid, may be responsible for the promoting activity while n-3 polyunsaturated fatty acids, eicosapentaenoic acid and docosahexaenoic acid, may act inversely [110].

An increase in the multiplicity of breast cancer is seen in long-term neuroleptics users [111]. A variety of drugs, such as reserpine and perphenazine, that decrease hypothalamic dopaminergic activity enhance the development, multiplicity, and growth of chemically-induced mammary carcinomas in rats [5, 112]. This effect is probably mediated by prolactin, since dopaminergic activity is primarily responsible for inhibition of prolactin release from the pituitary.

PROSPECTS

Since rat mammary tumors closely resemble the human counterparts in many aspects, genetically engineered rats may serve as a favorable model for human breast cancer research. A major disadvantage of the rat system is that the gene knockout technique has been unavailable. In contrast, almost 100 transgenes, targeted mutations, combinations of transgenes, and combinations of transgenes and targeted mutations have been used to study mammary cancer in mice. Genetically engineered mice tumors have: (1) phenotypes similar to those of non-genetically engineered mice tumors; (2) signature phenotypes specific to the transgene; and (3) some morphological similarities to human breast cancer [113, 114]. However, some investigators did not appreciate the relevance of the murine systems because mouse mammary tumors do not resemble most human breast cancers either morphologically or biologically. Nevertheless, the emergence of knockout and transgenic biologies has provided remarkable evidence that mouse tumors can be produced by the same genes implicated in human breast cancer [115]. The development of the gene knockout technique in rats will be a powerful tool in breast cancer research.

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Comprehensive screening for antigens overexpressed on carcinomas via isolation of human mAbs that may be therapeutic

Gene Kurosawa[a], Yasushi Akahori[a], Miwa Morita[a], Mariko Sumitomo[b], Noriko Sato[c], Chiho Muramatsu[b], Keiko Eguchi[b], Kazuki Matsuda[b], Akihiko Takasaki[d], Miho Tanaka[a], Yoshitaka Iba[a], Susumu Hamada-Tsutsumi[a], Yoshinori Ukai[e], Mamoru Shiraishi[e], Kazuhiro Suzuki[e], Maiko Kurosawa[a], Sally Fujiyama[f], Nobuhiro Takahashi[f], Ryoichi Kato[g], Yoshikazu Mizoguchi[h], Mikihiro Shamoto[i], Hiroyuki Tsuda[j], Mototaka Sugiura[k], Yoshinobu Hattori[l], Shuichi Miyakawa[l], Ryoichi Shiroki[c], Kiyotaka Hoshinaga[c], Nobuhiro Hayashi[d], Atsushi Sugioka[l], and Yoshikazu Kurosawa[a,m]

[a]Division of Antibody Project and [d]Department of Biomedical Polymer Science, Institute for Comprehensive Medical Science, [b]21st Century Center of Excellence Research Center, Departments of [c]Urology, [g]Radiology, [h]Pathology, [k]Internal Medicine, and [l] Surgery, School of Medicine, Fujita Health University, Toyoake, Aichi 470-1192, Japan; [e]Institute for Antibodies, Ltd., Toyoake, Aichi 470-1192, Japan; [f]Department of Biotechnology, United Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan; [i]Division of Clinical Laboratory, Yachiyo Hospital, Anjo, Aichi 446-8510, Japan; and [j]Department of Toxicology, Graduate School of Medical Sciences, Nagoya City University , Kawazumi, Mizuho, Nagoya 467-8601, Japan

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Although several murine mAbs that have been humanized became useful therapeutic agents against a few malignancies, therapeutic Abs are not yet available for the majority of the human cancers because of our lack of knowledge of which antigens (Ags) can become useful targets. In the present study we established a procedure for comprehensive identification of such Ags through the extensive isolation of human mAbs that may become therapeutic. Using the phage-display Ab library we isolated a large number of human mAbs that bind to the surface of tumor cells. They were individually screened by immunostaining, and clones that preferentially and strongly stained the malignant cells were chosen. The Ags recognized by those clones were isolated by immunoprecipitation and identified by MS. We isolated 2,114 mAbs with unique sequences and identified 21 distinct Ags highly expressed on several carcinomas. Of those 2,114 mAbs 356 bound specifically to one of the 21 Ags. After preparing complete IgG_1 Abs the *in vitro* assay for Ab-dependent cell-mediated cytotoxicity (ADCC) and the *in vivo* assay in cancer-bearing athymic mice were performed to examine antitumor activity. The mAbs converted to IgG_1 revealed effective ADCC as well as antitumor activity *in vivo*. Because half of the 21 Ags showed distinct tumor-specific expression pattern and the mAbs isolated showed various characteristics with strong affinity to the Ag, it is likely that some of the Ags detected will become useful targets for the corresponding carcinoma therapy and that several mAbs will become therapeutic agents.

phage Ab library | therapeutic Ab | tumor-associated antigen

Since the discovery of a method to produce mAbs numerous scientists have been trying to identify and produce mAbs that could be used for immunotherapy against various malignancies. The success for example of alemtuzumab against CD52, trastuzumab against HER2, and rituximab against CD20 for treatment of chronic lymphocytic leukemia, breast cancer, and non-Hodgkins lymphoma, respectively (1–3), suggests that mAbs are likely to become very important therapeutic agents also against a wider range of cancers. However, for the majority of the human cancers useful therapeutic Abs are not yet available because of our lack of knowledge of which antigens (Ags) are likely to become useful targets (4). Therefore, several groups of investigators have been trying to identify other potential Ags as targets for immunotherapy using microarray technology (5, 6). Although many differences in transcripts have been revealed between malignant cells and the normal counterpart cells, it will take more time and laborious work to examine which Ags could be targets and to prepare therapeutic Abs against them. Furthermore, the presence of a large amount of transcripts does not always indicate expression of a large amount of the proteins.

Our experimental approach was designed in the opposite way to the strategy with the microarray technology mentioned above and was based on the phage-display technology (7). First we isolated a large number of mAbs that bind to the surface of cancer cells using a huge phage Ab library and many kinds of cancer-derived cell lines. Then using fresh tumor tissues we selected clones that gave significant staining of malignant cells but were negative or very weakly positive on the normal cells in the histological sections. At the third step the Ags recognized by the respective clones were isolated by immunoprecipitation and identified by MS analysis. Finally mAbs were converted to complete human IgG_1 and the antitumor activity was examined. Thus, the procedure adopted in our study enabled us to succeed in comprehensive identification of tumor-associated Ags (TAAs) and simultaneous isolation of mAbs against them.

Recently several groups of investigators have been using the phage-display method to screen for tumor-specific Ags according to a procedure similar to ours (8, 9), but the number of TAAs identified by them was limited, and to the best of our knowledge none converted their clones to complete Abs, which are essential for further studies to try to evaluate their potential therapeutic effects.

Results

Isolation of mAbs That Differentially Bound to Cancer Cells. Using 33 different tumor cell lines from seven carcinomas, hepatocarcinoma, renal carcinoma, pancreatic carcinoma, lung carcinoma, colonic carcinoma, gastric carcinoma, and ovarian carcinoma, the phage Ab library was screened 51 times for isolation of mAbs that bound to molecules present on the cell surface. The number

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[m]To whom correspondence should be addressed at: Institute for Comprehensive Medical Science, Fujita Health University, 1-98, Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan. E-mail: kurosawa@fujita-hu.ac.jp.

Table 1. Summary of screenings

Cancer type	Screening no.	Cell	Isolated	Intact	Kinds	Select	Super Select
Hepatocarcinoma	035	HepG2	240	162	91		
	040	Nuk-1	286	254	100		
	041	OCTH-18	239	197	86		
	042	HepG2	96	21	20		
	044	Hep3B	190	120	112		
	045	HepG2	428	270	189		
	046	Clinical sample 0722*	190	156	14		
	047	Clinical sample 0722*	142	116	18		
	048	Clinical sample 0722*	142	137	8		
	049	Clinical sample 0722*	190	160	36		
	050	Clinical sample 0722*	190	138	51		
	051	HepG2	168	68	49		
	052	HepG2	208	187	94		
	053	HepG2	208	149	71		
	063	HLF	190	141	45		
	3172	Clinical sample 0317†	1	1	1		
	054	RBE	250	204	106		
	Total	17	3,358	2,481	1,091	967	
Renal carcinoma	057	Caki-1	190	168	90		
	059	CCF-RC1	190	148	80		
	061	Caki-1	190	146	53		
	062	CCF-RC1	190	140	111		
	060	ACHN	190	160	97		
	Total	5	950	762	431	341	
Pancreatic carcinoma	055	PANC-1	286	181	62		
	058	MIA PaCa-2	190	159	50		
	085	BxPC-3	190	145	61		
	087	Capan-1	190	44	27		
	Total	4	856	529	200	180	
Lung carcinoma	064	A549	189	172	56		
	065	PC-14	379	349	60		
	066	NCI-H441	190	167	71		
	068	Calu-3	48	34	22		
	067	EBC-1	285	210	107		
	079	RERF-LC-AI	190	172	73		
	080	LK-2	190	158	86		
	086	VMRC-LCP	190	177	69		
	Total	8	1,661	1,439	544	437	
Colonic cancer	028	Caco-2	190	170	102		
	029	CW-2	190	153	92		
	082	SW480	190	175	46		
	084	HT-29	190	177	70		
	Total	4	760	675	310	279	
Gastric cancer	031	MKN-45	190	159	90		
	075	NCI-N87	190	145	50		
	077	SNU-5	190	143	65		
	081	KATO III	190	162	79		
	Total	4	760	609	284	240	
Ovarian cancer	015	SKOv3	240	183	81		
	021	SKOv3	48	10	9		
	022	SKOv3	48	15	10		
	025	SKOv3	48	10	6		
	026	SKOv3	48	20	8		
	039	SKOv3	48	43	36		
	074	KF28	190	143	60		
	076	RMG-1	190	177	76		
	078	RMG-2	190	176	79		
	Total	9	1,050	777	365	287	
Total		51	9,395	7,272	3,225	2,731	2,114

*Clinical sample 0722 was derived from a male patient with hepato cell carcinoma HCV (+) stage II.
†Clinical sample 0317 was derived from a male patient with hepato cell carcinoma HBV (+) stage IV-B.

of clones that were picked up in each screening is indicated in the column "Isolated" in Table 1. A total of 9,395 clones were picked up. Those clones were then screened by ELISA using anti-cp3 Ab to examine expression of the intact single-chain Fv (scFv) molecules on the phage because scFv fused with a truncated cp3 was expressed in our system. The number of clones that were

judged to express the intact molecule is indicated in the column "Intact" in Table 1. A total of 7,272 clones turned out to express intact scFv molecules on the phage. Each one of those 7,272 clones was sequenced. The number of clones with different sequences isolated in respective screenings is indicated in the column "Kinds" in Table 1. Because the same clones were redundantly isolated from different screenings, the total number of different clones against the same carcinoma is shown in the column "Select" in Table 1. Because the same clones were also isolated from screenings against different types of carcinoma, 7,272 clones were composed of 2,114 different clones indicated as "Super Select" in Table 1. Of those 2,114 clones 406 were redundantly present in the 3,225 clones summed up in the column "Kinds" in Table 1, and 1,708 were isolated only once in all of the 51-time screenings performed in the present work. The number of times such redundant clones were isolated ranged from two to 27.

Those 2,114 mAbs were individually screened using at least three different fresh tumor tissues for each assay. They were classified into four groups based on the immunostaining patterns in the histological sections. When mAbs significantly stained only the surface of tumor cells but negatively or very weakly stained the other normal cells, they were classified to group A. When the strong staining by mAbs was localized on the surface of malignant cells but a part of the other normal tissue was also stained, they were classified to group B. When mAbs showed positive staining patterns both on malignant cells and on normal cells nonspecifically, they were classified into group C. The clones that did not give any positive signal were classified into group D. Of 2,114 mAbs 281 were classified to group A and 384 were classified to group B.

Identification of 21 TAAs. Of the 665 clones, 300 that strongly stained the malignant cells were chosen for further studies. Each of the 300 clones was screened against six different tumor cell lines by using flow cytometry (FCM). They were grouped according to their staining pattern on the basis of the following principle. In FCM analysis, the degree of peak shift should reflect the amount of Ag, the accessibility of Ab, and the strength of binding. The width and shape of peak should reflect the degree of homogeneity of expressed Ags in the cell population. Therefore, if the staining patterns against the six cell lines were identical or very similar among the clones examined, they were grouped together. It led to 40 groups made up of 150 clones. The other 150 clones could not be grouped because of a weak signal in the FCM.

The cell membrane proteins of carcinoma-derived cell lines were biotinylated and then individually immunoprecipitated by the mAbs of the same group and analyzed by SDS/PAGE. When several mAbs in each group gave rise to the same band on the gel the bands were cut out and subjected to MS analysis. This enabled us to identify 21 distinct membrane Ags, which are listed in Table 2. Those 21 Ags are recognized by 84 of the 300 mAbs that we studied.

We also synthesized the extracellular portions of nine of the 21 Ags. Using ELISA we tested the 2,114 mAb clones against those nine synthetic Ags. Of those, 272 clones gave a positive reading in addition to the 84 clones that had been already identified by the MS analysis. We are now in the process of synthesizing the remaining 12 Ags for further screening. To date 356 clones of the 2,114 mAbs isolated in the present study were revealed to specifically bind to one of the 21 TAAs. Of those 356 clones 156 belonged to the redundantly isolated 406 mAbs.

Expression of Fresh Cancer Tissues. Using representative clones that specifically bound to 18 TAAs except for three TAAs PTK7, CD9, and CDCP1, which were recently identified, the immunostaining analysis was performed against 24 fresh lung carcinomas. Table 3 summarizes the results of eight TAAs that gave simple patterns showing one of the following two cases: +, overexpression on malignant cells but no or very weak expression on the other normal cells; and −, no expression on either malignant or normal cells. In the case of the other 10 TAAs, although differential expression on malignant cells was distinct, expression on a part of the normal tissue was also observed. From these analyses we concluded the following. (*i*) TAAs identified in our study were overexpressed in fresh tumors at some frequency, but there is no TAA that was overexpressed in all of the fresh tumors. (*ii*) All of the fresh malignant cells analyzed to date overexpressed some of 18 TAAs in various combinations. (*iii*) Approximately half of them showed distinct tumor-specific expression pattern.

Table 2. Cell-surface Ags identified by the mAbs

Ag	MS*	ELISA†
Growth factor receptor		
EGFR	3	6
HER2	1	15
HGFR	3	84
PTK7/CCK-4	1	ND
Transmembrane protein-tyrosine phosphatase		
PTP-LAR	5	ND
Adhesion molecule		
Ig superfamily		
IGSF4	10	13
ALCAM	3	8
ICAM-1	5	17
Lu/BCAM	1	48
CEACAM6	1	ND
Non-Ig family		
CD44	3	ND
EpCAM	2	ND
Tetraspanin		
CD9	1	ND
Adenosine metabolism		
Ecto-5′-nucleotidase	1	ND
Complement inhibitor		
MCP	8	81
Protease inducer		
EMMPRIN	1	0
Iron metabolism		
TfR	6	ND
Anoikis regulator		
CDCP1	2	ND
Integrin family		
$\alpha 3\beta 1$	14	ND
$\alpha v\beta 3$	8	ND
$\alpha 6\beta 4$	5	ND

*Number indicates that of different clones identified by MS analysis.
†Clones identified by MS analysis are not included.

Antitumor Activity of Complete Human IgG mAbs. After preparing IgG_1 mAbs we performed an *in vitro* assay for Ab-dependent cell-mediated cytotoxicity (ADCC) using 22 clones against 10 Ags (EGFR, ALCAM, ICAM-1, EpCAM, HGFR, TfR, ITGA3, EMMPRIN, PTP-LAR, and CD44) using the cell lines listed in Table 4. As can be seen, those mAbs gave a positive reading that ranged between 5% and 95% for cell killing. The details using anti-EGFR Abs and anti-EpCAM Ab are in Fig. 1 *a* and *e*, respectively. The degree of ADCC by clone 059-152 was strong compared with that by cetuximab. Clone 067-153 showed ADCC activity even at an extremely low concentration such as lower than picomolar. We also performed an *in vivo* assay using three

Table 3. Hisotological analysis of lung carcinomas with mAbs against TAAs

		A			B	C	D											E						
Clinical sample		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Antigen	Stage Clone Ab	IA	IA	IIIB	IB	IIIB	IA	IA	IA	IB	IB	IB	IB	IB	IIB	IIIA	IIIA	IIIB	IB	IIB	IIIA	IIIA	IIIA	IIIB	IIIB
ITGA6	029-023	−	−	−	−	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
ITGAV	064-139	−	+	−	+	+	−	+	−	−	−	−	−	−	−	−	+	−	+	−	−	+	−	−	−
CD147	059-053	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	−	+	+	−	+	+	−	−	+
LAR	064-044	+	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	+	−
IgSF4	076-048	−	−	−	−	+	+	−	−	+	+	−	+	−	−	+	−	+	−	−	−	−	+	−	−
EGFR	048-006	+	+	−	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−
HER2	015-126	−	−	−	+	−	+	+	−	−	−	−	−	+	+	+	+	+	−	+	+	−	−	−	−
HGFR	067-133	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−

A, squamous cell carcinoma; B, adenosquamous carcinoma; C, bronchioloalveolar carcinoma; D, adenocarcinoma; E, large-cell carcinoma.

mAbs against two of the Ags (EGFR and EpCAM) in cancer-bearing athymic mice. As can be seen in Fig. 1*d* the anti-EGFR Abs showed a strong antitumor activity against tumor cell line A431. When we compared our mAbs (048-006 and 059-152) against EGFR with cetuximab it appeared that they had a very similar level of antitumor activity. The anti-EpCAM Ab also prevented the growth of HT29 (Fig. 1*f*).

The two anti-EGFR mAbs were used to analyze the mechanism of their antitumor activities. As can be seen in Fig. 1*b* mAb 048-006 was very effective in inhibiting the binding of EGF to the EGFR, whereas mAb 059-152 only partially prevented the binding reaction. The phosphorylation assay (Fig. 1*c*) showed that mAb 048-006 was effective in the inhibition of phosphorylation. The mAb 059-152 also gave inhibitory effects on phosphorylation, although less effective than 048-006. It suggests that mechanisms of antitumor activity mediated by these two mAbs might be different from each other.

Discussion

In the present study we used a phage Ab library that had been constructed from human B cells (10). It has been suspected that majority of the clones isolated from phage Ab libraries may not show high affinity to the Ags because they should be naïve to the Ags (11). However, as shown in Fig. 1 two anti-EGFR Abs and one anti-EpCAM Ab showed strong ADCC activity at the concentration of 0.01–0.1 μg/ml, which corresponds to 0.06–0.6 nM. This strength appeared to be practically strong enough to be therapeutic agents whereas comparison of nucleotide sequences of V_H genes encoding theses three Abs with those of germ-line genes indicated that mutations had not been introduced (data not shown). In the screenings of Ab library there were many cases where the same clones were redundantly isolated from different screenings against the same carcinoma as well as against different types of carcinoma. The reason why specific clones were redundantly isolated might be as follows: (*i*) amounts of Ags recognized by them were relatively abundant on the cells used in the screenings, and (*ii*) the binding activity to the Ags was stronger than that of other clones. This interpretation could be at least partly correct. For example, while anti-EGFR Ab 048-006 was isolated by six time screenings the dissociation constant (K_d) of the Ag/Ab complex measured by the BIAcore instrument was 0.025 nM (data not shown).

Table 4. ADCC activities of mAbs that have been converted to IgG

Ag	Clone	Target cells	Cell killing,* %
EGFR	048-006	NCI-H1373, CCF-RC1, A431, ACHN	36–80
	055-147	CCF-RC1, HT-29, A431	25–95
	059-152	NCI-H1373, CCF-RC1, A431, ACHN	35–75
	059-173	CCF-RC1, HT-29, A431	35–85
ALCAM	035-234	NCI-H1373, SKOv3, CW-2	8–19
	041-118	NCI-H1373, EBC-1	14–18
	066-174	NCI-H1373, SKOv3, CW-2	45–59
	083-040	NCI-H1373	10
ICAM1	053-042	NCI-H1373	16
	053-051	NCI-H1373, NCI-H441, HepG2	5–31
	053-059	NCI-H1373, NCI-H441, HepG2	8–39
	053-085	NCI-H1373, NCI-H441, HepG2	7–26
EpCAM	067-153	NCI-H1373, MKN45, HT-29, EBC-1	23–80
HGFR	067-133	NCI-H1373, MKN45, EBC-1	19–42
TfR	028-178	MIA Paca2	65
	052-138	MIA Paca2	80
	041-288	MIA Paca2	30
ITGA3	015-003	ACHN	20
EMMPRIN	059-053	CCF-RC1, ACHN	40
PTP-LAR	064-044	PC14	10
	079-085	PC14	32
CD44	064-003	PC14	84

*Percentage of cell killing increased dose-dependently. When it reached plateau, the percentage for cell killing was indicated.

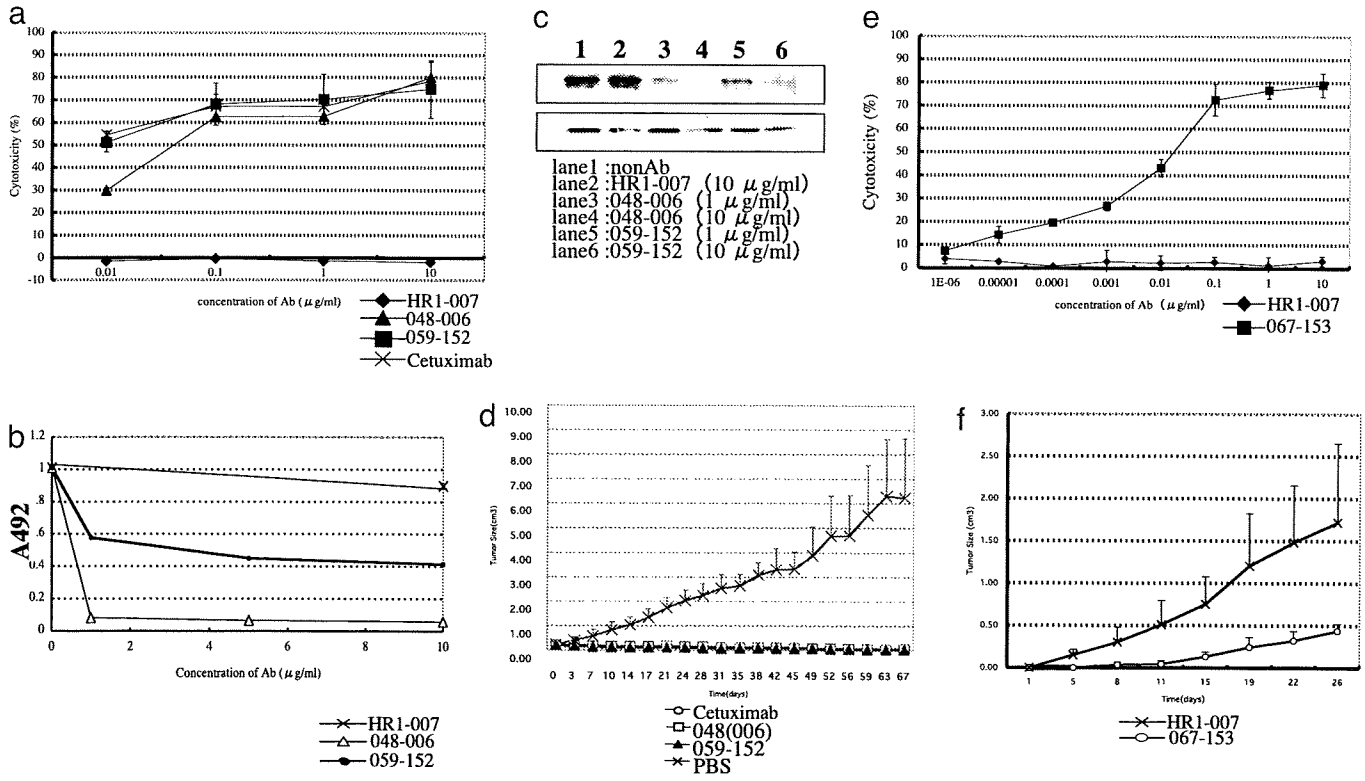

Fig. 1. Antitumor activities of two anti-EGFR mAbs (clone 048-006 and clone 059-152) and an anti-EpCAM mAb (clone 067-153). (*a*) ADCC. Target cells: NCI-1373. Ab: HR1-007 (negative control), mAb 048-006, 059-152, and cetuximab (positive control). (*b*) Inhibitory effects of mAbs on the binding of EGF to EGFR on cell line A431. Ab: HR1-007 (negative control), mAb 048-006, and 059-152. (*c*) Inhibitory effects of mAbs on phosphorylation of EGFR induced by EGF. Upper bands: Western blot by anti-phosphotyrosine mouse mAb. Lanes 1–6: incubated for 30 min after addition of EGF at 1 μg/ml; lane 1, without Ab; lanes 2–6, incubated with Ab for 30 min, then EGF was added; lane 2, HR1-007 (negative control) at 10 μg/ml; lanes 3 and 4, 048-006; lanes 5 and 6, 059-152; lanes 3 and 5, 1 μg/ml; lanes 4 and 6, 10 μg/ml. Lower bands: control Western blot with rabbit antiserum against β-actin. (*d*) Inhibitory effects of mAbs on the growth of tumor cell line A431 in athymic nude mice assayed by the first method described in *Antitumor Activity in Vivo*. Ab: HR1-007 (negative control), mAb 048-006, 059-152, and cetuximab. (*e*) ADCC. Target cells: HT-29. Ab: HR1-007 (negative control) and mAb 067-153. (*f*) Inhibitory effects of mAb on the growth of a tumor cell line in athymic nude mice assayed by the alternative method described in *Materials and Methods*. Ab: HR1-007 (negative control) and mAb 067-153.

In this study we isolated 2,114 mAbs with unique sequences that bound to molecules on the surface of tumor-derived cells and selected 665 clones that gave tumor-specific immunostaining patterns. To identify TAAs recognized by the tumor-specific mAbs we developed two strategies. The grouping of mAbs by FCM enabled us to achieve efficient identification of TAAs by the following reasons. (*i*) FCM analyses against several cell lines taught us which cell expressed most abundantly the target molecules. (*ii*) Because multiple mAbs in each group turned out to bind to the same Ag in most cases, many clones have been treated very efficiently by a limited number of experiments. (*iii*) Detergent for solubilization of membrane proteins may destroy the structural integrity with the results of losing the antigenic structure. When several clones classified into the same group were analyzed together, some of them could bind to a relatively detergent-resistant epitope. Screenings of all of the 2,114 mAbs by ELISA with the polypeptides that correspond to the extracellular portions of the TAAs already identified by MS analysis also enabled us to efficiently identify the Ags recognized by respective Abs. Now it is likely that we have already revealed more than half of TAAs potentially identified according to this procedure.

We characterized two anti-EGFR mAbs, clone 048-006 and clone 059-152. Whereas clone 048-006 inhibited both the binding of EGF to EGFR and the phosphorylation of EGFR, clone 059-152 partly inhibited the binding of EGF to EGFR and gave inhibitory effects on the phosphorylation of EGFR less effectively. However, both clones showed a strong antitumor activity in cancer-bearing athymic mice. The characteristics of clone 048-006 appeared to be similar to that of cetuximab (12). However, to the best of our knowledge there has been no report describing mAb whose characteristics was similar to that of 059-152. In the present study multiple Abs have been isolated against respective TAAs. It is possible that these mAbs may have various characteristics as shown by anti-EGFR mAbs.

As indicated in Table 3, half of the TAAs identified in this study gave a tumor-specific staining pattern that is overexpression on malignant cells but no or very weak expression on the other normal cells in the histological sections. These TAAs could be candidates to be useful targets for therapeutic Abs. Furthermore, as indicated in Table 4 and Fig. 1, IgG form of mAbs that bound to them showed strong antitumor activity *in vitro* and *in vivo*. Therefore, we believe that some Ags detected will be useful targets for cancer therapy and that several mAbs will become useful therapeutic agents in the foreseeable future.

Materials and Methods

Ab Library and Screening. AIMS-5 library constructed by using a phage-display system was employed (10). Screenings were performed by a method similar to the one developed by Giordano *et al.* (13). In brief, the phages (2×10^{13} cfu) were mixed with cells ($0.2–1 \times 10^{8}$) in 1.6 ml of solution A (1% BSA, MEM, and 0.1% NaN_3), and Ag–Ab complexes on the cell surface were formed. The cell and phage suspension was overlaid on the organic solution in an Eppendorf tube. After the tube was centrifuged, water and organic layers were discarded. The collected cells were suspended in solution A. This process was repeated three times. Finally, the cells were suspended in PBS and frozen in liquid nitrogen. The frozen cells were thawed and mixed with *Escherichia coli*

DH12S. The phages were prepared. This screening round was performed repeatedly three times. After three rounds of screenings, *E. coli* DH12S infected with recovered phages was spread on plates. Approximately 200 colonies were picked up. Thirty-three cancer cell lines listed in Table 1 were used as Ags.

Immunostaining of Fresh Tumors. Tumor tissues and the neighboring normal tissues resected by operation were used for immunostaining. They were fixed with 4% paraformaldehyde in 0.1 M cacodylic buffer (pH 7.4) by microwave irradiation as described previously (14).

Identification of Ag. Membrane protein analysis was performed according to Zhao *et al.* (15). Proteins present on the cell surface were biotinylated according to the manufacturer's instruction by using the EZ-Link Sulfo-NH-LC Biotinylation kit (Pierce). After the cells were homogenized with a Dounce homogenizer, the protein–membrane complexes were banded between 0.25 M and 1.25 M sucrose layers by centrifugation. The complexes were dissolved in a detergent mixture: 50 mM Hepes (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1% β octyl glucoside. scFv-C_L fused with cp3 was converted to scFv-C_L fused with protein A domains (scFv-C_L-PP) (16). scFv-PP form was covalently bound to beads that were CNBr-activated Sepharose 4B (GE Health Care Bioscience). Ab-bound beads were used for immunoprecipitation as described by David *et al.* (17). MS analysis was performed according to Geuijen *et al.* (8).

Preparation of IgG_1. ScFv was converted to IgG_1 and prepared by using a high-level expression vector (18). Using IgG_1 mAbs we examined ADCC, effects on binding of EGF to EGFR, effects on phosphorylation of EGFR, and antitumor activity in athymic nude mice.

ADCC. The enzymatic activity of lactic dehydrogenase released from the target cells was measured for estimation of ADCC (19). Various cell lines were used as targets for the mAbs. Cells were derived from the following cancers: NCI-H1373, lung adenocarcinoma; CCF-RC1, renal clear cell carcinoma; A431, vulva epidermoid carcinoma; ACHN, renal adenocarcinoma; HT-29, colorectal adenocarcinoma; SKOv3, ovarian adenocarcinoma; CW-2, colorectal adenocarcinoma; EBC-1, lung squamous cell carcinoma; NCI-H441, lung papillary adenocarcinoma; HepG2, hepatocellular carcinoma; MKN45, gastric adenocarcinoma; MIAPaca-2, pancreatic carcinoma; PC14, ling carcinoma. Effector cells were prepared from blood of healthy volunteers and used in a ratio of 100:1 (10^6 to 10^4 in 200 μl) (20).

Effects of Anti-EGFR mAbs on the Function of EGFR. Binding of EGF to EGFR on the cell surface was estimated according to Yang *et al.* (21). Phosphorylation of EGFR induced by EGF was measured according to Matar *et al.* (22).

Antitumor Activity *in Vivo*. Two different methods were adopted. In the first method each mouse was injected with 5×10^6 cells, and when the tumor grew to 0.2 cm^3 mAb therapy was initiated. Treatments consisted of twice-weekly i.p. injections of mAb for 3 weeks. One milligram in 0.5 ml of PBS was used in each injection. Control animals received injection of PBS. Six mice were used for each treatment. The alternative method: 1 day after injection of cells mAb therapy was started. Treatments consisted of twice-weekly i.v. injections of mAb for 2 weeks. A total of 50 μg was used in each injection.

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

Yoichiro Matsuoka,[1,3] Katsumi Fukamachi,[2] Norihisa Uehara,[1] Hiroyuki Tsuda[2] and Airo Tsubura[1]

[1]Second Department of Pathology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506; [2]Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

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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.

[3]To whom correspondence should be addressed. E-mail: matsuoyo@takii.kmu.ac.jp

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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.

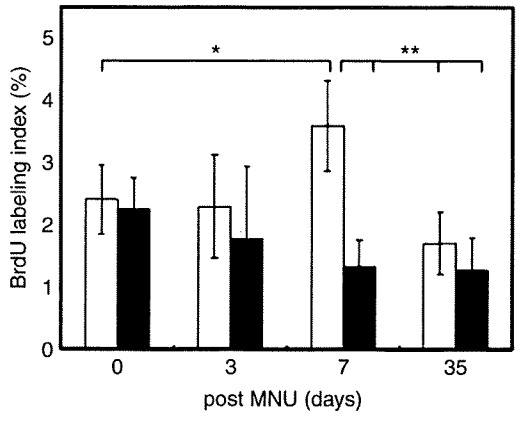

Fig. 1. Comparison of cell proliferation between age-matched virgin (AMV; open columns) and parous (solid columns) rat mammary glands after *N*-methyl-*N*-nitrosourea (MNU) inoculation. Bromodeoxyuridine-labeling indices in the mammary ductal epithelium of AMV and parous rats at 0, 3, 7, and 35 days 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

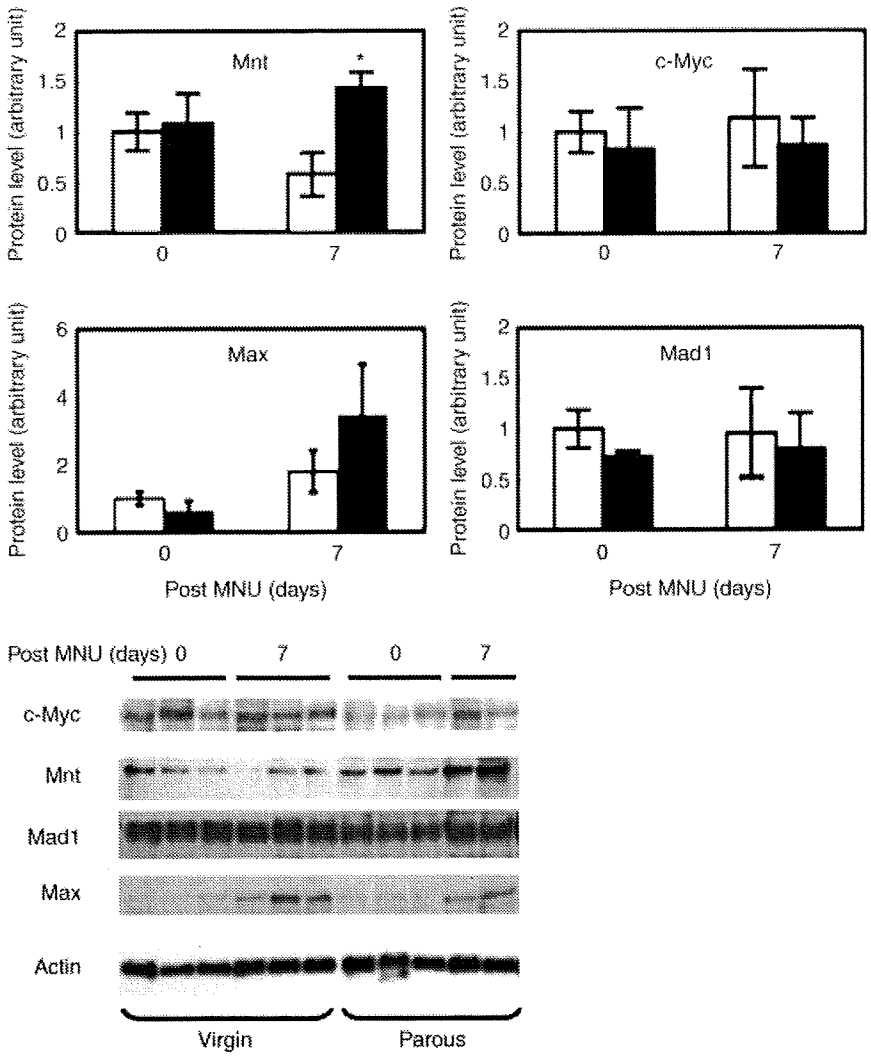

Fig. 2. Immunoblot analyses of levels of the c-Myc regulators in age-matched virgin (AMV; open columns) and parous (solid columns) rat mammary glands after *N*-methyl-*N*-nitrosourea exposure. Changes in the protein levels of the E-box-interacting proteins were monitored with immunoblots at 0 and 7 days 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.

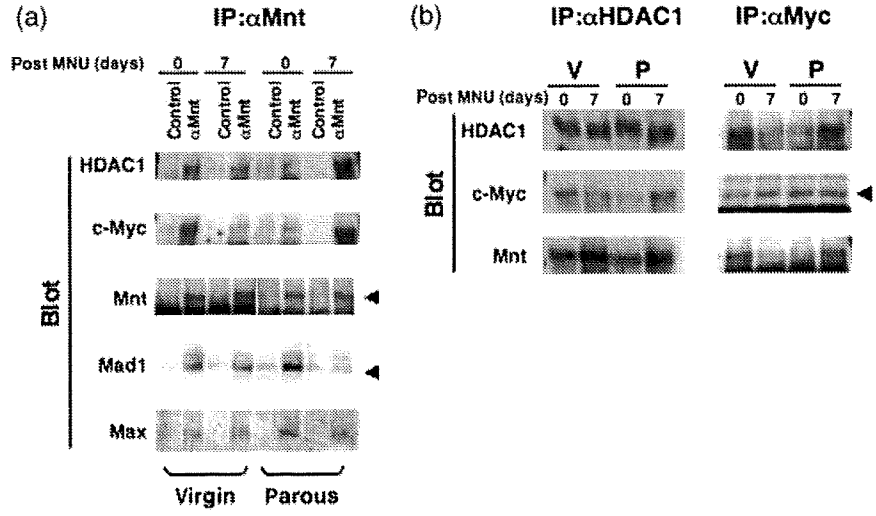

Fig. 3. Induction of HDAC1/c-Myc/Mnt/Max complex formation in *N*-methyl-*N*-nitrosourea (MNU)-treated parous rat mammary glands. (a) Western blot data from immunoprecipitation with anti-Mnt antibody from each combined lysate of age-matched virgin (V) and parous (P) mammary glands ($n = 5$ each) at 0 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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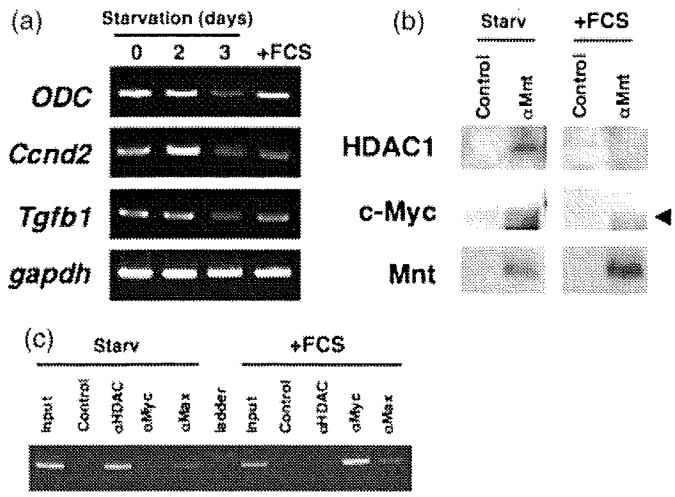

Fig. 4. Assembly and disassembly of HDAC1/c-Myc/Mnt/Max complexes on the promoter of Myc-target gene in fibroblasts. (a) Reverse transcription–polymerase chain reaction analysis of mRNA expression of Myc-target genes during serum starvation. The suppression of target gene expressions was relieved 3 h 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.

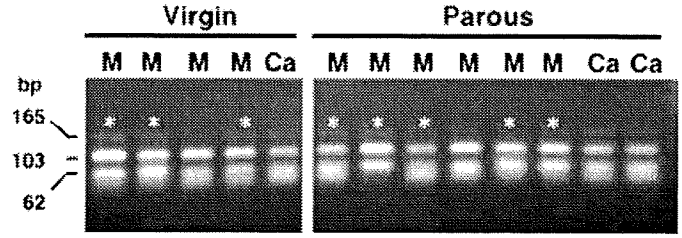

Fig. 5. Mutations in the human *c-Ha-ras* genes in the mammary glands of transgenic (Tg) age-matched virgin (AMV) and parous rats after dimethylbenz(a)anthracene treatment. Representative restriction fragment length polymorphism analysis data for codon 12 of the human *c-Ha-ras* gene. DNA samples were 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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