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14. ABSTRACT A mammary derived growth inhibitor related gene (MRG) was previously identified and characterized. 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. To further define the role of MRG on mammary differentiation, a MRG transgenic mice model under the control of MMTV promoter was established and investigated. While there was no lobulo-alveolar structure in control virgin mice, expression of MRG transgene in the mammary gland 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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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 β -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).

B. WORK ACCOMPLISHED

Specific Aim 1: Does MRG overexpression in MMTV/MRG transgenic mice induce a pregnancy/lactation-like mammary gland differentiation? Finished.

SA1-1. Does MRG induce functional differentiation in mammary epithelial cells? FINISHED (J. Biol. Chem., 278: 47319-47325, 2003)

Our in vitro studies suggest a differentiation-associated function of MRG on breast epithelial cells. In the current study, 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.

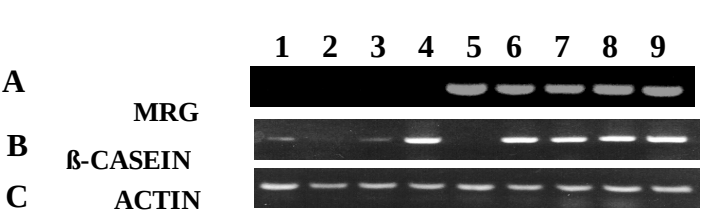


Fig. 1. RT-PCR analysis of MRG transgene and β -casein expression. Eight-week old fourth generation virgin MM-H1 and MM-H2 mice, and age matched control virgin mice and control pregnant mouse were scarified and the third pare thoracic mammary glands

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 $\frac{1}{2}$ 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).

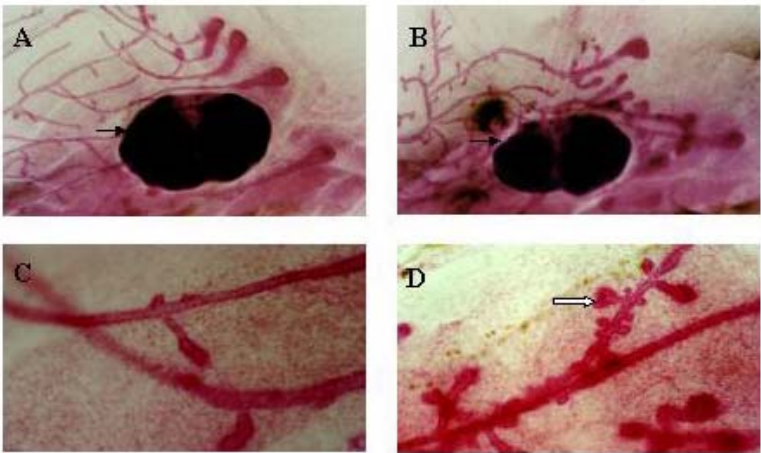


Fig. 2. Whole mount histological analysis of mammary gland from female MM-H2 transgenic mouse and wild-type littermate. A 32-day old virgin MM-H2 mouse and a age-matched virgin wild-type littermate mouse were sacrificed, the right inguinal gland were removed and subjected to whole mount gland fix, defat,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

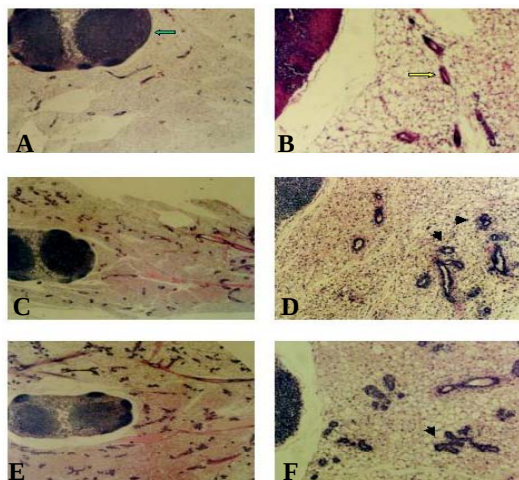


Fig. 3. Histological analysis of alveoli structure. Third pairs of mouse whole thoracic mammary glands were isolated from 7-week old female virgin mice. All the sections were stained with H&E for histological analysis. **A&B**, control mouse mammary gland. A, 2x10, an arrow indicates lymph nodes. B,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

SA2. Does overexpression of MRG in the transgenic mice prevent breast cancer? Not finished but modified.

While MMTV/MRG model is still in process of characterization, we are on the way to study the preventative effect of MRG on mammary tumorigenesis. We will **(1)** determine if overexpression of MRG in the transgenic mice will prevent breast cancer induced by carcinogen DMBA; **(2)** determine if overexpression of MRG in the transgenic mice will further enhance human placental hormone chorionic gonadotropin (hCG)-induced mammary tumor prevention in DMBA model; **(3)** compare the pregnancy-induced prevention vs. the MRG-induced prevention in DMBA model. We anticipate having another year to finish these objectives. In addition to previous two specific aims, we also studied the molecular mechanisms underlying the MRG-induced mammary gland differentiation.

Induction of Stat5 activation. The transcriptional activation of the β -casein gene expression in the mammary gland is mediated at least in part by Stat5. The stimulation of β -casein gene expression in the mammary gland from the transgenic mice promoted us to investigate Stat5 activation. Phosphorylation on tyrosine is essential for Stat 5 binding and its transcriptional activity. We

examined tyrosine phosphorylation of Stat5 in mammary glands from virgin control mice, pregnant control mice, and virgin transgenic mice (Fig. 4). While limited phosphorylated Stat5 protein was detectable in the gland from the non-differentiated virgin control mice, both pregnancy and expression of MRG transgene in the gland significantly stimulated Stat5 phosphorylation, resulting in a 5.1-fold and 4.7-fold increase over the control gland, respectively. These data demonstrated that expression of MRG results in a phosphorylation of Stat5, indicating a potential role of MRG in activating of Stat5 in the mammary gland from the transgenic mice.

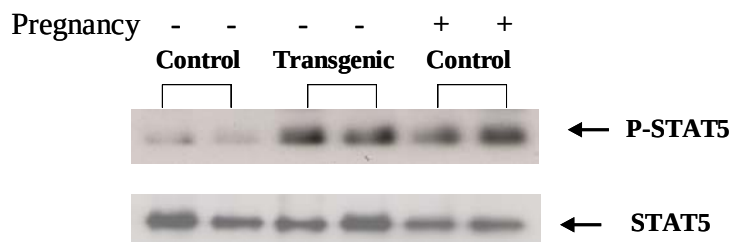


Fig. 4. Induction of Stat5 phosphorylation in the mammary glands by pregnancy and MRG transgene expression. Ten-week old virgin control mice (lane 1-2), age-matched transgenic MM-H1 mice (lane 3-4), and age matched control 4-day pregnant mice (lane 5-6) were sacrificed,

inguinal mammary glands were removed. Total protein was isolated, normalized, and 300 μ g of total protein was subjected to immunoprecipitation with anti-Stat5 antibody followed by Western analysis. The expression of phosphorylated Stat5 was determined by using a specific anti-phosphorylated Stat5 antibody (A) and normalized for total Stat5 expression (B).

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. Expression of MRG transgene in the mammary gland in the transgenic mouse resulted in differentiated gland morphology with increased formation of lobulo-alveoli.
4. Consistent with the morphological change, MRG stimulated milk protein β -casein expression in the gland of the transgenic mice.
5. Expression of MRG in mammary gland activates Stat5. While activated Stat5 was expressed at the minimal level in the non-differentiated control virgin gland, a significant Stat5 phosphorylation was observed in the virgin transgenic gland.

D. REPORTABLE OUTCOMES AND CONCLUSIONS

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.

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