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A mammary derived growth inhibitor related gene and a fatty acid binding protein MRG was previously identified and characterized. The present study is to test the hypothesis that MRG is a candidate mediator of the differentiating effect of pregnancy and lactation on breast epithelial cells and up-regulation of MRG expression in young nulliparous females can mimic pregnancy- and lactation-induced mammary gland differentiation and prevent breast cancer incidence. Overexpression of MRG in human breast cancer cells induced differentiation with changes in cellular morphology and a significant increase in the production of lipid droplets. Treatment of mouse mammary gland in organ culture with MRG protein resulted in a differentiated morphology and stimulation of β-casein expression. While there was no lobulo-alveolar structure in control virgin mice, expression of MRG transgene in the mammary gland of MRG transgenic mice resulted in the formation of alveolar-like structure. Consistent with the morphological change, expression of MRG also increased milk protein β-casein expression in the gland. Our results suggest that MRG is a candidate mediator of the differentiating effect of pregnancy on breast epithelial cells.

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I. INTRODUCTION

I-1. Mammary derived growth inhibitor (MDGI) Related Gene MRG. Mammary gland development is controlled by systemic hormones and by local growth factors that might complement or mediate hormonal actions. In an effort to search growth regulators in the human mammary gland, we generated cDNA libraries from a breast cancer biopsy specimen and a normal breast and analyzed these libraries by differential cDNA sequencing (1). We identified, cloned, and characterized a novel tumor growth inhibitor and named it the Mammary derived growth inhibitor-Related Gene MRG (2). The predicted amino acid sequence of MRG has a significant sequence homology to previously identified mouse mammary derived growth inhibitor **MDGI** (3). MDGI is a mammary epithelial cell growth inhibitor and differentiation factor initially identified and purified from Ehrlich ascites mammary carcinoma cells (3), and then from the lactating bovine mammary gland (4-5) and from cows milk (6). Studies of **mouse and bovine** MDGI suggest several functions of MDGI on growth and differentiation of mammary gland. MDGI specifically inhibit the growth of normal mouse mammary epithelial cells (MEC), and promote morphological differentiation: the appearance of bulbous alveolar end buds and formation of fully developed lobuloalveolar structures (7). Selective inhibition of endogenous MDGI expression in mouse MEC by use of antisense oligonucleotides suppresses alveolar budding and impairs ß-casein synthesis in organ cultures (7). Increasing amounts of MDGI mRNA were detected in terminal parts of ducts and lobuloalveolar epithelial cells of differentiated glands and maximally expressed in the terminally differentiated state found just prior to lactation (8). MDGI expression in mouse mammary epithelium cells is hormonally regulated (9-10). Many of these growth inhibition and differentiating effects of MDGI are **conserved** in MRG.

II-2. Fatty acid binding protein (FABP). Interestingly, MRG and MDGI revealed no homology to any other known growth inhibitors; rather, they revealed extensive sequence homology to FABP (11-12). A striking homology was evident between bovine MDGI and Heart type (H-) FABP, which differ only in seven positions of the amino acid sequence (13). In fact, it turned out that the originally described MDGI is a mix of H-FABP and adipocyte type (A-) FABP both expressed in mammary gland (14-15). H-FABP fully replaced the MDGI effect and inhibited the growth of mammary epithelial cells (14). Like MDGI and H-FABP, the sequence of MRG was found to be identical to the recently deposited sequences of human brain type **(B-) FABP** in GenBank (accession #AJ002962) (12). Cellular FABPs are a highly conserved family of proteins consisting of several subtypes and have been suggested to be involved in intracellular fatty acid metabolism and trafficking. Among them, only H-FABP/MDGI and the recently identified B-FABP/MRG have a differentiating effect on mammary epithelial cells and tumor suppressing activity against breast cancer. In this regard, we suggest to keep the names of MDGI and MRG when referring their functions on mammary gland and use H-FABP and B-FABP when referring their well-accepted FABP family phylogenetic tree (12).

I-3. The roles of MRG/B-FABP on mammary gland differentiation and suppression of breast cancer growth. FABPs comprise a well-established family of **cytoplasmic** hydrophobic ligand binding proteins and are thought to be involved in lipid metabolism by binding and intracellular transport of long-chain fatty acids. However, from other studies on role for FABPs in cell signaling, growth inhibition and differentiation has also been implied (12,16-17). In particular, H-FABP and B-FABP are abundantly expressed in differentiated mammary gland. It has been suggested that in heart and brain, FABPs regulate the supply of fatty acids to the mitochondria for beta-oxidation (18-19). The mammary gland, however, is a highly **lipogenic** tissue and fatty acids are not likely to be a major fuel for its metabolism. Within the phylogenetic tree of FABPs, B-FABP and H-FABP belong to a closely related subfamily of proteins that act as tumor suppressors for breast cancer (12). Therefore, MRG and MDGI could fulfill different functions in brain and heart compared with mammary gland.

MDGI/H-FABP protein was mainly detected in myocardium, skeletal and smooth muscle fibres, lipid, and steroid synthesizing cells adrenals, lactating mammary gland, and terminally differentiated epithelia of the respiratory, intestinal and urogenital tracts (20). Within the similar content, the expression of MRG was mainly detected in **brain**, **heart**, and **skeletal muscle**, which are in the postmitotic status (2). Abundant MRG protein expression was also detected in human lactating mammary epithelial cells by immunohistochemical staining (21). These results provide evidence that expression of MRG and MDGI are associated with an irreversibly **postmitotic and terminally differentiated** status of cells. It has been previously demonstrated that the expression of B-FABP (mouse MRG) is correlated with neuronal differentiation in many parts of the mouse central nervous system (22-23) and blocking antibody to B-FABP can block glial cell differentiation in mixed primary cell cultures prepared during the first postnatal week (22). In mammary epithelium, MRG also induces mammary differentiation (21). These include that (a) overexpression of MRG in human breast cancer cells induced differentiated cellular morphology and a significant increase in the production of lipid droplets and (b) treatment of mouse mammary gland in organ culture with MRGp resulted in a differentiated morphology and production of β-casein (Appendix 1). Therefore, it seems clear that a differentiation-associated function is a common property of these structurally related subfamily of FABPs. Being the members of FABP family, the most characterized biological functions for MRG/B-FABP are tumor suppressing activities against breast cancer and differentiating effect on mammary cells. These include:

1). The loss of B-FABP/MRG expression (2) and H-FABP/MDGI (24) is associated with breast cancer progression.

2). Both MRG (21) and MDGI (11,25) are highly expressed in the fully differentiated lactating mammary gland and induce mammary differentiation.

3). MRG and MDGI have been mapped at the chromosome 6q22-23 (12) and 1p35 (26) that harbor the putative tumor suppressor genes for breast cancer (27-28).

4). Both MRG and MDGI strongly suppress the growth of breast tumors (2,26).

I-4. Hypotheses

1. MRG is a candidate mediator of the differentiating effect of pregnancy and lactation on breast epithelial cells; its expression is maximal in the differentiated state and hormonally regulated.
2. Up-regulation of MRG expression in the young nulliparous MRG transgenic female mice can mimic pregnancy-and lactation-induced mammary gland differentiation and prevent breast cancer incidence.

I-5. Specific aims

SA1. MRG expression and hormonal regulation during mammary gland differentiation and lactation. We will first confirm and extend preliminary studies, which suggest that MRG is a differentiation factor and its expression is associated with mammary gland differentiation and lactation. We will then determine if differentiation-related regulation of MRG expression is specific to hormonal regulation during pregnancy and lactation or MRG in general is also regulated by non-hormone associated differentiating agents.

SA2. Prevention of breast cancer in MRG transgenic mice. We will determine if overexpression of MRG in the transgenic mice will prevent breast cancer induced by carcinogen 7,12-Dimethylbenz-[*a*]-anthracene (DMBA). We also compare the pregnancy- and lactation-induced prevention vs. the MRG-induced prevention.

II. WORK ACCOMPLISHED

II-1. Specific Aim 1: MRG expression and hormonal regulation. FINISHED (Cancer Res, Appendix 1)

A. Screening of MRG expression in clinical breast specimens. In an attempt to evaluate the potential biological significance of MRG on differentiation and lactation of human mammary gland, we studied MRG protein expression in the formalin-fixed and paraffin-embedded clinical human biopsy specimens from normal breast reduction mammoplasty specimens, lactating mammary glands, and malignant breast carcinomas. As shown in the Fig.1 of the Cancer Res paper, we found a strongly positive MRG protein staining in the alveolar mammary epithelial cells from the lactating mammary gland. The expression of MRG protein was clearly detectable in the alveolar epithelial cells in all 5 lactating mammary glands. In contrast, either no detectable MRG protein staining or very weak MRG protein expression was visualized in 8 cases of the non-pregnant normal breast reduction mammoplasty specimens from nulliparous women. Expression of MRG protein was absent in all 10 cases of malignant breast carcinomas.

B. Effects of MRG overexpression on the expression of differentiation-related milk protein genes. To investigate if the high level of MRG expression in the lactating alveolar mammary epithelial is an instigator or merely a by-product during mammary gland differentiation leading to the milk production, we investigated whether overexpression of MRG gene could induce differentiation. We transfected MDA-MB-231 human breast cancer cells with full-length MRG cDNA and established several MRG expressing clones (MRG-231 clones) (1). Fig. 3A shows the MRG protein expression in MRG-231-10 and MRG-231-6 cells, two MRG positive clones, but not in parental MDA-MB-231 and neo-231-1 MRG negative cells.

It is well established that the extracellular matrix is required for normal functional differentiation of mammary epithelia. Striking changes in cell morphology were observed when MRG-231 cells were cultured in the Matrigel coated dish. MRG-231-10 cells were aggregated to form spheroids on a reconstituted basement membrane gel (Fig. 3B), a typical differentiated phenotype for mammary epithelial cells (28). In contrast, neo-231-1 cells showed considerable heterogeneity in cell size, and many cells had "fibroblast-like" spreading morphology (Fig.3C).

We examined whether MRG-induced morphological changes are consistent with differentiation. Because the maturation of breast cells is characterized by the presence of lipid droplets that are milk components, we examined the lipid accumulation on MRG-231 cells compared with the control cells. Droplets containing neutral lipid were readily detectable in MRG-231-6 clones cultured in the non-coated culture plates; in contrast, no obvious lipid droplet could be observed in the neo-231-1 cells. When the lipid-producing cells were counted, 2 % and 5 % of MRG-231-6 and MRG-231-10 cells produced lipid droplets, respectively, but virtually no lipid producing cells were observed in MDA-MB-231 and neo-231-1 cells. When the cells cultured in the Matrigel-coated plates, a significant increase in lipid accumulation was observed in both MRG-231 cells and MRG negative control cells. Representatives of lipid staining in MRG-231-6 and neo-231-1 cells were shown in Fig. 4. Fifteen % of MRG-231-6 and 21% of MRG-231-10 cells produced lipid droplets, but only 4 % of MDA-MB-231 cells and 3 % of neo-231-1 contained lipid droplets, which were much smaller size than that of MRG positive cells (Table 1).

Induction of differentiation of mouse mammary gland by MRG recombinant protein (MRGp). Tissue-specific expression of milk protein in mammary epithelial cells depends on contact with stromal cells and matrix proteins. To further confirm the differentiating effect of MRG on mammary gland, we used the mouse whole-organ culture of mammary gland to study whether MRGp can regulate milk protein β-casein. The glands from virgin mice were cultured for 6 days with or without 50 nM MRGp. In mammary gland development, the alveolar buds represent a developmental pathway that eventually leads to secretory alveoli during functional differentiation. Histological examination of MRGp-treated glands revealed the appearance of secretory active alveoli with enlarged luminal spaces and the induction of lipid accumulation (Fig. 5, A & B). In consistent with these changes, which are characteristic for the differentiated phenotype, functional differentiation with stimulation of β-casein was also observed. While no detectable β-casein mRNA was observed in control mammary glands, expression of β-casein mRNA was significantly increased in MRGp treated glands (Fig. 5, C & D). Therefore, treatment of mouse mammary gland in organ culture with MRGp resulted in a histologically differentiated phenotype as well as functional differentiation.

C. Regulation of MRG expression by hormones. **Stimulation of MRG expression by prolactin**. Since mammary differentiation is controlled by systemic hormones, we were interested to see whether MRG expression is regulated by the hormones such as prolactin. In this regard, we tested effects of prolactin on MRG expression in T47D cells. Treatment of the cells with prolactin resulted in a 5.8-fold increase in the MRG expression (Fig. 1).

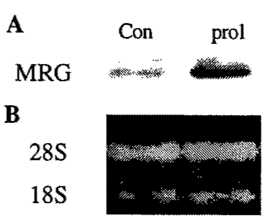


Fig. 1. Stimulation of MRG expression by prolactin. T47D cells were culture collagen coated dishes in DMEM containing 5% FCS and 5 μg/ml of insulin. Cells were treated with or without 80 IU of prolactin for 12 hr. Total RNA was isolated and analyzed (20 mg/lane) by Northern blot. The integrity and the loading control of the RNAs were ascertained by direct visualization of the 28 S and 18 S rRNA in stained gel.

II-2. Specific Aim 2: Prevention of breast cancer in MRG transgenic mice.

A. To generate transgenic mice overexpressing human MRG under the control of MMTV promoter (FINISHED).

A1. Screening, identification, and maintenance of mice heterozygous and homozygous for the transgene. Mating founder animals to wild-type (FVB/n background) males and females generated four 1st-generation transgenic lines. Transgenic males and females from the same family were mated to generate homozygous mice. If a mouse produced two or more litters of offspring that were transgenic, the mouse was considered to carry the transgene. Homozygous male and female mice from the same family were mated to each other to maintain the homozygous lines. Among the four lines, MRG mRNA expressions in mammary gland was highest in family of MM16, and progressively lower levels of MRG expression were observed in families of MM4 and MM3. Two homozygous MMTV/MRG lines from MM16 and MM4 families were generated and named as MM-H1 and MM-H2. Fig. 2 shows the transgene mRNA and protein expression in these two homozygous lines as well as two control littermates. As expected, the transgene and its protein were highly expressed in MM-H1 and MM-H2 lines. Since the transgene is driven by the MMTV promoter, which is maximally active during the pregnancy, the transgene expression could be further increased. However, as we demonstrated in Fig. 3, the expression of endogenous mouse MRG/B-FABP in pregnant mammary gland also increased considerably.

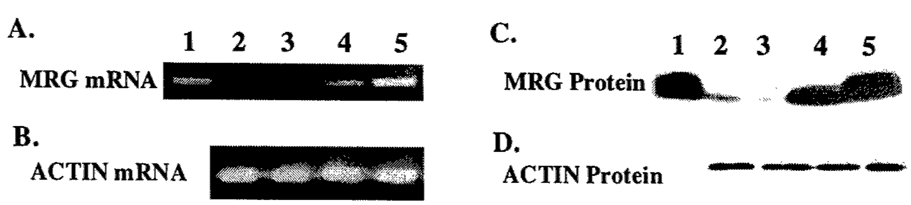


Fig. 2. MRG transgene expression in control and homozygous transgenic lines. Eight-week old virgin MM-H1 and MM-H2 mice, and age matched control virgin mice were scarified and the third pare

thoracic mammary glands were removed. The left gland was subjected to RNA isolation and RT-PCR analysis and the right gland was subjected protein isolation and Western analysis. (**A**). RT-PCR analysis of MRG using primers within MRG cording sequence (5'-GTGGAGGCTTTCTGTGCTACCTGG-3' and 5'-TGCCTTCTCATAGTGGCGAACAG-3'). The 393-bp PCR product is a specific indication of the presence of **human** MRG transgene. The integrity and the loading control of the RNA samples were ascertained by actin expression with a set of primers (5'-GCTGTGCTATCCCTGTACGC-3' and 5'-TGCCTCAGGGCAGCGGAACC-3') for 314-bp β-actin (**B**). Lane 1, T47D cells as a positive control; lane 2-3, control mice; lane 4, MM-H2; lane 5, MM-H1. Each reaction consisted of 25 cycles in the GeneAmp PCR System 2400 (Perkin Elmer). The parameters for PCR were: denaturation at 94^{0}C for 30 s; annealing and elongation at 55^{0}C for 30 s. and at 72^{0}C for 30 s. One third of the PCR products were electrophoresed through 1% agarose-TAE-gel. (**C-D**). Western analysis of MRG protein and actin expression. Western blot using the specific anti-MRG antibody was carried out as we previously described. Lane 1, 10 ng of purified recombinant MRG protein; lane 2-3, control mice; lane 4, MM-H2; lane 5, MM-H2. Lanes 2-5 contained 50 μg of cellular protein. Please note that our antibody did cross-react with mouse MRG.

<u>A2. Expression of endogenous mouse MRG/B-FABP in mammary gland of control mice</u>. To address the role of endogenous versus the transgenic MRG in breast epithelial differentiation, we analyzed the endogenous MRG expression in control virgin mouse vs. control pregnant mouse by RT-PCR (**Fig. 3**). As expected, the 550-bp endogenous **mouse** MRG was clearly present in the mammary gland during pregnancy. However, there was **a very weak** endogenous mouse MRG expression in the gland from non-pregnant virgin mouse. In a similar pattern, while expression of β-casein was abundant in the gland from pregnant mouse, it was barely detectable in the gland from control virgin mouse.

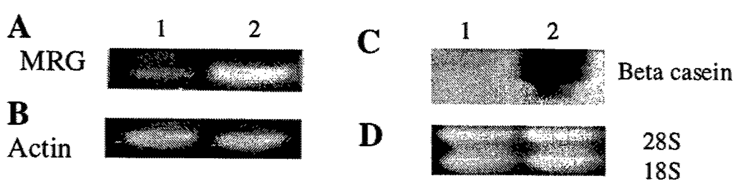


Fig. 3. Expression of mouse MRG/B-FABP and β-casein in control non-transgenic mice. Third thoracic mammary glands were isolated from 9-week old non-transgenic virgin and pregnant mice. Expression of mouse MRG mRNA (**A**) was analyzed by RT-PCR and normalized for β-actin expression (**B**). Four μg total RNA were used for RT reaction using a First-Strand cDNA synthesis kit (Boehringer Mannheim), and one tenth of this reaction was used for the PCR. The 550-bp of the mouse MRG gene was amplified by PCR with a set of primers (5'TGG TAG ATG CTT TCT GCG CA-3' and 5'TCA AAA GCA AGT TCC CAT TCA A-3'). To control for cDNA quality and quantity, a 314-bp β-actin fragment was analyzed. Densitometric scan indicates that MRG expression is increased 10-fold during pregnancy. Expression of β-casein was analyzed by Northern blot (**C**) and normalized by direct visualization of the ribosomal RNAs in stained gel (**D**).

<u>B. To determine if the effects of MRG overexpression on mammary gland development</u>.

<u>B1. Stimulation of β-casein expression</u> . To determine if the expressed transgene stimulates the functional differentiation, we developed a quick screening assay for analysis of MRG and β-casein expression by RT-PCR. **Fig. 4** shows a representative MRG transgene and β-casein expression in four virgin control mice and four randomly picked fourth generation virgin transgenic mice from MM-H1 and MM-H2 lines. While control mice did not have the transgene, all picked four transgenic pups had

transgene expression. Most importantly, all four transgenic mice also have β-casein expression, which was not detectable in control virgin mice. These results indicate that the mammary glands of the established MMTV/MRG transgenic lines MM-H1 and MM-H2 have functional expression of the transgene, which stimulates mammary gland differentiation.

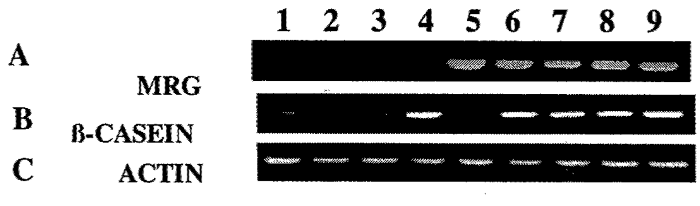
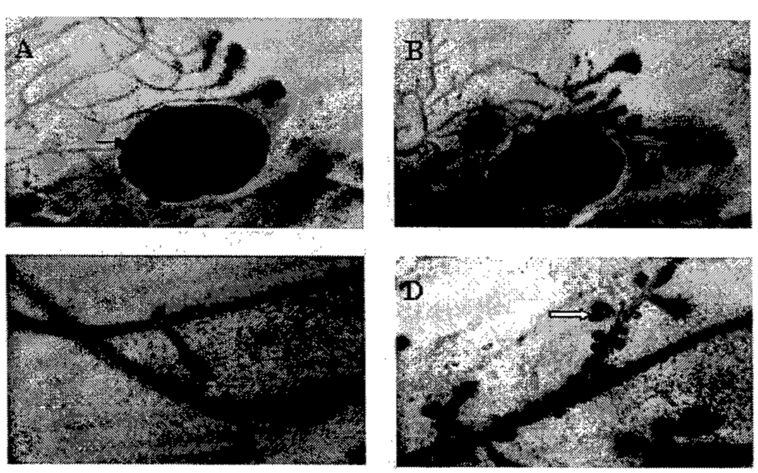


Fig. 4. RT-PCR analysis of MRG transgene and β-casein expression. Eight-week old fourth generation virgin MM-H1 and MM-H2 mice, and age matched control virgin mice and control pregnant mouse were scarified and the third pare thoracic mammary glands were removed. Expression of MRG transgene (**A**) and β-casein mRNA (**B**) was analyzed by RT-PCR and normalized for β-actin expression (**C**). RNA from T47D cells was used as a positive control for MRG expression (lane 5). RT-PCR was conducted as described in Fig. 3. The 393-bp of the human MRG was amplified by PCR with a set of primer as described in Fig. 2. The 480-bp of the mouse β-casein gene was amplified by PCR with a set of primers (5'-GTC TCT TCC TCA GTC CAA AGT-3' and 5'-TTG AAA TGA CTG GAA AGG AAA TAG-3'). **Lanes 1-4**, control mice; **lane 4**, control pregnant mouse; **lane 5**, T47D breast cancer cell; **lane 6**, MM-H1 #2; **lane 7**, MM-H1 #4; **lane 8**, MM-H2 #1, **lane 9**, MM-H2 #2.

<u>*B2. Induction of differentiated gland morphology with increased lobulo-alveoli in the gland from the transgenic line .*</u>

Because MRG protein expression was associated with human mammary gland differentiation with the highest expression observed in the differentiated alveolar mammary epithelial cells from the lactating gland, we were interested in studying whether MRG is an instigator of mammary gland differentiation or merely a correlative product during mammary gland development. The effect of transgene expression on mammary gland development and functional differentiation was assayed by morphological analyses of ductal elongation and appearance of a differentiated alveolar branching morphogenesis. While the mammary gland development starts at about 3-week old in wild-type mice with ductal elongation and development of the initial branching structure, the functional differentiation starts at the onset of pregnancy with the expansion of secretory lobulo-alveolar architect. Whole mount preparations of the mammary glands from virgin wild-type and virgin transgenic mice were examined to determine the effect of MRG on mammary gland development. Fig. 5 shows a representative mammary gland analysis of 40-day old transgenic mouse vs. wild-type control littermate. Mammary ducts in the transgenic virgin as well as in the control virgin littermate filled the typical ½ length of the inguinal gland and appeared normal (Fig. 5, compare A and B), indicating that expression of the transgene did not alter the ductal outgrowth during the early mammary gland development. However, an alternation in the developmental pattern of the distal cells of ducts in transgenic virgin mice (Fig. 5D) was observed compared with the control littermate (Fig. 5C). While the limited budding was developed in the wild-type gland (Fig. 5C), transgenic gland exhibited multiplicity



of budding (Fig. 5D).

Fig. 5. Whole mount histological analysis of mammary gland from female MM-H2 transgenic mouse and wild-type littermate. A 40-day old virgin MM-H2 mouse and a age-matched virgin wild-type littermate mouse were sacrificed, the right inguinal gland were removed and subjected to whole mount gland fix, defat, and staining. **A** & **C**, wild-type control mouse. **B** & **D**, MM-H2 transgenic mouse. **A** & **B**, lower magnification images from (Nikon, 2X10). Arrows indicate the inguinal lymph node and the direction for duct extension (from left to right). **C** & **D**, higher magnification pictures from (10X10). An open arrow indicates budding.

Using whole mount histological analysis, we performed a histological analysis of formation of lobulo-alveoli. As shown in **Fig. 6**, while there is limited lobulo-alveolar structure in the 7-week old control virgin mice (A & B), a significant increase in the formation of lobulo-alveolar structure was observed in the gland from MMTV/MRG mice (C & D). Giving the fact that mammary gland development and differentiation is controlled by **systemic hormones** and by a variety of different local growth factors that might complement or mediate hormonal actions, we are interested in comparison of the magnitude of this MRG-induced formation of alveoli to that of hormone stimulated alveoli formation. As we mentioned in the grant (p27), Russo has demonstrated that treatment of rat with human placental hormone chorionic gonadotropin (hCG) resulted in a similar effect on mammary differentiation as pregnancy. Control virgin mice were treated with hCG 20 U/day for 8 days and then the glands were histologically analyzed. As expected, hCG treatment resulted in a tremendous increase in the formation of alveoli (E & F). Although, the magnitude of MRG effect is less than that of hCG on the formation of alveoli, the MRG-induced formation of alveoli is compatible to that of hCG and is significant vs. the control virgin mice.

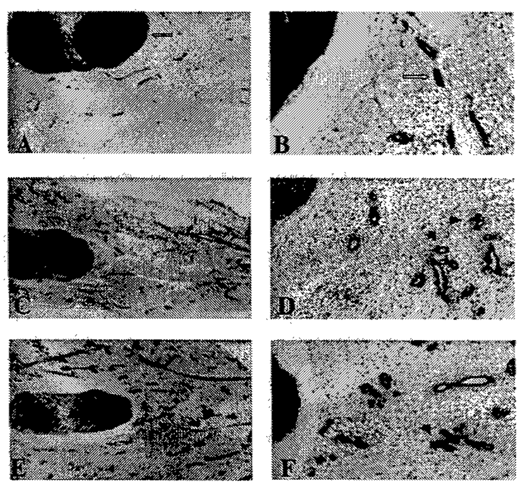


Fig. 6. Histological analysis of alveoli structure. Third pairs of mouse whole thoracic mammary glands were isolated from 7-week old female virgin mice. All the sections were stained with H&E for histological analysis. **A&B**, control mouse mammary gland. A, 2x10, an arrow indicates lymph nodes. B, 10x10, an arrow indicates ductal structure. **C&D**, MMTV/MRG mouse mammary gland. C, 2x10. D, 10x10, arrowheads indicate alveolar structure. **E&F**, mammary gland from hCG treated mouse. Six-week old mice were treated with hCG 20 U/day for 8 days and then the glands were isolated for histological analysis. E, 2x10. F, 10x10, arrowheads indicates alveolar structure.

III. KEY RESEARCH ACCOMPLISHMENTS:

1. MRG protein expression was associated with human mammary gland differentiation, with the highest expression observed in the differentiated alveolar mammary epithelial cells from the lactating gland.
2. Transfection of human breast cancer cells with MRG gene resulted in differentiated phenotypes.
3. Treatment of mouse whole mammary gland in organ culture with purified recombinant MRG protein induced gland differentiation with β-casein expression and differentiated morphology.
4. Overexpression of MRG in the mammary gland of transgenic mice resulted in β-casein expression and an increased formation of lobulo-alveoli in the gland.

IV. REPORTABLE OUTCOMES AND CONCLUSIONS:

There is an increasing public interest in the impact of pregnancy-induced differentiation on breast cancer incidence. As a hormonally related process, the evidence is now convincing, and it is widely accepted that early pregnancy and breastfeeding reduce the risk of breast cancer. Manipulation of pregnancy-like differentiation is a novel and broad approach for breast cancer prevention. Little is known about the regional and developmental expression of locally acting differentiating factors in the mammary epithelium during pregnancy. Within this content, we have previously identified, cloned, and characterized a novel growth inhibitor and a fatty acid binding protein MRG in human mammary gland.

We now report that MRG, which is highly expressed in differentiated lactating human mammary gland, induces the functional differentiation of mammary epithelial cells in cell culture and in mammary gland organ culture. We also investigated the *in vivo* role of MRG in mammary gland development and differentiation in the MMTV/MRG transgenic mice model. We demonstrated that 1) exogenous expression of MRG resulted in differentiated gland morphology with increased formation of lobulo-alveoli-like structure; and 2) consistent with the morphological change, MRG stimulated milk protein β-casein expression in the gland of the transgenic mice. MRG is a candidate mediator of the differentiating effect of pregnancy and lactation on breast epithelial cells and up-regulation of MRG expression in young nulliparous females may mimic pregnancy- and lactation-induced mammary gland differentiation and prevent breast cancer incidence. MRG can also be used as a surrogate endpoint to guide for breast cancer prevention.

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Induction of Mammary Differentiation by Mammary-derived Growth Inhibitor-related Gene That Interacts with an ω-3 Fatty Acid on Growth Inhibition of Breast Cancer Cells[1]

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ABSTRACT

We previously identified and characterized a novel tumor growth inhibitor and a fatty acid-binding protein in human mammary gland and named it the mammary-derived growth inhibitor-related gene (MRG). Here, the effects of MRG on mammary gland differentiation and its interaction with ω-3 polyunsaturated fatty acids (ω-3 PUFAs) on growth inhibition were investigated. MRG protein expression was associated with human mammary gland differentiation, with the highest expression observed in the differentiated alveolar mammary epithelial cells from the lactating gland. Overexpression of MRG in human breast cancer cells induced differentiation with changes in cellular morphology and a significant increase in the production of lipid droplets. Treatment of mouse mammary gland in organ culture with MRG protein resulted in a differentiated morphology and stimulation of β-casein expression. Treatment of human breast cancer cells with the ω-3 PUFA docosahexaenoic acid resulted in a differential growth inhibition proportional to their MRG expression. MRG-transfected cells or MRG protein treated cells were much more sensitive to docosahexaenoic acid-induced growth inhibition than MRG-negative or untreated control cells. Our results suggest that MRG is a candidate mediator of the differentiating effect of pregnancy on breast epithelial cells and may play a major role in ω-3 PUFA-mediated tumor suppression.

INTRODUCTION

MRG[3] has been cloned in normal human mammary gland by differential cDNA sequencing aimed at the identification of growth inhibitory factors of the normal mammary gland (1). The sequence of MRG was found to be identical to the recently identified human B-FABP (Ref. 2). FABPs constitute a well-established family of cytoplasmic hydrophobic ligand-binding proteins and are thought to be involved in lipid metabolism by binding and transporting long-chain fatty acids intracellularly. However, other studies have implicated different roles for FABPs in cell signaling, growth inhibition, and differentiation (3–6). In particular, H-FABP, also known as MDGI, is abundantly expressed in differentiated lactating mammary gland and has been shown to inhibit growth of breast cancer cells (7–9). Among several subtypes of FABPs, only MRG/B-FABP and the previously identified H-FABP/MDGI have tumor-suppressing activity against breast cancer (2). These include the loss of MDGI (10) and MRG expression (1) during breast cancer progression, an inhibitory effect on proliferation of breast cancer cells (1, 7–10), and suppression of breast tumor growth in the mammary fat pad nude mouse model (1, 11). In addition, the expression of both MRG (1) and MDGI (6) was mainly detected in myocardium, brain, and skeletal muscle, which are associated with an irreversibly postmitotic and terminally differentiated status of cells.

It is well established that ω-3 PUFAs, primarily DHA and EPA in fish oil, suppress mammary tumorigenesis *in vivo* and breast cancer cell proliferation *in vitro* (12–21). As a member of FABP, it has been reported that ω-3 PUFA DHA is the physiological ligand for mouse MRG (B-FABP), based on its high binding affinity (K_d = 10 nM; Ref. 22). We have demonstrated that the gene encoding MRG has a strong tumor suppressor activity (1). The magnitude of the tumor-suppressing activity of MRG on mammary tumor is comparable to that observed previously for *Rb* and *p53* (23). In the current study, we investigated the effects of MRG on mammary differentiation and its interaction with DHA on the growth of breast cancer cells. Our data suggest that MRG is a differentiation factor for breast epithelial cells and that it may play a major role in DHA-mediated growth suppression of breast cancer cells.

MATERIALS AND METHODS

Cell Culture. Human breast cancer cell lines MDA-MB-231, MDA-MB-436, and MDA-MB-468 were maintained in DMEM containing 5% FCS.

Preparation of Anti-MRG Antibody. A peptide sequence corresponding to amino acids 43–57 (1) was chosen for developing of the antibody because of its unique sequence for MRG. The peptide synthesis, purification, conjugation, and immunization of rabbits were conducted as we described previously (24). For final purification, a MRG peptide affinity column was made by conjugating 20 mg of MRG peptide to 5 ml of Aminolink resin (Pierce Chemical Co.), using sodium cyanoborohydride (Sigma).

Immunohistochemical Staining. As we described previously (1, 25), deparaffinized, rehydrated, and acid-treated human breast sections (5 μm thick) were treated with H_2O_2 and trypsin, and blocked with normal goat serum. Sections were incubated with a specific anti-MRG polyclonal antibody (1 μg/ml) at 4°C overnight, followed by incubation with biotin-conjugated secondary antirabbit antibodies (DAKO). The colorimetric detection was performed using a standard indirect streptavidin-biotin immunoreaction method with DAKO's Universal LSAB Kit according to the manufacturer's instructions. There were some variations in staining intensity for MRG expression among the specimens. The negative cases were confirmed with at least two independent experiments. All stainings were reviewed by two pathologists.

Preparation of MRGp. The full-length MRG was amplified using standard PCR techniques with primers corresponding to the 5′ and 3′ sequences of the gene (5′ primer, GGATCCCGTGGAGGCTTTCTGT; 3′ primer, GGTACCCCAGGGACATTTTTA). The amplified fragment was gel-purified, and the DNA sequence was confirmed. As we described previously (24), a baculovirus expression vector, pA2-GP, was used to transform Sf9 cells. The purification of MRGp was performed as follows: (*a*) Medium supernatant, adjusted to pH 5.5, was first applied to tandem Poros HS/HQ columns (PerSeptive Biosystems) preequilibrated with 50 mM NaOAc (pH 5.5). (*b*) MRGp, collected in the flowthrough fraction, was adjusted to pH 8.0 and reapplied to the tandem Poros HS/HQ column preequilibrated with 20 mM Tris-HCl (pH 8.0). (*c*) MRGp, collected in the flowthrough fraction, was concentrated 50-fold, using a Filtron 3000 M_r cutoff tangential-flow system and then separated on a Superdex-75 size-exclusion column equilibrated with 10 mM NaOAc (pH 6.5). (*d*) Pooled

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[3] The abbreviations used are: MRG, mammary-derived growth inhibitor-related gene; B-FABP, brain-type fatty acid-binding protein; H-FABP, heart-derived FABP; MDGI, mammary-derived growth inhibitor; PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MRGp, recombinant MRG protein.

MRGp fractions were applied to a hydroxyapatite column equilibrated with 10 mM NaOAc (pH 6.5); the weakly bound MRGp was eluted with 7.5 mM K_2HPO_4 (pH 6.8). (*e*) MRGp fractions were then separated on a Superdex-75 size-exclusion column equilibrated with 65 mM Na_2HPO_4, 100 mM NaCl (pH 7.2). MRGp fractions were pooled and found to be >98% pure by SDS-PAGE with an endotoxin level <0.5 endotoxin units/mg. Purified MRGp was identified as a single band at 18 kDa in the SDS-PAGE by silver staining. The protein was analyzed for glycosylation by determining the monosaccharide content in a purified preparation, and the N-linked sugar chains were confirmed.

Cell Morphology on Matrigel. Cell morphology was determined using Matrigel-coated wells. Briefly, 6-well culture plates were coated with growth factor-reduced Matrigel (Collaborative Research) at 0.5 ml/well. Cells were then cultured in the coated wells with DMEM containing 5% fetal bovine serum. The cell morphology was observed under the microscope after 4 days.

Detection of Cytoplasmic Lipids in Breast Cancer Cells. Lipid accumulation was detected by oil red *O*-isopropanol staining as described previously (26). The cells were cultured on either Matrigel-coated plates or regular uncoated plates. After 4 days, the cells were fixed by 10% formaldehyde and subjected to oil red *O*-isopropanol staining. Accumulated lipids in the cells were stained red, and nuclei were stained blue by hematoxylin. Three independent observers counted the positive cells, and each observer randomly counted three fields (×40). The numbers represent the average percentage of lipid accumulate cells from nine fields (×40).

Western Analysis. Western blot analysis was conducted as we described previously (24). Briefly, the blot was incubated with anti-MRG primary antibody (1:800 dilution) overnight at 4°C, and then incubated with goat antirabbit IgG-horseradish peroxidase (1:6000 dilution) for 1 h, washed, and visualized by chemiluminescence.

Mammary Gland Organ Culture. Whole second thoracic mammary glands were removed from 7- and 10-week-old virgin female mice (FVB/n background) as described previously (27). The glands were cultured in medium 199 containing 5% FCS, with medium changed every 2 days. The medium was supplemented with following components from Clonetics: bovine pituitary extract (52 μg/ml), insulin (5 μg/ml), epidermal growth factor (10 ng/ml), and hydrocortisone (1 μg/ml).

***In Vitro* Assay for Cell Growth.** Cells were seeded in triplicate at 3000 cells/well (24-well plate) in 1 ml of DMEM-5% serum. For treatments with DHA or MRGp, cells were cultured in DMEM-1% serum. Cell growth was measured using the CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay Kit (Promega Corporation, Madison, WI).

Statistical Analysis. Values were expressed as means ± SD. Statistical comparisons were made using the two-tailed Student's *t* test.

RESULTS

Association of MRG Expression with Mammary Gland Lactation. In an attempt to evaluate the potential biological significance of MRG on the differentiation and lactation of the human mammary gland, we studied MRG protein expression in formalin-fixed, paraffin-embedded clinical human biopsy specimens from normal breast reduction mammoplasty specimens, lactating mammary glands, and malignant breast carcinomas.

Fig. 1 shows a representative immunohistochemical staining for MRG. The terminally differentiated lactating mammary gland is characterized by ducts branching into distended and large lipid-rich active secretory lobuloalveolar structures. An increase in cell volume as a

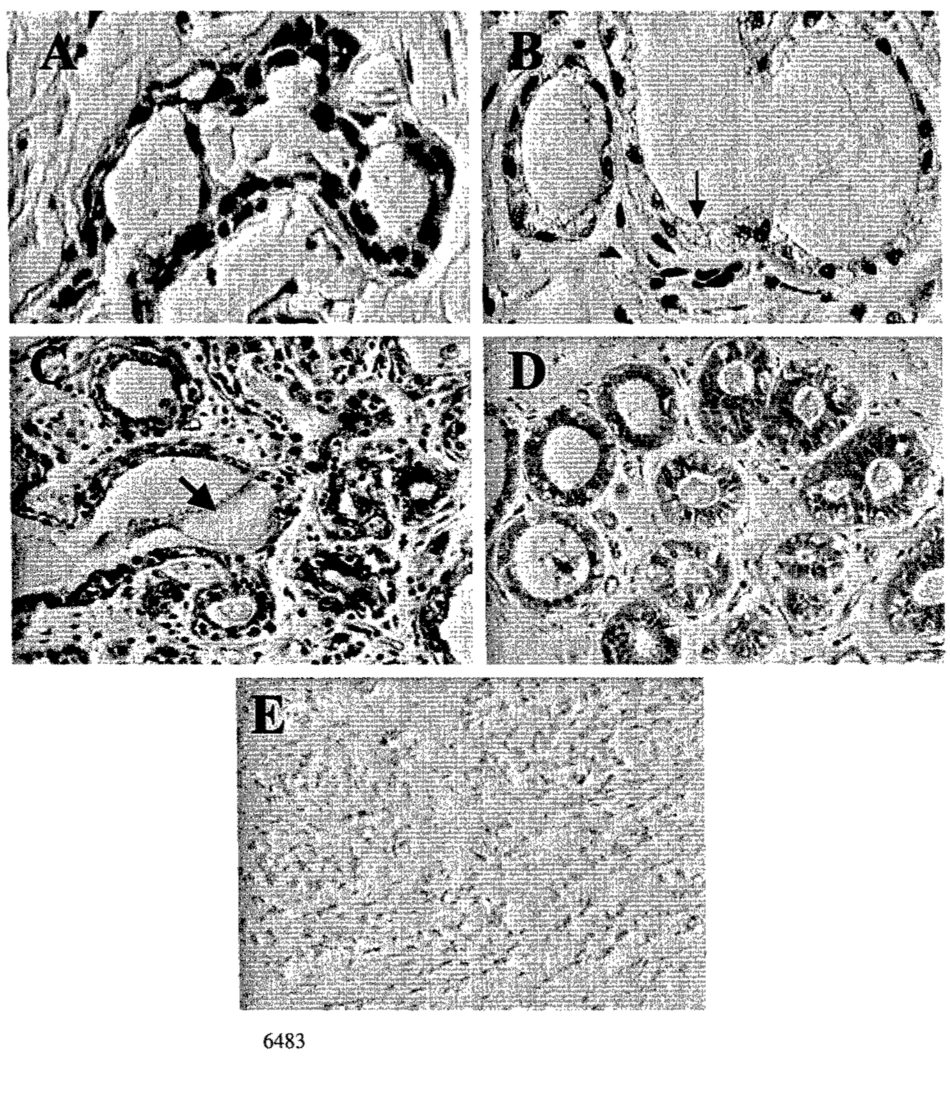

Fig. 1. Analysis of MRG protein expression on human breast tissues by immunohistochemical staining. Sections in *panels A* and *B* were stained with H&E with no immunohistochemical staining. Sections in *panels C–E* were stained immunohistochemically, with *brown* indicating MRG protein expression in mammary epithelial cells. All sections in *C–E* were also counterstained lightly with hematoxylin for viewing non-MRG-stained cells. *A*, epithelial cells in normal nonlactating lobules from a normal breast reduction mammoplasty specimen (×40). *B*, epithelial cells in lactating lobules from a needle biopsy specimen (×40). The differentiated lactating mammary epithelial cells have much diluted cytoplasm containing large lipid-rich secretory vacuoles (*arrow*). *C*, epithelial cells from lactating lobules showed very strong MRG staining (×10). The specimen was from a 32-year-old lactating woman. The presence of vesicles containing milk protein (*arrow*) was noted. A serial slide from the same block was also incubated with nonimmunized control IgG, and no detectable background staining was observed at the same conditions as for the anti-MRG antibody. *D*, negative staining of normal lobular epithelial cells from a 25-year-old nulliparous woman with breast reduction mammoplasty (×10). *E*, negative staining of MRG in a highly infiltrating breast carcinoma (×10). A total of 23 clinical breast specimens were analyzed: 5 of 5 lactating samples were strongly positive; 10 of 10 infiltrating breast cancer samples were negative; 5 of 8 normal breast reduction mammoplasty samples were negative, and the remaining 3 normal breast samples were weakly positive.

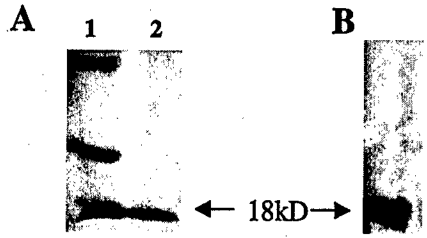

Fig. 2. Purity and immunoreactivity of the purified MRGp. *A*, SDS-PAGE of purified MRGp. *Lane 1*, molecular mass markers; *Lane 2*, MRGp (50 ng). The homogeneity of the purified MRGp was revealed by silver staining. *B*, immunoblot with a specific anti-MRG antibody. The gel contained 30 ng of MRGp.

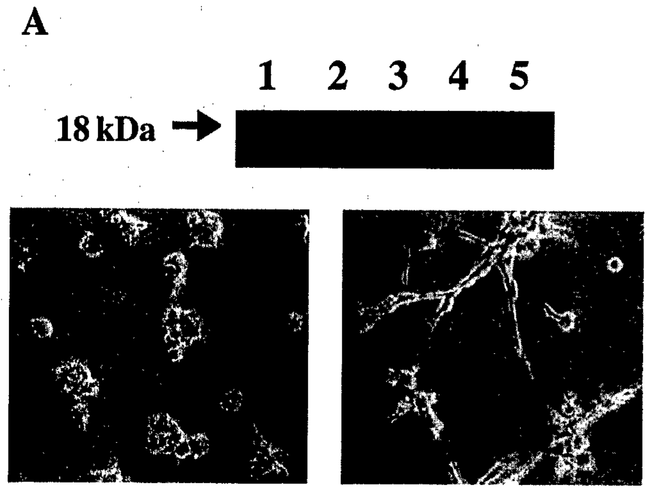

Fig. 3. Analysis of MRG expression and cell morphology. *A*, Western blot analysis of MRG protein expression. Total protein was isolated and normalized, and 25 μg of total cellular protein were subjected to Western analysis with a specific MRG antibody. *Lane 1*, 60 ng of purified recombinant MRG protein; *Lane 2*, MRG-231-10; *Lane 3*, MRG-231-6; *Lane 4*, parental MDA-MB-231; *Lane 5*, neo-231-1. For morphology analysis, cells were culture on Matrigel-coated chamber slides for 6 days. *B*, MRG-231-10 cells were aggregated and formed spheroids. *C*, neo-231-1 cells had spreading morphology.

result of cytoplasmic vacuolation and the presence of secretory vesicles containing milk proteins was clearly noted in the lactating gland (Fig. 1*B*). We found strongly positive MRG protein staining in the alveolar mammary epithelial cells from the lactating mammary gland (Fig. 1*C*). The expression of MRG protein was clearly detectable in the alveolar epithelial cells in all five lactating mammary glands. In contrast, either no detectable MRG protein staining or very weak MRG protein expression was visualized in eight of the nonpregnant normal breast reduction mammoplasty specimens from nulliparous women (Fig. 1*D*). Expression of MRG protein was absent in all 10 cases of malignant breast carcinomas (Fig. 1*E*).

Expression and Purification of MRGp. Active MRGp is required to test its function on mammary epithelial cells. We expressed and purified MRGp prepared from baculovirus-infected Sf9 cells (see "Materials and Methods"). When analyzed by SDS-PAGE, the purified protein showed a single band at molecular mass of 18 kDa (Fig. 2*A*). The purified 18-kDa protein was confirmed as MRG by Western blot using a specific anti-MRG antibody (Fig. 2*B*).

Induction of Differentiation of Breast Cancer Cells. To investigate whether the high level of MRG expression in the lactating alveolar mammary epithelial is an instigator or merely a by-product of mammary gland differentiation leading to milk production, we investigated whether overexpression of the MRG gene could induce differentiation. We transfected MDA-MB-231 human breast cancer cells with full-length MRG cDNA and established several MRG-expressing clones (MRG-231 clones; Ref. 1). Fig. 3*A* shows the MRG protein expression in MRG-231-10 and MRG-231-6 cells, two MRG-positive clones, but not in parental MDA-MB-231 and neo-231-1 MRG-negative cells.

It is well established that the extracellular matrix is required for normal functional differentiation of mammary epithelia. Striking changes in cell morphology were observed when MRG-231 cells were cultured in the Matrigel-coated dish. MRG-231-10 cells were aggregated to form spheroids on a reconstituted basement membrane gel (Fig. 3*B*), a typical differentiated phenotype for mammary epithelial cells (28). In contrast, neo-231-1 cells showed considerable heterogeneity in cell size, and many cells had "fibroblast-like" spreading morphology (Fig. 3*C*).

We examined whether MRG-induced morphological changes are consistent with differentiation. Because the maturation of breast cells is characterized by the presence of lipid droplets that are milk components, we examined lipid accumulation in MRG-231 cells compared with the control cells. Droplets containing neutral lipid were readily detectable in MRG-231-6 clones cultured in the uncoated culture plates; in contrast, no obvious lipid droplet could be observed in the neo-231-1 cells. When the lipid-producing cells were counted, 2 and 5% of MRG-231-6 and MRG-231-10 cells, respectively, produced lipid droplets, but virtually no lipid-producing cells were observed in MDA-MB-231 and neo-231-1 cells. When the cells were cultured in the Matrigel-coated plates, a significant increase in lipid accumulation was observed in both MRG-231 cells and MRG-negative control cells. Representative samples of lipid staining in MRG-231-6 and neo-231-1 cells are shown in Fig. 4. Fifteen percent of MRG-231-6 and 21% of MRG-231-10 cells produced lipid droplets, but only 4% of MDA-MB-231 cells and 3% of neo-231-1 contained lipid droplets, which were much smaller in size than those of MRG-positive cells (Table 1).

Induction of Differentiation of Mouse Mammary Gland by MRGp. Tissue-specific expression of milk protein in mammary epithelial cells depends on contact with stromal cells and matrix proteins. To further confirm the differentiating effect of MRG on mammary gland, we used whole-organ culture of mouse mammary glands to study whether MRGp can regulate milk protein β-casein. The

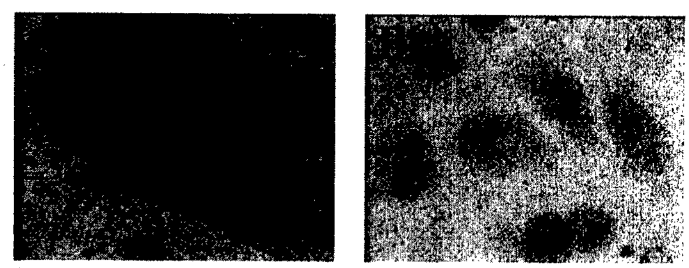

Fig. 4. Stimulation of lipid accumulation by MRG. Cells were cultured on Matrigel-coated dishes for 4 days. *A*, a representative field for MRG-231-10 cells (×40). *B*, a representative field for neo-231-1 cells (×40). *Darker areas* indicate lipid staining.

Table 1 *Effects of MRG on the lipid accumulation of MDA-MB-231 cells*

Cells were cultured either on Matrigel-coated plates or uncoated plates for 4 days, fixed, and subjected to oil red *O*-isopropanol staining. All slides were also counterstained lightly with hematoxylin for viewing nuclei. The positive cells were counted randomly in three fields (×40), with each field containing 150 cells. Three observers counted a total of 1350 cells. The numbers represent the average percentage ± SE of lipid accumulated cells from nine fields.

Cell lines	Lipid droplets in uncoated dish, lipid-producing cells/total (%)	Lipid droplets in Matrigel-coated dish, lipid-producing cells/total (%)
MDA-MB-231	0.2 ± 0.02	4 ± 0.9
neo-231-1	0.08 ± 0.01	3 ± 0.8
MRG-231-6	2 ± 0.4	15 ± 3.2
MRG-231-10	6 ± 1.8	24 ± 4

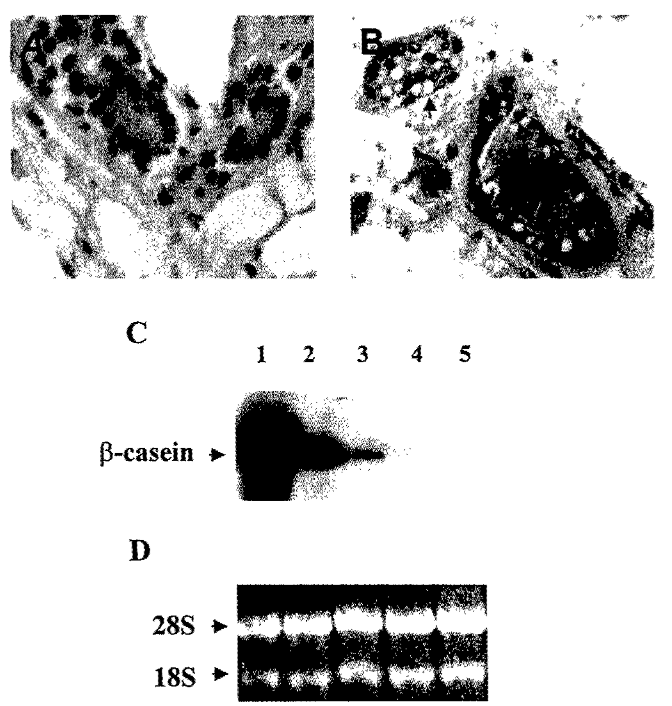

Fig. 5. Effects of MRGp on mammary gland morphology and β-casein expression. Second pairs of mouse whole thoracic mammary glands were cultured for 6 days with or without 50 nM MRGp in medium supplemented with bovine pituitary extract, insulin, epidermal growth factor, and hydrocortisone as described in "Materials and Methods." Fresh medium containing MRGp was added every 2 days. Half of the gland was subjected to fixing, sectioning, and histological analysis (*A* and *B*), and the other half was subjected to RNA extraction for Northern analysis of β-casein expression (*C* and *D*). Mammary gland histological analysis: *A*, control (×20); *B*, MRGp-treated (×20). The fat droplets accumulated in MRGp-treated alveolar epithelial cells were observed (*arrows*). Expression of β-casein mRNA (*C*) was analyzed by Northern blot and normalized by visualization of ribosomal bands (*D*). *Lane 1*, mammary gland from pregnant mouse as a positive control for β-casein; *Lanes 2* and *3*, MRGp-treated mammary glands in organ culture; *Lanes 4* and *5*, control untreated glands in organ culture. Mammary glands in *Lanes 2* and *4* were derived from a 10-week-old virgin mouse; mammary glands in *Lanes 3* and *5* were derived from a 7-week-old virgin mouse.

glands from virgin mice were cultured for 6 days with or without 50 nM MRGp. In mammary gland development, the alveolar buds represent a developmental pathway that eventually leads to secretory alveoli during functional differentiation. Histological examination of MRGp-treated glands revealed the appearance of secretory active alveoli with enlarged luminal spaces and the induction of lipid accumulation (Fig. 5, *A* and *B*). Consistent with these changes, which are characteristic for the differentiated phenotype, functional differentiation with stimulation of β-casein was also observed. Although no detectable β-casein mRNA was observed in control mammary glands, expression of β-casein mRNA was significantly increased in MRGp-treated glands (Fig. 5, *C* and *D*). Therefore, treatment of mouse mammary gland in organ culture with MRGp resulted in a histologically differentiated phenotype as well as functional differentiation.

Interaction of the ω-3 PUFA DHA and MRG on Cell Growth. Because MRG is a fatty acid-binding protein with the highest binding affinity to the ω-3 PUFA DHA, we were interested in studying whether the growth-suppressing effect of DHA is mediated in part by MRG. We first studied the effects of DHA on MRG-negative MDA-MB-231 cells. The cells were treated with DHA at doses of 2, 4, 6, 8, and 12 μg/ml for 4 days, with fresh DHA added every 2 days. A very narrow dose-dependent growth inhibition was observed for DHA (Fig. 6*A*). Although no significant growth inhibition was observed for DHA at a dose of 2 μg/ml, 71 and 92% growth inhibition was observed at doses of 8 and 12 μg/ml, respectively. We therefore chose the non-inhibiting DHA dose of 2 μg/ml to test its growth-regulatory effect on MRG-positive *versus* MRG-negative cells. As demonstrated in Fig. 6*B*, when the cells were treated with 2 μg/ml DHA, 55 and 47% growth inhibition was observed in MRG-231-6 and MRG-231-10 MRG-transfected cells, respectively. However, no growth inhibition was observed in MRG-negative parental MDA-MB-231 cells and neo-231-1 cells. We also studied the effect of the ω-6 fatty acid linoleic acid on the growth of MDA-MB-231 cells. At the same conditions as for the ω-3 fatty acid DHA, no significant growth effect was observed at the similar dose range between 4 to 20 μg/ml (data not shown).

To further confirm the synergistic interaction of MRG expression and DHA on growth inhibition, we treated MRG-negative MDA-MB-436 and MDA-MB-468 cells with DHA and MRGp. MRGp treatment induced dose-dependent growth inhibition in MDA-MB-436 breast cancer cells (Fig. 7*A*). Although no significant growth inhibition was observed when the MRGp dose was <50 nM, 10 and 14% growth inhibition was observed when cells were treated with 50 and 80 nM MRGp, respectively. At 150 nM MRGp, growth was inhibited 58%. A submaximal MRG dose of 80 nM was used to test the interaction between MRG and DHA. Treatment of MDA-MB-436 (Fig. 7*B*) and MDA-MB-468 (Fig. 7*C*) cells with 80 nM MRGp resulted in either a slight inhibition or a slight stimulation of cell growth, respectively. When the cells were treated with MRGp together with DHA, a significant synergistic growth inhibition was observed. The growth of MDA-MB-436 cells was inhibited by 63% when the cells were treated with DHA and MRGp, compared with 18% inhibition with DHA alone. Similarly, the growth of MDA-MB-468 cells was inhibited by 80% with DHA and MRGp, compared with 22% inhibition with DHA alone.

DISCUSSION

MRG, identified and cloned by a differential cDNA sequencing approach as a novel human breast cancer growth inhibitor (1), has

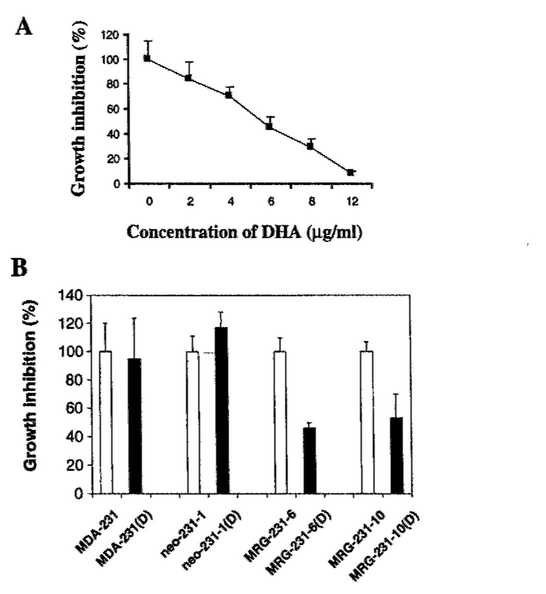

Fig. 6. Differential growth inhibition by DHA on MRG-positive and MRG-negative breast cancer cells. Cells were cultured in DMEM containing 1% FCS and treated with DHA at different concentrations for 4 days. Medium containing fresh DHA was added every 2 days. Cell growth was measured as described in "Materials and Methods." *A*, dose-response curve of DHA on MDA-MB-231 cells. *B*, effect of DHA on MRG-positive and -negative cells. The cells were treated (*filled columns*) or not treated (*open columns*) with 2 μg/ml DHA. All values were normalized to the percentage of untreated control cells, which was taken as 100%. The numbers in both *A* and *B* represent the means of three cultures; *bars*, SE.

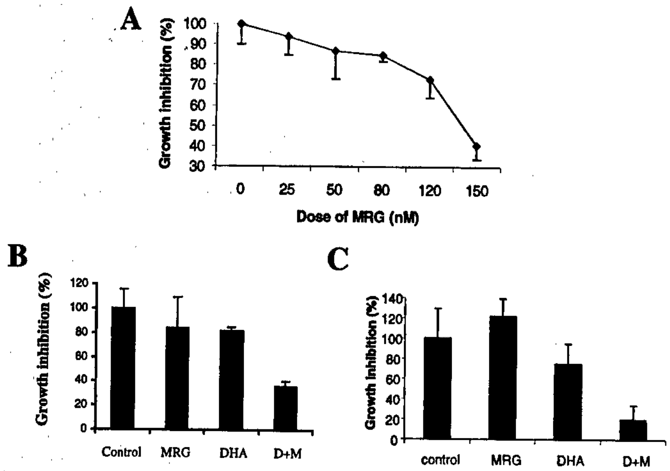

Fig. 7. Synergistic effects of MRGp and DHA on growth inhibition. All cells were cultured in DMEM containing 1% FCS. *A*, MDA-MB-436 cells were cultured with different doses of MRG for 4 days. MDA-MB-436 (*B*) and MDA-MB-468 (*C*) cells were treated with 80 nM MRGp, 2 μg/ml DHA, or MRGp plus DHA (*D* + *M*) for 4 days. Medium containing fresh MRGp and DHA was added every 2 days. All values were normalized to the percentage of untreated control cells, which was taken as 100%. The numbers in both represent the means of three cultures; *bars*, SE. Statistical comparisons for both cell lines treated with DHA and MRGp relative to the cells treated with DNA alone indicated $P < 0.001$ for growth inhibition.

sequence identical to that of the recently identified B-FABP (2). MRG/B-FABP has no sequence homology to any of the hitherto known growth inhibitors. The exact function of B-FABP has not been identified. Cellular fatty acid-binding proteins are a highly conserved family of proteins involved in intracellular fatty acid metabolism and trafficking. It has been suggested that in brain and heart, B-FABP and H-FABP regulate the supply of fatty acids to the mitochondria for β-oxidation (29, 30). The mammary gland, however, is a highly lipogenic tissue, and fatty acids are not likely to be a major fuel for its metabolism. Therefore, MRG/B-FABP and MDGI/H-FABP could fulfill different functions in mammary gland compared with brain and heart. We demonstrated that (*a*) MRG expression was associated with human mammary gland differentiation, with the highest expression in the terminally differentiated alveolar mammary epithelial cells from the lactating gland, and (*b*) that MRG induced differentiation of mammary epithelial cells.

MRG protein expression was undetectable in breast carcinomas by immunohistochemical staining, which is consistent with the previous *in situ* hybridization data on the loss of MRG transcription in breast carcinomas. Although in the previous *in situ* hybridization analysis, MRG transcripts could be detected in the epithelial cells from normal mammary glands (1), in the current immunohistochemical analysis of MRG protein expression, MRG protein staining was either very weak or undetectable in nondifferentiated mammary glands from nulliparous women. This discrepancy may reflect the different sensitivities of the more sensitive *in situ* hybridization *versus* the less sensitive immunohistochemical staining. Alternatively, the tested different normal breast specimens may represent different stages of differentiation. It is also possible that this discrepancy between the *in situ* hybridization and immunohistochemical staining is attributable to the fact that the message may not be translated. Nevertheless, addition of MRGp to cultures of breast cancer cells and to organ cultures of mouse mammary gland induced growth inhibition and gland differentiation. Although the mechanism for cellular uptake of MRGp is not clear, it is likely that MRGp diffuses through the membrane because of its very hydrophobic and lipogenic nature. In fact, some FABPs such as H-FABP (MDGI) can be secreted and detected in milk (9).

In addition to the differentiating effect on mammary gland, the expression of MRG also correlates with neuronal differentiation in many parts of the mouse central nervous system (31, 32). Furthermore, blocking antibody for MRG/B-FABP can block glial cell differentiation (31). MDGI/H-FABP protein has been detected mainly in myocardium, skeletal and smooth muscle fibers, lipid and steroid-synthesizing cells of adrenals, lactating mammary gland, and terminally differentiated epithelia of the respiratory, intestinal, and urogenital tracts (6). The results provide evidence that expression of MDGI is associated with an irreversibly postmitotic and terminally differentiated status of cells. Therefore, it seems clear that a differentiation-associated function is a common property of this structurally related subfamily of FABPs.

It is well established that the ω-3 fatty acids DHA and EPA, found in fish oil, have a suppressive effect on tumor growth and particularly on mammary tumorigenesis. Epidemiological studies (33–37) support a role for ω-3 fatty acids as adjunct therapy in the prevention and treatment of breast cancer. This protective effect of ω-3 PUFAs can be demonstrated in animal models with carcinogen-induced mammary tumors in mouse and rat and mammary xenografts in nude mice (14–19). Various mechanisms have been proposed to explain the tumor-suppressive activity of ω-3 PUFAs; of special interest are alteration of the oxidative metabolism of arachidonic acid via the cyclooxygenase pathway (35) and changes in lipoxygenase activity (reviewed in Ref. 36). Lipid peroxidation, the oxidation of long-chain PUFAs, can produce an array of secondary products of lipid oxidation that may possess cytostatic or cytolytic capacity. It has been proposed that DHA and EPA can both directly and indirectly modulate gene expression (38). The direct effects of DHA and EPA are most probably mediated by their ability to bind to positive and/or negative regulatory transcription factors, whereas the indirect effects appear to be mediated through alterations in the generation of intracellular lipid second messengers.

At present, the mechanisms by which DHA exerts its tumor suppressing activity remain controversial and unknown. As a newly identified fatty acid-binding protein and a growth differentiation factor for mammary cells, we have demonstrated here that treatment of human breast cancer cells with DHA resulted in differential growth inhibition proportional to the MRG expression in the cells: MRG-positive cells or MRGp-treated cells were much more sensitive to DHA-induced growth inhibition than MRG-negative cells or control, untreated cells. Our data suggest that the growth-suppressing activity of DHA on breast cancer cells may be mediated in part by MRG and presumably by MRG-induced differentiation. This hypothesis is also supported by a previous report that DHA has the highest binding affinity for mouse B-FABP (MRG), suggesting that the physiological ligand for MRG is the ω-3 PUFA DHA (22).

The impact of pregnancy and lactation on breast cancer risk recently has been of great interest in terms of breast cancer prevention. As hormonally related processes, it is widely accepted that pregnancy at an early age and breastfeeding reduce the risk of breast cancer (39–42). The possibility of preventing breast cancer by manipulation of these processes with hormones or dietary factors such as ω-3 PUFAs that mimic the differentiating effect is a novel and manipulable approach to breast cancer intervention and prevention. However, little is known about the regional and developmental expression of locally acting growth factors and differentiating factors in the mammary epithelium during pregnancy and lactation. Within this context, MRG could play a role in both mammary gland differentiation and ω-3 PUFA-mediated antitumor effect. The potential application of MRG as a biomarker for mammary gland differentiation to assess the efficiency of differentiation-based breast cancer chemoprevention and to predict tumor-suppressive response to ω-3 PUFAs warrants further investigation.

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