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Award Number: DAMD17-00-1-0309

TITLE: Omega-3 Fatty Acids and a Novel Mammary Derived Growth Inhibitor Fatty Acid Binding Protein MRG in Suppression of Mammary Tumor

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REPORT DATE: July 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual (1 Jul2000 - 30 Jun 2003)

4. TITLE AND SUBTITLE
Omega-3 Fatty Acids and a Novel Mammary Derived Growth Inhibitor Fatty Acid Binding Protein MRG in Suppression of Mammary Tumor

5. FUNDING NUMBERS
DAMD17-00-1-0309

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

A mammary derived growth inhibitor related gene (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 a candidate mediator of the tumor suppressing effect of n-3 fatty acid DHA on mammary tumors. MRG induces differentiation of mammary epithelial cells *in vitro* and its expression is associated with mammary differentiation. 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 in the transgenic mouse 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. Treatment of human breast cancer cells with ω-3 PUFA DHA resulted in a differential growth inhibition proportional to their MRG expression. MRG transfected cells or MRG protein treated cells were much more sensitive to DHA-induced growth inhibition compared with MRG negative or control non-treated 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.

14. SUBJECT TERMS
MRG, n-3 fatty acid, mammary differentiation

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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A. INTRODUCTION

A-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.

A-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).

A-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 3). 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).

A-4. High affinity binding of DHA to B-FABP/MRG. Being a number of FABP family, among several PUFAs, DHA has highest ligand binding affinity for mouse MRG/B-FABP (K_d 10 nM) (29). These data suggest that DHA is the physiological ligand for MRG/B-FABP, since its binding affinity is the highest yet reported for B-FABP/ligand interaction, exceeding even the affinity of retinoic acid for its binding proteins (29). Although n-3 PUFAs DHA has been suggested as an adjunct therapy in prevention and treatment of breast cancer (30), its cellular interaction is currently unknown. We demonstrated a differential inhibitory effects of DHA on human breast cancer cells in respect to MRG expression: MRG positive cells or MRG treated cells are more sensitive to DHA-induced growth inhibition than MRG negative and control non-treated cells . These results suggest that the tumor suppressing activity of DHA on mammary gland may be mediated in part by MRG.

B. WORK ACCOMPLISHED

Specific Aim 1: *In vitro* study of differential growth inhibition of DHA and EPA on human breast cancer cells in respect to their MRG expression. FINISHED (See Figs 6 & 7 in Cancer Res. 60,6482-6487, 2000)

Interaction of ω-3 PUFA DHA and MRG on cell growth. Since MRG is a fatty acid binding protein with the highest binding affinity to 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 the doses of 2, 4, 6, 8, and 12 μg/ml for four days with fresh DHA added every two days. A very narrow dose-dependent growth inhibition was observed for DHA (Fig. 6A). While no significant growth inhibition was observed for DHA at the doses of 2 μg/ml, 71% and 92% of growth inhibition was observed at the doses of 8 and 12 μg/ml, respectively. We therefore chose the non-inhibiting DHA dose of 2 μg/ml for testing its growth-regulatory effect on MRG positive vs. MRG negative cells. As demonstrated in Fig. 6B, when the cells were treated with 2 μg/ml of DHA, 55% and 47% of growth inhibition were 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 ω-6 fatty acid linoleic acid on the growth of MDA-MB-231 cells. At the same conditions as we did for 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 a dose-dependent growth inhibition in MDA-MB-436 breast cancer cells (Fig. 7A). While no significant growth inhibition was observed when MRGp dose was below 50 nM, 10% and 14% of growth inhibition was observed when cells were treated with 50 nM and 80 nM of MRGp, respectively. At 150 nM of MRGp, the growth was inhibited 58%. A sub-maximal MRG dose of 80 nM was used to test the interaction between MRG and DHA. Treatment of MDA-MB-436

(Fig. 7B) and MDA-MB-468 (Fig. 7C) cells with 80 nM of MRGp resulted in either a slight inhibition or a slight stimulation on cell growth, respectively. When the cells were treated with MRGp and together with DHA, a significantly 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 to 18% inhibition with DHA alone. Similarly, the growth of MDA-MB-468 cells was inhibited by 80% with DHA and MRGp compared to 22% inhibition with DHA alone.

Specific Aim 2: Induction of mammary epithelial cell differentiation by MRG (FINISHED)

Effects of MRG on mammary cell differentiation in vitro. (See Figs. 3-5 and Table 1 in paper of Cancer Res.).

Induction of differentiation of breast cancer cells. 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 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.

Specific Aim 3: In vivo study of tumor suppressing activities of DHA and EPA on MRG positive tumors vs. MRG negative tumors (Modified).

We hypothesize that MRG is a **candidate mediator** of the tumor suppressing effect of DHA and EPA on human mammary tumors. This hypothesis is generated based on three rationales: (a) MRG, suppressing cell and tumor growth and inducing differentiation, is a candidate mediator of the differentiating effect of pregnancy and lactation on breast epithelial cells; (b) MRG positive breast cancer cells is much more sensitive to DHA-induced growth

inhibition; and (c) DHA, which has highest ligand binding affinity for mouse B-FABP, is proposed to be the physiological ligand for MRG. To test the hypothesis, we previously thought to take the approach of injection of genetically MRG transfected cells (MRG-231-6 and MRG-231-10 vs. control neo-231-1 and neo-231-2) into the nude mice and then determine if DHA and EPA have differential tumor suppressing effects on MRG positive MRG-231-6 and MRG-231-10 tumors vs. MRG negative neo-231-1 and neo-231-2 tumors. However, this xerograph approach was not successful. One of the biggest challenges is the low tumorigenic phenotype of MRG transfected MRG-231 clones due to the tumor suppressing effect of MRG. As we previously demonstrated, the tumor growths (but not the tumor incidence) of MDA-MB-231 cells were significantly inhibited by MRG expression (2). This low tumor growth undermined the further tumor suppressing effect of fish oil on MRG-231-6 and MRG-231-10 tumors.

MRG transgenic mouse provides an excellent in vivo model to study the interaction between n-3 fatty acid DHA and its binding protein MRG on regulation of mammary differentiation and mammary tumorigenesis. In this regard, we established MRG transgenic mouse under the promoter of mouse mammary tumor virus (MMTV) and investigated the role of MRG on mammary gland differentiation. Our data indicate that MRG is a mediator in the differentiation effect of pregnancy on breast epithelial cells.

Stimulation of β-casein expression. 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. 1** 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.

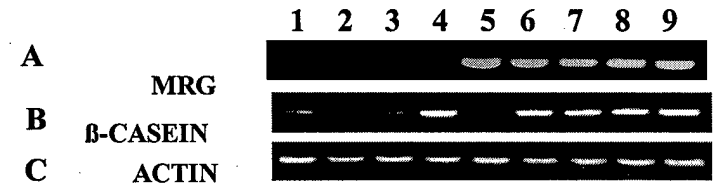

Fig. 1. RT-PCR analysis of MRG transgene and β-casein expression. Eight-week old fourth generation virgin MM-H1 and MM-H2 mice, and age matched control virgin mice and control pregnant mouse were scarified and the third pare thoracic mammary glands were removed. Expression of MRG transgene (**A**) and 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). The 393-bp of the human MRG was amplified by PCR with a set of primer. 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.

Effects of expression of MRG transgene on mammary gland development and differentiation

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. 2 shows a representative mammary gland analysis of 32-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. 2, 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. 2D) was observed compared with the control littermate (Fig. 2C). While the limited budding was developed in the wild-type gland (Fig. 2C), transgenic gland exhibited multiplicity of budding (Fig. 2D).

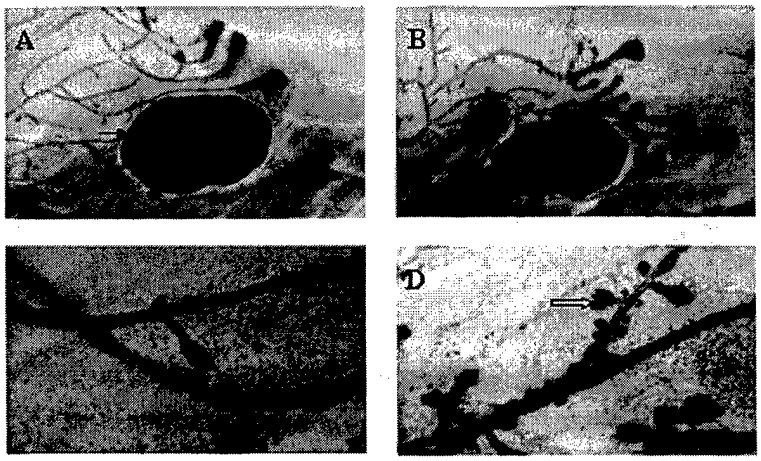

Fig. 2. Whole mount histological analysis of mammary gland from female MM-H2 transgenic mouse and wild-type littermate. A 32-day old virgin MM-H2 mouse and a age-matched virgin wild-type littermate mouse were sacrificed, the right inguinal gland were removed and subjected to whole mount gland fix, defat, 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.

We also performed a histological analysis of formation of lobulo-alveoli. As shown in **Fig. 3**, 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.

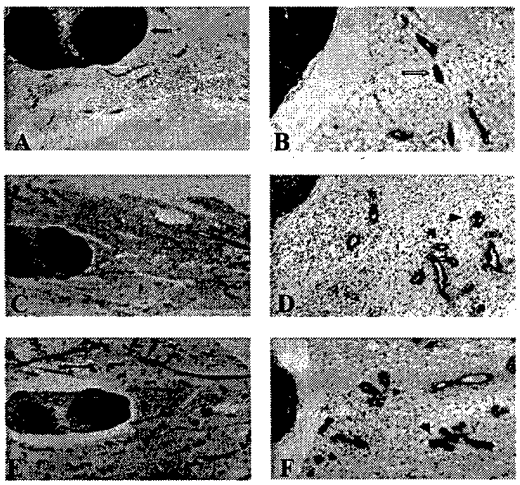

Fig. 3. Histological analysis of alveoli structure. Third pairs of mouse whole thoracic mammary glands were isolated from 7-week old female virgin mice. All the sections were stained with H&E for histological analysis. **A&B**, control mouse mammary gland. A, 2x10, an arrow indicates lymph nodes. B, 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.

While this model is still in process of characterization, we are on the way to study the interactions between MRG and n-3 fatty acid DHA on mammary tumorigenesis. Mouse DMBA model is a well-established carcinogen-induced mammary tumorigenesis and has been extensively used for evaluation of chemopreventive agents including vitamin D analogue, retinoids, soy compound genistein, indole-3-carbinol, and n-3 PUFA DHA and EPA. The protective effect of pregnancy and hCG treatment against DMBA-induced mammary tumor were also investigated and confirmed. We will use this DMBA-induced mammary tumorigenesis model to study the anti-tumor effects of MRG

and DHA.

C. KEY RESEARCH ACCOMPLISHMENTS

1. Transfection of human breast cancer cells with MRG gene resulted in differentiated phenotypes.
2. Treatment of mouse whole mammary gland in organ culture with purified recombinant MRG protein induced gland differentiation with β-casein expression and differentiated morphology.
3. Transfection of breast cancer cells with MRG gene or treatment of the cells with MRG protein significantly enhanced DHA-induced growth inhibition.
4. Expression of MRG transgene in the mammary gland resulted in differentiated gland morphology with increased formation of lobulo-alveoli.
5. Consistent with the morphological change, MRG stimulated milk protein β-casein expression in the gland of the transgenic mice.

D. REPORTABEL OUTCOMES AND CONCLUSIONS

1. The protective effect of pregnancy against breast cancer can be attributed to the transition from undifferentiated mammary epithelial cells in the nulliparous to differentiated mature cells during the pregnancy and lactation. The realization that specific reproductive endocrine events alter breast cancer risk in a predictable fashion raises the possibility that events known to decrease breast cancer risk might be mimicked pharmacologically. Unfortunately, the biological basis of parity-induced protection against breast cancer is unknown. A stumbling block in chemoprevention has been the prolonged and costly clinical trials required to determine the efficacy of chemoprevention regimens due to reliance on the development of breast cancer as a clinical end point. As such, the identification and use of intermediate **molecular end points** that accurately identify changes in the breast associated with parity would facilitate the development of such chemopreventive regimens. Within these contents, we have demonstrated that MRG, which are highly expressed in the differentiated pregnant mammary gland, induces the gland differentiation both morphologically and functionally. The potential application of MRG as a pregnancy-like differentiation factor for mammary gland and served as one of the intermediate molecular end points for chemoprevention warrant further investigation.
2. There is an increasing public interest in the dietary supplement of n-3 polyunsaturated fatty acids (PUFA) with respect to their beneficial effects on reduction of certain types of cancer and particular breast cancer. It is well established that n-3 PUFAs such as DHA and EPA have a tumor suppressing effect and prevents mammary tumors in the animal models. Currently, the cellular interactions of n-3 PUFAs are poorly understood. Being identified as a member of fatty acid binding protein (FABP), MRG has a highest binding affinity to n-3 PUFA DHA (Cancer Res 60: 6482-6487, 2000). Demonstration of MRG as a mammary differentiation factor will potentially bring the well-established epidemiological observations and animal studies of the decreased risk of breast cancer in association with n-3 PUFA, to point to an under-explored area mechanistically linking n-3 PUFA-induced prevention to MRG-induced mammary gland differentiation.

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F. PAPER PUBLISHED

Wang M, Liu YE, Ni J, Aygun B, Goldberg ID, Shi YE. Induction of mammary differentiation by MRG that interacts with ω-3 fatty acid on growth inhibition of breast cancer cells. ***Cancer Res.*** 60: 6482-6487, 2000.