Tumor Suppressor Activity of the Gene Encoding Mammary-derived Growth Inhibitor[1]

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Abstract

The gene encoding mammary-derived growth inhibitor (MDGI), a protein previously purified from bovine mammary gland and shown to have modest antiproliferative activity for human breast cancer cells *in vitro*, is demonstrated to function as a potent tumor suppressor gene. Human breast cancer cells transfected with a MDGI expression construct exhibited differentiated morphology, reduced proliferation rate, reduced clonogenicity in soft agar, and reduced tumorgenicity in nude mice relative to mock-transfected or untransfected controls. We mapped the human homologue of this gene to chromosome 1p33–35, a locus previously shown to exhibit frequent loss of heterozygosity in human breast cancer. MDGI immunoreactivity was detected in epithelial cells of human breast tissue, but not on ductal carcinoma cells on the same sections. Our results suggest that MDGI is a strong candidate for the distal 1p breast tumor suppressor gene. Furthermore, as prior reports have demonstrated that MDGI is hormonally regulated in breast epithelial cells and maximally expressed at the time of maximal differentiated function (just prior to lactation), MDGI is a candidate mediator of the differentiating effect of pregnancy on breast epithelial cells, which may be involved in the protective effect of early parity on subsequent breast cancer incidence.

Introduction

Genes encoding growth inhibitory proteins are candidate tumor suppressor genes (1). MDGI[3] is a 14.4-kDa protein purified from lactating bovine mammary gland (2). It has modest antiproliferative activity for breast cancer cells when present in culture media at nM concentrations (3, 4). However, a cell surface receptor for MDGI has not been described and it has not been established with certainty that MDGI functions physiologically as an extracellular growth inhibitory protein.

MDGI is a member of the fatty acid-binding protein family which includes retinoid-binding proteins and other lipophilic intracellular proteins (2–7). Expression of MDGI in mammary epithelium has been shown to be hormonally regulated, and is maximal in the terminally differentiated state found just prior to lactation (8, 9). MDGI is structurally unrelated to previously characterized growth inhibitory or tumor suppressor proteins (2–4).

The published peptide sequence of MDGI is virtually identical to that of a bovine fatty acid-binding protein originally designated "heart fatty acid-binding protein" (6, 10). hFABP is now known to be expressed in many differentiated tissues in addition to cardiac muscle (11–14). There is evidence that MDGI and hFABP are products of the same gene (15), and may either be identical or closely related isoforms of a single protein. Recombinant bovine hFABP (16) has been characterized structurally by nuclear magnetic resonance spectroscopy as a β-barrel consisting of 10 antiparallel β strands and a helix-turn-helix motif (17).

In order to evaluate the hypothesis that the gene encoding MDGI has tumor suppressor properties, we transfected an MDGI expression construct into two human breast cancer cell lines that do not express MDGI and characterized phenotypic changes associated with MDGI expression.

Materials and Methods

Transfections. Bovine MDGI cDNA (8) (671-bp fragment containing a 38-bp 5′ UTR, 397-bp coding sequence, and 236-bp 3′ UTR digested with *Hin*dIII to preserve the 5′ UTR, and with *Xba*I downstream of a polyadenylation signal, to generate a 680-bp fragment) was subcloned into the eukaryotic expression vector pRc/CMV (Invitrogen) downstream of the human cytomegalovirus promoter and enhancer to generate the pRc/CMV-MDGI expression vector. (Evidence for biological similarity of the human and bovine MDGI/hFABP proteins includes >80% amino acid sequence homology (10, 18), as well as the hybridization and immunological cross-reactivity data given in "Results.") Ten μg pRc/CMV-MDGI or the control vector pRc/CMV were used for transfections. Briefly, 1×10^6 MCF7 or T47D human breast cancer cells were plated 24 h prior to transfection on 100-mm dishes and then incubated with DMEM supplemented with 10% FCS (GIBCO) and 5 μg/ml bovine insulin (Sigma) 6 h prior to transfection. Transfection was carried out using the calcium phosphate coprecipitation method (19). DNA was removed 12 h later by replacing the incubation medium. Forty-eight h later, the cells were subcultured to five 100-mm dishes containing DMEM supplemented with 10% FCS, 5 μg/ml bovine insulin, and 0.8 mg/ml G418 (Geneticin; GIBCO). After colonies of about 10^4 cells had grown, 30 G418-resistant individual clones were picked, subcloned, and characterized with respect to MDGI expression. Stable transfection was confirmed by Southern blotting and by demonstrating that the proliferation of pRc/CMV and pRc/CMV-MDGI transfectants after 10 passages was identical in the presence or absence of 0.8 mg/ml G418, while untransfected clones were inhibited by G418 (data not shown).

Northern Blot Analysis. Total cellular RNA from cells was isolated as described (20) using a RNAZol B premixed solution (Tel-Test, Friendswood, TX). Thirty μg total RNA were used for Northern blot analysis on 1.2% agarose gels containing 2.2% formaldehyde in 1X morpholinopropanesulfonic acid buffer. Gels were treated with 50 mM NaOH for 45 min and transferred for 16 h to a Zeta-probe membrane (Bio-Rad) in 50 mM NaOH. The blots were hybridized with a 680-bp MDGI insert (8) as previously described (20), using 1×10^7 cpm of the probe prepared by random primer synthesis (Pharmacia). After 24-h hybridization, the blot was washed as described (20). Integrity and equal loading of RNA were verified by hybridizing the blots to a human β-actin insert (21).

Immunoblot Analysis. Cells were washed twice with ice-cold PBS (140 mM NaCl, 2.5 mM KCl, and 15 mM KH_2PO_4, pH 7.2) and incubated on ice with 1 ml lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride, and 0.5% NP40) for 20 min. The lysate was clarified by centrifugation at 10,000 $\times$ *g* for 15 min. One hundred fifty μg

protein were electrophoresed on 16% SDS-polyacrylamide gels using the Protein II system (Bio-Rad) and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad) in 25 mM Tris-base, 190 mM glycine, and 20% methanol. The blots were blocked with 3% gelatin in TBS (25 mM Tris-HCl, pH 7.5, 500 mM NaCl) for 2 h at room temperature and incubated with a polyclonal rabbit antiserum against bovine hFABP (Ref. 22; 1:1000 dilution in TBST) for 3 h. After extensive washing in TBST, the filters were incubated for 1 h in an alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (1:3000; Bio-Rad). Filters were washed three times with TBST and once with TBS before visualization with *p*-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt (Bio-Rad) according to the manufacturer's directions. Three hundred ng recombinant hFABP (16) were used as a positive control.

Cell Proliferation. Cell lines were plated (2.5×10^4 cells/15-mm well in DMEM supplemented with 10% FCS, 5 μg/ml bovine insulin, and 50 μg/ml garamycin in a 37°C 5% CO_2 humidified environment. Cell number was determined with a hemocytometer at indicated times. Mean values of quadruplicates are shown; in no case was the SD >15% of the mean. The experiments were repeated three times with similar results, and the cell number on day 5 was always significantly less (Mann-Whitney U test, $P < 0.05$) in MDGI transfectants than controls.

Anchorage-independent Growth. Mean number of colonies $\geq$ 16 cells were counted in soft agar plates 15 days after seeding 8×10^2 cells suspended in 1.5 ml of a 0.33% (w/v) agar solution containing DMEM supplemented with 10% FCS and 5 μg/ml bovine insulin over a 1% (w/v) agar solution in 35-mm dishes. Quadruplicate replicates were used to determine mean values, and in no case was the SD >18% of the mean.

***In Vivo* Tumor Formation.** This was assayed using 4–8-week-old athymic nude mice (CD1 *nu/nu*; Charles River Breeding Laboratories) given estrogen supplementation by a surgically placed (lower back) s.c. 0.25-cm silastic tube (inside diameter, 0.0635 cm; outside diameter, 0.12 cm) containing 17-β estradiol on the day of injection of tumor cells. Each cell line was assayed in four mice, and each mouse received an injection of 5×10^6 cells into an inframammary fat pad, and another identical injection of the same cell line into a contralateral fat pad. Animals were inspected twice weekly for 3 months following tumor cell injection. All tumors appeared in the fourth week.

Fluorescence *in Situ* Hybridization Analysis. A human cosmid (pWE15) library prepared from placental DNA was screened with the insert of the bovine MDGI cDNA clone (8). Clones positive in the first screening were rescreened by standard techniques until pure, and then verified by Southern blot analysis after *Eco*RI cleavage. Slides of human metaphase chromosomes were prepared from standard lymphocyte cultures from healthy individuals and used for fluorescence *in situ* hybridization analysis essentially as described (23). The slides were postfixed, RNase treated, and denatured. The cosmid clone cMDGI was used as a probe after being labeled with biotin-16-dUTP using a nick translation kit, as suggested by the manufacturer (BRL). The probe (50 ng) was preannealed with Cot-1 DNA (4–8 μg) for 30 to 60 min at 37°C. Hybridization was performed in 50% formamide and 2X SSC at 42°C overnight. The slides were then washed three times for 5 min in 50% formamide, 2X SSC at 42°C, and three times in 0.1X SSC at 60°C. After washing, the probe was coupled to fluorescein-isothyocyanate-avidin D, and the fluorescent signal was amplified by three successive treatments with biotinylated anti-avadin antibodies alternated with FITC-avidin D. The chromosomes were counterstained with propidium iodide, and the results were analyzed and photographed under a confocal laser scanning microscope (Zeiss). The chromosomal localization of the signal was identified by quinacrine (QFQ) banding. The position was assigned using statistical criteria as previously described (23).

Immunostaining of Normal and Neoplastic Human Breast Tissue. Human tissue sections containing regions of both normal ductal epithelium and invasive ductal carcinoma were fixed in 10% neutral-buffered formalin for 24 h, dehydrated in serial ethanol, then cleared and embedded in paraffin. Sections (3–5 μm) were mounted on glass slides, cleared in toluene, rehydrated in serial ethanol, washed twice in PBS, and permeabilized with 1% Triton X-100 in PBS for 2 min followed by two washes in PBS. Slides were incubated with the rabbit anti-hFABP antibody (1:300 v/v; Ref. 22) in PBS at 4°C overnight, and then washed three times in PBS at room temperature. Following the last wash, sections were incubated with a goat anti-rabbit IgG-FITC conjugate (Boehringer Mannheim) in PBS (1:60 v/v) using conditions similar to those for the primary antibody and washed in PBS. Slides were visualized with a Jenalunar microscope equipped with epifluorescence optics and appropriate filters for FITC and photographed. Specificity of staining was demonstrated by lack of signal when serum was substituted for the primary antibody.

Results

Phenotypic Characterization of Human Breast Cancer Cells Transfected with an MDGI Expression Vector. We first established that an MDGI cDNA isolated from a bovine mammary gland expression library (8) hybridizes with MDGI-related nucleic acid sequences in rodent and human tissues, and that an anti-bovine hFABP antiserum (22) detects a 14.4-kDa protein only in tissues that express the mRNA species that hybridize with the MDGI cDNA probe (8). These findings are consistent with the near-identity (6, 10) of MDGI and bovine hFABP and with the >80% homology between human (18) and bovine (10) hFABPs.

Expression of the gene encoding MDGI and MDGI-related immunoreactivity has been observed in a well-differentiated untransformed breast epithelial cell line (24) in mammary gland organ culture (9) and *in vivo* (8). However, expression of MDGI-related mRNAs and hFABP immunoreactivity were undetectable in 8 of 8 human breast cancer cell lines and in 10 of 14 dimethylbenz(*a*)anthracene-induced rat mammary tumors (data not shown).

To address the possibility that the gene encoding MDGI is a tumor suppressor gene, we first transfected MCF-7 and T47D human breast cancer cells with an expression vector containing full-length MDGI cDNA (pRc/CMV-MDGI), and then compared the phenotype of transfectants expressing MDGI mRNA with that of controls. Fig. 1 shows high levels of expression of MDGI mRNA (~0.8 kb) in transfected cell lines MCF7/6 and MCF7/44, but that MDGI was not expressed in MCF7 cells or the mock-transfected cell line MCF7/79. Western blotting with a polyclonal anti-hFABP antiserum (22) was used to detect hFABP-related proteins in the various clones. A 14.4-kDa protein which comigrated with recombinant hFABP was

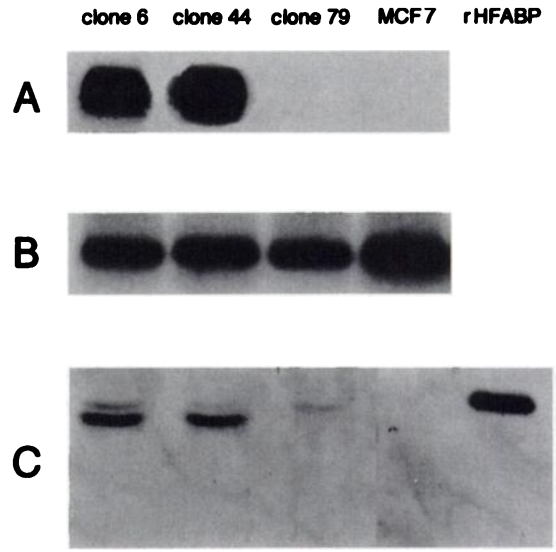

Fig. 1. MDGI mRNA abundance and immunoreactivity in MCF7 human breast cancer cells, and in representative subclones transfected with an MDGI expression vector. Northern blot analysis of total RNA extracted from transfected and control cell lines was carried out using a 680-bp MDGI insert (Ref. 15; *A*) or β-actin (Ref. 36; *B*) probes. Immunoblotting of cell lysates (*C*) was carried out as described in "Materials and Methods."

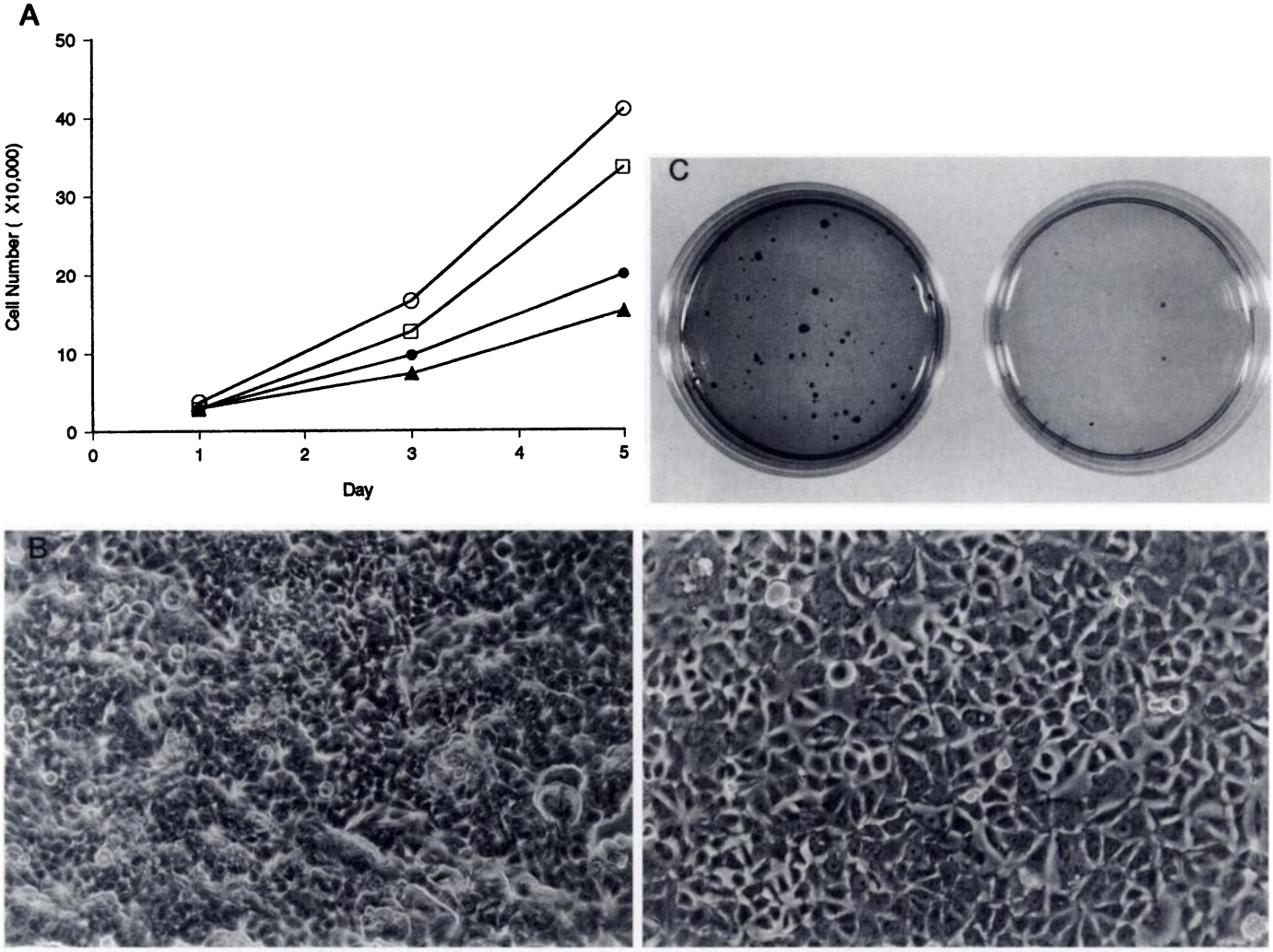

Fig. 2. Effect of MDGI expression on proliferation, morphology, and soft agar colony formation of breast cancer cells. *A*, growth curves of representative control (□, parental MCF7; ○, mock-transfected clone MCF7/79) and MDGI-expressing (●, clone MCF7/6; ▲, clone MCF7/44) cell lines. *B*, phase-contrast photomicrographs (× 250) of the mock-transfected clone MCF7/79 (*left*) and a representative MCF7 transfectant expressing MDGI (clone MCF7/44, *right*). Mock-transfected cell cultures were morphologically identical to parental MCF7 cells. *C*, soft agar colony formation by MCF7 transfectants. *Left*, clone MCF7/79 (mock transfectant). *Right*, clone MCF7/44 (transfected with pRc/CMV-MDGI). The appearance of untransfected MCF7 cells was indistinguishable from that of MCF7/79 cells.

Table 1 *Characteristics of MDGI-transfected clones and controls*

Relative MDGI mRNA abundance in cell lines was determined by densitometric scanning of Northern blots. In comparison, *MDGI* mRNA abundance in lactating rat breasts ranged from 3.0 to 6.0. Cell number refers to mean cell number counted by hemocytometer 5 days after seeding 2.5×10^4 cells. Colony formation refers to mean number of colonies ≥16 cells were counted in soft agar plates 15 days after seeding 8×10^2 cells. In vivo tumor formation refers to number of grossly visible tumors present in nude mice following 8 independent injections of 5×10^6 cells into mammary fat pads. Differences in cell number, colony formation, and tumor number between parental lines and transfectants were tested using the Mann-Whitney *U* test.

Clone	Description	Relative MDGI mRNA abundance	Cell number ($\times 10^4$)	Colony formation	*In vivo* tumor formation
MCF7	Parental neoplastic	0	40.8	190	8/8
MCF7/6	MDGI transfectant	7.23	20.0[a]	80[b]	2/8[a]
MCF7/11	MDGI transfectant	3.13	34.8[a]	104[a]	ND[c]
MCF7/44	MDGI transfectant	8.06	15.0[b]	38[b]	0/8[a]
MCF7/56	MDGI transfectant	3.23	36.2	50[b]	ND
MCF7/79	Mock transfectant	0	40.9	187	7/8
MCF7/81	MDGI transfectant	10.53	18.8[a]	ND	ND
T47D	Parental neoplastic	0	16.5	ND	ND
T47D/31	MDGI transfectant	0.38	12.0[a]	ND	ND
T47D/32	MDGI transfectant	1.48	7.8[b]	ND	ND
T47D/44	MDGI transfectant	3.48	10.1[a]	ND	ND
T47D/47	MDGI transfectant	2.91	7.6[a]	ND	ND

[a] $P < 0.05$, Mann-Whitney *U* test.
[b] $P < 0.005$, Mann-Whitney *U* test.
[c] ND, not determined.

detected only in cells transfected with pRc/CMV-MDGI. Together, these data demonstrate that MDGI transfectants express MDGI cDNA and exhibit MDGI-related immunoreactivity while MDGI gene expression and MDGI-related immunoreactivity are absent in control clones.

We initially evaluated the effect of MDGI transfection on cell proliferation by determining cell number on plastic dishes after 5-day incubation. The number of cells was significantly less in MDGI-expressing transfectants than in controls ($P < 0.05$, Mann-Whitney *U* test; Table 1). Cell number and MDGI mRNA abundance were negatively correlated in the MCF7 clones (Spearman's $r = -0.9$, $P < 0.05$). Fig. 2 presents growth curves of representative MDGI-transfected and control cell lines and illustrates the effect of MDGI expression on the appearance of cells cultured on plastic and in soft agar. Transfectants expressing MDGI exhibited significant reductions in both colony formation in soft agar and in *in vivo* tumorgenicity relative to controls ($P < 0.05$, Mann-Whitney *U* test; Table 1). In nude mice, the rate of tumor formation following injection of MCF7 cells was 100% (8/8), that of MCF7/79 mock-transfected cells 88% (7/8), and that of the MDGI-transfected clones MCF7/6 and MCF7/44 25% (2/8) and 0% (0/8), respectively.

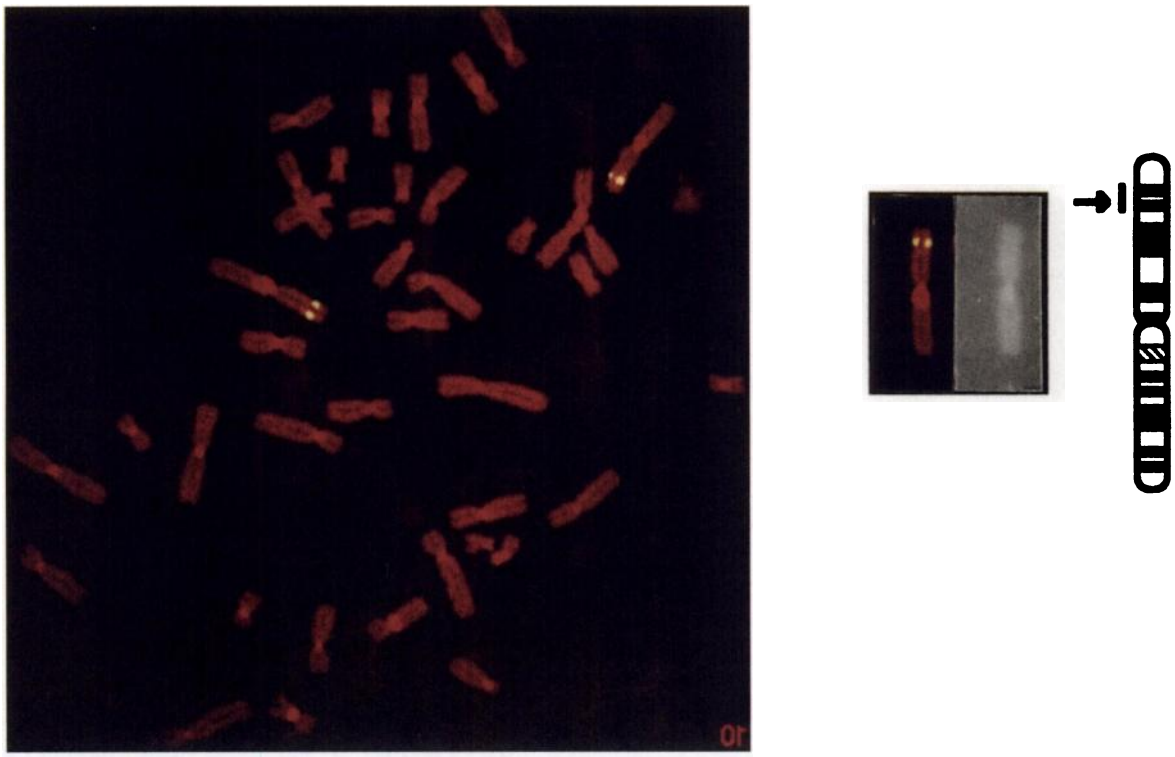

Fig. 3. Chromosomal localization of the human homologue of the bovine MDGI gene by fluorescence *in situ* hybridization analysis. *Left*, normal human metaphase with labeling of both chromosomes 1 at position p35-p33. *Right*, an enlarged chromosome 1 (before and after QFQ banding) shown next to a schematic chromosome 1 indicating the position of the human MDGI homologue.

The effect of MDGI expression on proliferation of T47D cells was similar to that seen for MCF7 cells, as shown in Table 1. However, untransfected T47D cells did not consistently display anchorage-independent growth or tumor formation in immunodeficient mice under our experimental conditions, so these end points were not determined for T47D transfectants.

Localization of the Human Gene Homologous to the Bovine *MDGI* Gene. We mapped the chromosomal location of the human homologue of bovine MDGI by fluorescent *in situ* hybridization to human chromosomes using a probe obtained by screening a human genomic library with the bovine MDGI cDNA. A single signal was detected on chromosome 1p33–35 (Fig. 3). This locus had previously been identified as a common site of loss of heterozygosity in primary human breast cancer (25–27), suggesting the presence of an important tumor suppressor gene.

Immunostaining of Normal and Neoplastic Human Breast Tissue for MDGI. As an initial step toward determining the relevance of our observations to clinical breast cancer, we immunostained sporadic human breast cancers for MDGI using the antibody utilized for the immunoblots presented in Fig. 1. In keeping with prior reports (3, 8), MDGI was present in normal epithelia, but absent from stromal elements. Immunostaining for MDGI was absent from 8 of 8 invasive ductal carcinomas. Representative photomicrographs in Fig. 4 show strong staining of normal human breast ductal epithelia and an absence of staining in neoplastic duct epithelial cells. These results motivate studies of the frequency and mechanism of silencing of *MDGI* in human neoplasia. Initial investigation of the mechanism underlying silencing of *MDGI* in experimental rat mammary cancers demonstrated hypermethylation of the gene in DNA extracted from carcinomas relative to DNA extracted from normal rat mammary epithelial cells (data not shown), suggesting that methylation analysis as well as mutational analysis will be necessary to detect abnormalities of *MDGI* in primary neoplasms, as recently demonstrated for other suppressor genes (28).

Discussion

The magnitude of the *in vivo* and *in vitro* tumor suppressor activity of *MDGI* is comparable to that previously observed for *Rb*, *p53*, and *H19* (29–33). The *1p33–35* locus to which we have mapped *MDGI* is frequently (~40% of tumors) a site of loss of heterozygosity in sporadic breast cancers (25–27), and the pathophysiological significance of loss of heterozygosity at *1p33–35* in sporadic breast cancer is supported by the finding that allele loss at the distal *1p* loci is strongly correlated with metastasis to regional lymph nodes (34).

Lack of expression of a suppressor gene can be due to mutation, deletion, inappropriate hypermethylation, or combinations of these processes (1, 28, 35). While data presented here demonstrate tumor suppressor activity of *MDGI* and show lack of expression in carcinoma cells relative to normal human breast epithelium, the frequency and mechanism of silencing of *MDGI* in human breast neoplasms remain to be defined. This will require more precise mapping of MDGI relative to distal 1p markers and description of mutations, methylation, and expression of the gene in a larger series of human cancers. Because stromal and epithelial cell populations of breast tissue clearly differ with respect to MDGI expression, it will be

necessary to compare breast carcinoma cells to normal breast epithelial cells in these studies.

The classic model predicts that tumor suppressor genes are likely to be involved in hereditary cancer syndromes. Although our results demonstrate tumor suppressor activity of *MDGI*, and the *1p33–35* locus is frequently a site of loss of heterozygosity in sporadic breast cancers, there are few data suggesting linkage of inherited breast cancer to a locus on chromosome 1p. An early study suggesting linkage between breast-ovarian cancer susceptibility and the *Rh* locus on distal 1p (36) has not been confirmed. A separate study (26) associated loss of heterozygosity on 1p in breast neoplasms with positive family history, with young age of onset, and with multiple primary tumors. However, recent linkage studies suggest that approximately two thirds of hereditary breast cancer families are linked with either the recently cloned *BrCa1* gene on chromosome 17q21 or with the *BrCa2* locus on chromosome 13 (37, 38). Evaluation of the possibility that *MDGI* is involved in a subset of hereditary breast cancers will require analysis of a group of susceptible families that are not linked to *BRCA1* or *BRCA2*. Although *BRCA1* is implicated in a majority of breast-ovarian cancer families, it has not been shown to be mutated somatically in sporadic tumors (39), despite the fact that some of these show loss of heterozygosity in the 17q21 region (40). These data may be interpreted either as calling into question the hypothesis that genes involved in hereditary breast cancer are important in suppression of sporadic tumors and *vice versa*, or as indicating that mutational analysis alone is insufficient to document molecular pathology of suppressor genes.

The growth inhibitory effects of *MDGI* transfection are greater than those previously observed (3, 4) when breast cancer cells were incubated in media containing MDGI. This may be because the physiological site of activity of MDGI is predominately intracellular, a hypothesis supported by the lack of a signal peptide for export (2), by immunolocalization studies (22, 41), and by the lack of evidence for a plasma membrane receptor for MDGI. It is possible that the modest growth inhibitory activity observed when MDGI was assayed as an extracellular growth inhibitor (3, 4) is related to passive diffusion of this lipophilic molecule across the plasma membrane.

Dietary fatty acids are ligands for hFABP (42) and have been shown to strongly influence breast cancer behavior by unknown mechanisms (43, 44). Evaluation of the possibility that binding of these fatty acids to MDGI modulates its tumor suppressor activity must await elucidation of the molecular mechanisms underlying this activity. However, in this context, it is interesting to note that MDGI and hFABP have certain properties compatible with regulatory or signal transduction functions. These include an AsnPheAspAspTyr consensus site for phosphorylation by tyrosine kinases (45), a putative DNA binding domain (45), immunocytochemical evidence of nuclear localization (22, 41), a differentiation-promoting effect on BLC6 murine pluripotent stem cells (46), and an inhibitory effect of expression of the cDNA encoding hFABP on yeast (*Saccharomyces cerevisiae*) proliferation (47).

In contrast to previously characterized tumor suppressor genes, there is evidence that, in normal breast epithelial cells, *MDGI* expression is hormonally regulated (8, 9) and highest at the time of maximum differentiated function. Therefore, *MDGI* is a candidate mediator of the differentiation-promoting effect of pregnancy on breast epithelial cells, which may underlie the protective effect of early parity on subsequent breast cancer incidence (48).

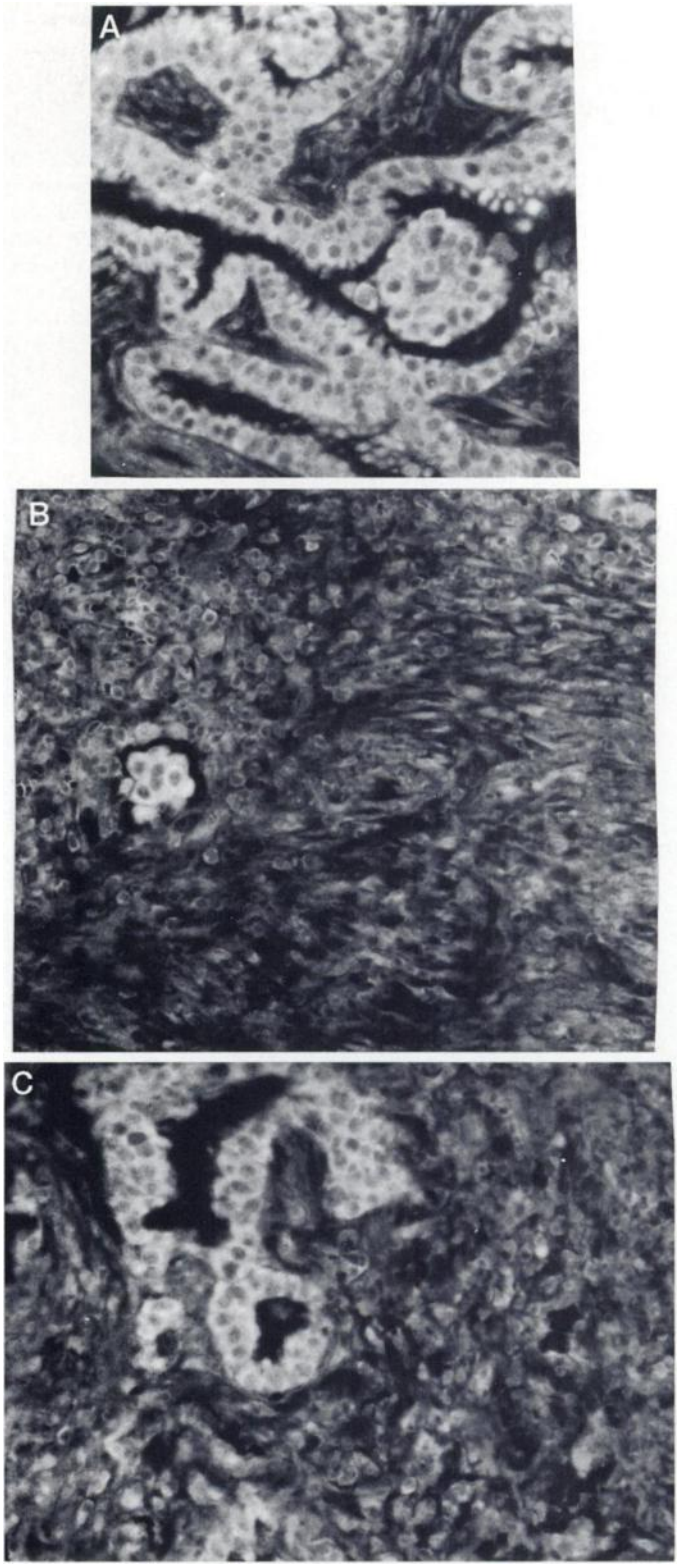

Fig. 4. Immunostaining of normal and neoplastic human breast tissue for MDGI. *A*, normal human breast, showing staining of epithelial cells. *B* and *C*, invasive ductal carcinoma, showing lack of staining of the morphologically disorganized carcinoma cells in contrast to residual areas of normal epithelial cells, which serve as internal positive controls. × 400.

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