Induction of Mammary Gland Differentiation in Transgenic Mice by the Fatty Acid Binding Protein MRG

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Abbreviations: FABP, fatty acid binding protein; A-FABP, adipocyte derived FABP; B-FABP, brain type FABP; H-FABP, heart derived FABP; hCG, human chorionic gonadotropin; MDGI, mammary derived growth inhibitor; MRG, mammary derived growth inhibitor related gene
Summary

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. To further define the role of MRG on mammary gland differentiation, a MRG transgenic mice model under the control of MMTV promoter was established and the effect of MRG on mammary gland differentiation was investigated at histological and molecular levels. Expression of endogenous mouse MRG gene was significantly increased from the non-differentiated gland of control virgin mice to the functionally differentiated gland of pregnant control mice. Whole mount analyses demonstrated that ductal development was not affected by MRG transgene expression. While there was no lobulo-alveolar structure in control virgin mice, expression of MRG transgene in the mammary gland resulted in the development of lobuloalveolar-like structure, which mimics the gland from early pregnancy. Consistent with the morphological change, expression of MRG also increased milk protein β-casein expression in the gland. To study the mechanism of MRG-induced mammary differentiation, we investigated the Stat5 activation in the glands from the transgenic mouse vs. virgin control mouse. 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. Our data indicate that MRG is mediator of the differentiating effects of pregnancy on breast epithelium and overexpression of MRG in young nulliparous mice can induce differentiation.
Introduction.

In an effort to search for growth regulators in the human mammary gland development, we generated cDNA libraries from a breast cancer biopsy specimen and a normal breast and analyzed these libraries by differential cDNA sequencing (1-2). We identified, cloned, and characterized a novel tumor growth inhibitor and named it as a Mammary derived growth inhibitor-Related Gene MRG (3). The predicted amino acid sequence of MRG has a significant sequence homology to previously identified mammary derived growth inhibitor MDGI (4). Interestingly, MRG and MDGI revealed no homology to any other known growth inhibitors; rather, they revealed extensive sequence homology to fatty acid binding protein (FABP) (5-6). A striking homology was evident between MDGI and Heart type (H-) FABP, which differ only in seven positions of the amino acid sequence (5). In fact, it turned out that the originally described MDGI is the same protein of H-FABP, which is also expressed in mammary gland (7-8). H-FABP fully replaced the MDGI effect and inhibited the growth of mammary epithelial cells (7). Thus, MDGI was also named as H-FABP. Interestingly, similar to the story of MDGI and H-FABP, subsequent to our isolation of MRG, human brain type (B-) FABP was independently cloned from human fetal whole-brain cDNA library (9). In fact, the sequence of MRG was found to be exactly identical to the recently deposited sequences of human B-FABP in GenBank (accession #AJ002962). Thus, while the names MRG and MDGI are used when referring their functions on mammary gland, the names of B-FABP and H-FABP are also used when referring their well-accepted FABP family phylogenetic tree (10).

Cellular 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. It has been suggested that in heart and brain, FABPs regulate the supply of fatty acids to the mitochondria for beta-oxidation (11-12). The mammary gland, however, is a highly lipogenic tissue and fatty acids are not likely to be a major fuel for its metabolism. However, from other studies on role for FABPs in cell signaling, growth inhibition and differentiation has also been implied (3,13-14). MDGI was mainly detected in myocardium, skeletal and smooth muscle fibres, lipid, and steroid synthesizing cells adrenals, and terminally differentiated epithelia of the respiratory, intestinal and urogenital tracts (15). Within the similar content, the expression of MRG was mainly detected in brain, heart, and skeletal muscle, which are in the postmitotic status (3). In particular, MDGI (14,16) and MRG (17) are abundantly expressed in the mammary gland during functional differentiation. These results provide evidence that expression of MRG is associated with an irreversibly postmitotic and terminally differentiated status of cells. Within the phylogenetic tree of FABPs, MRG and MDGI belong to a closely related subfamily of proteins that act as growth inhibitor
for breast cancer (18). Therefore, MRG and MDGI could fulfill different functions in brain and heart compared with mammary gland. Being the members of FABP family, the most characterized biological functions for MRG and MDGI are differentiating effect on mammary cells and tumor-suppressing activities against breast cancer. These include (a) the loss expression of MRG (3) and MDGI (19) is associated with breast cancer progression; (b) Both MRG (17) and MDGI (14,16) are highly expressed in the fully differentiated lactating mammary gland and induce mammary differentiation; (c) MRG and MDGI have been mapped at the chromosome 6q22-23 (18) and 1p35 (20) that harbor the putative tumor suppressor genes for breast cancer (21-22); and (d) both MRG and MDGI strongly suppress the growth of breast tumors (3,20).

It has been previously demonstrated that the expression of mouse MRG is correlated with neuronal differentiation in many parts of the mouse central nervous system (23-24) and blocking antibody to mouse MRG can block glial cell differentiation in mixed primary cell cultures prepared during the first postnatal week (24). In mammary epithelium, MRG also induces mammary differentiation (17). 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 MRG recombinant protein resulted in a differentiated morphology and production of β-casein (17). Therefore, it seems clear that a differentiation-associated function is a common property of this structurally related subfamily of FABPs. 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 and the MRG-induced differentiation is mediated by JAK-Stat5 signaling pathway.
**Materials and Methods**

**DNA constructions and generation of transgenic mice.** The MMTV regulatory sequences were derived from the plasmid pMMTV/STR (kindly provided by Lynn Matrisian) that previously used for stromelysin-1 transgenic mice (25). The full-length MRG cDNA sequence from pCI-MRG (3) was subcloned into the Bam HI and Apa I sites of the MMTV plasmid. To ensure proper expression of this cDNA, SV40 splicing and polyadenylation signals were added to the 3’ portion of the construct. A 2.5 kb MMTV-MRG transgene was separated from the vector and isolated from an agarose gel. The DNA fragments were injected into fertilized eggs (5 ng/µl) of FVB/N mouse at the Transgenic Core Facility at Albert Einstein College of Medicine. Injected cells were transferred into the oviduct of pseudopregnant ICR female mice and allowed to develop to term. Six weeks after microinjections, 34 mice were screened by PCR and Southern blot.

**Identification of founder transgenic mice.** Among 34 mice, a total of 4 (2 male and 2 female) founder transgenic mice containing the fusion gene were identified by Southern blot analysis of BamHI-digested tail DNA hybridized with the full length MRG cDNA under the high stringency conditions: MM.F16, MM.F31, MM.M3, and MM.M4 (MM represents MMTV/MRG). Mating founder animals to wild-type (FVB/n background) males and females generated four 1st-generation transgenic lines: F1-MM16, F1-MM31, F1-MM3, and F1-MM4. We have screened the presence of the transgene in third generation of all four lines by a PCR analysis of isolated tail DNA using primers within MRG coding sequence (5’-GTGGAGGCTTTCTGTGCTACCTGG-3’ and 5’-TGCCCTTCTCATAGTGCAAGCAG-3’). The 393-bp PCR product is a specific indication of the presence of human MRG transgene. Approximately 45% of F1-MM3 and F1-MM4 transgenic mice expressed MRG transgene; 30% of F1-MM16 mice expressed MRG transgene; and less than 8% of F1-MM31 mice expressed the transgene.

**Detection of Stat5 tyrosine phosphorylation.** Whole tissue extracts were subjected to immunoprecipitation with anti-Stat5 antibody followed by Western analyses with an anti-phosphotyrosine Stat5 antibody (Cell Signaling). The levels of tyrosine phosphorylated Stat5 protein were monitored by reprobing the membranes with the anti-Stat5 antibody.

**Western analysis of MRG protein in mammary gland.** Western blot was conducted as we previously described (17). Briefly, the blot was incubated with anti-MRG primary antibody (1:200 dilution) overnight at 4°C, and then incubated with goat anti-rabbit IgG-horseradish peroxidase (1:1200 dilution) for 1 h, washed, and visualized by chemiluminescence.

**RT-PCR analyses.** RT-PCR analysis was performed by using a standard reversed transcription–PCR with the primers specific for mouse actin, β-casein, and human MRG. Total RNAs were isolated
from tissue using the Rnasy MINI Kit (Qiagen). One µg total RNAs were used for RT reaction using Oligo dT 15 primers (Roche), and one fifth of this reaction was amplified by PCR using Roche PCR kit. Each reaction consisted of 30 cycles in the GeneAmp PCR System 2400 (Perkin Elmer). The parameters for PCR were: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 90 seconds. One third of the PCR products were electrophoresed through 1.2% agarose-TAE-gel. Following primers were synthesized and used for amplifying the corresponding genes: 1) Mouse actin: 5’-GCTGTGCTATCCCTGTACGC-3’ and 5’-TGCCTCAGGGCAGCGGAACC-3’; 2) Mouse β-casein: 5’-GTC TCT T CC TCA GTC CAA AGT-3’ and 5’-TTG AAA TGA CTG GAA AGG AAA TAG-3’; and 3) Human MRG: 5’-GTGGAGGCTTTTCTGTGCTACCTGG-3’ and 5’-TGCCTTCTCATAGTGCGGAACAG-3’.

**Whole mount histological analysis of mammary gland.** Whole inguinal mammary glands were removed from virgin control as well as virgin transgenic mice as we previously described (17). The removed gland was subjected to whole mount fix, defat, and staining as previously described (26). Briefly, the inguinal mammary glands were fixed in 75% EtOH, 25% HoAC, and stained with alum carmine (0.1% w/v). Whole mount glands were destained in 70%, 90%, and 100% EtOH, respectively, defatted in xylenes, and stored in methyl salicylate.

**Morphological assessment of mammary gland.** Whole inguinal mammary glands were removed from virgin control as well as transgenic mice, fixed in 4% paraformaldehyde, and routine 5-µm sections were stained with hematoxylin and eosin. The characteristic lymph node in each gland was observed.

**Treatment of mouse with hCG.** Treatment of virgin mouse with hCG was performed as previously described by Russo (27-29) with some modifications. Briefly, virgin mice were injected i.p. with hCG 20 U/day for 8 days. The animals were sacrificed; the inguinal mammary glands were removed and subjected to histological analysis.
RESULTS

Screening, identification, and maintenance of mice heterozygous and homozygous for the transgene. Six weeks after microinjections of MMTV/MRG transgene, 34 newborn mice were generated and screened by Southern blot. As shown in Fig. 1, 4 transgenic mice were identified and named as MM3, MM4, MM16, and MM31. 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, MM3, and MM31 (data not shown). Two homozygous MMTV/MRG lines from MM16 and MM4 families were generated and named as strain MM-H1 and MM-H2. Both MM-H1 and MM-H2 mice developed normally compared with their nontransgenic littermates.

Transgene expression. Transgene expression in the mammary gland was assayed by RT-PCR and Western analyses. In the mammary glands from virgin MM-H1 and MM-H2 mice, the expression of the transgene was detected by RT-PCR using the primers specific for human MRG but not for mouse endogenous MRG (Fig. 2A). No signal was detected in the mRNA isolated from mammary glands of virgin wild-type females. Consistent with the transgene mRNA expression, in the Western blot using the antibody cross reacting with both mouse and human MRG, while either no or limited amount of MRG protein were detected in the gland from the virgin control females, MRG protein was highly expressed in MM-H1 and MM-H2 lines (Fig. 2C). Tissue expression of the transgene was assayed by hybridizing RNA samples extracted from various tissues from female mice with MRG full-length cDNA probe. Transgene mRNA was clearly detectable in the mammary gland, no MRG transgene mRNA was detectable in the RNA samples isolated from heart, liver, kidney, lung, and brain (Fig. 3). As expected, the endogenous mouse MRG gene was present in the heart and brain as the lower band, which is consistent with the MRG expression in the human tissue (3).

Expression of endogenous mouse MRG in mammary gland of control mice. To address the role of endogenous versus the transgenic MRG in breast epithelial differentiation, we analyzed the endogenous MRG protein expression in control virgin mouse vs. control pregnant mouse by Western blot (Fig. 4A). As expected, while there were limited amounts of endogenous mouse MRG in the gland
from virgin mouse, expression of endogenous mouse MRG was significantly increased in the mammary gland during pregnancy. The amount of mouse MRG in the functionally differentiated gland from the pregnant mouse was 5-fold of that in the non-differentiated gland from virgin mice. 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. 4C).

Effects of expression of MRG transgene on ductal development. Because MRG protein expression was associated with human mammary gland functional differentiation with the highest expression observed in the differentiated alveolar mammary epithelial cells from the lactating gland (17), 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 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 architecture (30). Whole mount preparations of the mammary glands starting at 4-week to 12-week 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 5-week and 10-week old transgenic mouse vs. wild-type control littermate. Mammary ducts in the transgenic virgin (Fig. 5B) as well as in the control virgin littermate (Fig. 5A) filled the typical ½ length of the inguinal gland and appeared normal. At the 10-week old, the mammary gland was completely filled with the ducts in both control (Fig 5C) and transgenic mouse (Fig. 5D). Similar ductal developments were also observed at different time points, 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 branching points of ducts in transgenic virgin mice was observed compared with the control littermate. While the limited budding was developed in the wild-type gland (Fig. 5E), transgenic gland exhibited multiplicity of budding (Fig. 5F).

Effects of expression of MRG transgene on gland differentiation and lobuloalveolar development. The increased budding in the transgenic mice suggests a potential effect of MRG on mammary gland differentiation leading to lobuloalveolar development. Two sets of experiments were done to exam the presence of differentiation of mammary alveolar epithelium in the transgenic mice. First, histological
evaluation of H & E stained mammary sections revealed the presence of multiple budding structures in the transgenic mice. There were no morphological differences observed in the younger (4-6-week old) transgenic mice compared to the age-matched control mice. However, starting at 8-week old, a significantly different morphology was observed in the transgenic mice vs. the control mice. As shown in Fig. 6, whereas no lobuloalveolar structures in the branching points of ducts were present in the 10-week old control virgin mouse (A&B), formation of the alveolar-like structure were observed in the gland from a transgenic mouse (C&D). Since the observed morphology did not look like the fully differentiated phenotype with expanded alveolar lumina containing lipid droplets, we further investigated if the observed phenotype resembles the characteristic alveolar-like structure in the gland during early pregnancy. We compared the morphology of the glands from virgin transgenic mice to the glands from early pregnant (6 days) and the late pregnant (15 days) control mice. The gland from a transgenic mouse exhibited an alveolar-like structure similar to the gland from the 6-day pregnant mouse (Fig. 6F). In contrast to the glands with alveolar-like structure in the transgenic mouse and the early pregnant mouse, a fully functionally differentiated gland with typical alveolar lumina contained milk was observed in the late pregnant mouse (Fig. 6H).

Given the fact that mammary gland development and differentiation is controlled by systematic hormones and by a variety of different local growth factors that might complement or mediate hormonal actions, hormone treatment of virgin mice has been used to mimic the effect of early pregnancy on alveolar development. It was previously well established by Russo that treatment of rat with human placental hormone chorionic gonadotropin (hCG) resulted in a similar effect on mammary differentiation as pregnancy (27-29). Since MRG-induced alveolar-like structure resembles the phenotype from early pregnant mice, we further compared the histology of MRG-induced alveoli-like structure to that of hormone stimulated alveoli formation. We treated control virgin mice 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-like structure (Fig. 7 E & F). Although, the magnitude of MRG effect is less than that of hCG on the formation of alveoli-like structure, the MRG-induced formation of alveoli-like structure (Fig. 7C & D) is compatible to that of hCG and is significantly different from the control virgin mice.

**Effects of expression of MRG transgene on the gland morphology during pregnancy, lactation, and involution.** Female mice that expressed MRG exhibited normal mammary glands function during pregnancy and lactation. Histological analyses of the mammary glands at early pregnancy (6-day), later pregnancy (15-day), and lactation indicated no phenotype difference among control and the
transgenic mice (data not shown). The glands from late pregnant and lactating transgenic mice exhibited a normal fully functionally differentiated phenotype with a marked increase in glandular lumen with significant accumulation of milk and secretory material. Transgenic female mice nursed their pups normally and the transgenic pups developed as normal as their nontransgenic littermates. We also examined the gland morphology at day 2 and day 6 of involution and no distinct morphological differences were apparent between the glands from control and the transgenic mice (data not shown). At day 2 of involution, typical alveolar structures with a single layer of epithelial cells surrounding a lumen are observed in mammary glands from both normal and transgenic mice. At day 6 of involution, the alveoli in mammary glands from both normal mice and transgenic mice have collapsed, and numerous apoptotic bodies were apparent in the ductal lumens.

**Stimulation of β-casein expression.** To determine if the mammary epithelial cells were functionally as well as morphologically differentiated, the expression of the early differentiation marker milk protein gene β-casein was analyzed by RT-PCR. Fig. 8 shows a representative MRG transgene and β-casein expression in four virgin control mice and four randomly picked virgin transgenic mice from MM-H1 and MM-H2 lines. RT-PCR analysis revealed the expression of the transgene MRG and β-casein in all four transgenic mice (Fig. 8, lines 6-9). However, no detectable β-casein transit was observed in age-matched control virgin mice (Fig. 8, lines 1-3). As expected, expression of β-casein was detected in an 8-day pregnant of normal mouse (Fig. 8, line 4). We also investigated the late marker whey acidic protein (WAP) expression in the transgenic mouse. No WAP RNA expression was observed by Northern blot in both virgin control and virgin transgenic mice (data not shown). These results indicate that the mammary glands of the established MMTV/MRG transgenic mice have functional expression of the transgene, which stimulates the morphological formation of alveolar-like structure and functional expression of the early differentiation marker β-casein. The histological as well as molecular change observed in the gland from the transgenic mice resembles the differentiated phenotype in the gland from the early pregnant mice.

**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. 9). 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.
Discussion.

Mammary gland differentiation requires the coordinated action of systematic hormones and local growth factors that promote morphological development and milk protein production in the lactating gland (29). Although much is known about systematic hormonal effect on mammary differentiation, little is known about the regional and developmental expression of locally acting differentiating factors in the mammary epithelium during pregnancy and lactation. In order to understand the molecular events contributing to mammary gland differentiation, in vitro cell culture system have proven invaluable. We have previously identified, cloned, and characterized a novel growth inhibitor and a fatty acid binding protein MRG in human mammary gland (3) and demonstrated its in vitro differentiating effect on mammary epithelial cells and mammary gland in organ culture (17). In the present study we 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; 2) consistent with the morphological change, MRG stimulated milk protein β-casein expression in the gland of the transgenic mice; 3) MRG expression resulted in Stat5 phosphorylation in the gland of the transgenic mice.

There are two distinct patterns of growth and development in the mouse mammary gland. The first involves the penetration of the mammary fat pad by branching ductal morphogenesis and is under the control of ovarian hormones and local growth factors. The second involves the growth and expansion of the secretory lobules at the onset of pregnancy, which is dependent upon the hormonal status, induced by pregnancy and continues until parturition. Each of these periods of expansion involves the production of epithelial progenitor cells, which are responsive to specific inductive and growth signals (31). While the ductal elongation is the normal mammary development before the onset of pregnancy, development of secretory lobules and formation of lobule alveoli is the consequence of functional differentiation induced by pregnancy. In the MMTV/MRG transgenic line, whole mount mammary gland histological analysis revealed no alteration in the ductal branching compared with that in control mice, indicating that MRG expression did not alter the first pattern of growth and development in mammary gland. However, an alternation in the second developmental pattern of the glands in transgenic virgin mice was observed compared with the control littermate. While the limited budding was developed in the wild-type gland, the transgenic gland exhibited multiplicity of budding and the formation of alveolar-like structures, indicating an initiation of functional differentiation.

The differentiation status of mammary tissue from the transgenic mice was further monitored by analyzing the steady state levels of milk protein β-casein. While the expression of β-casein gene
was not detectable in the gland from virgin control mice, β-casein gene expression was clearly activated in the glands from the virgin transgenic mice. Since activation of β-casein gene occurs earlier during gestation (30), expression of the β-casein gene in the transgenic gland indicates these mammary epithelial entered into the differentiated stage, which partially mimics the early stage of pregnancy.

The full spectrum of intracellular events regulated by MRG is currently unknown. In the present study, we did not attempt to identify all critical cell differentiation-related factors that are induced by MRG; rather in an initial effort to study the mechanisms underlying MRG-induced differentiation in the mammary cells, we focused on Stat5. Prolactin (Prl) plays a central role in the differentiation of the mammary gland. The Prl receptor transmits signals in part via activation of the JAK/Stat pathway. The general paradigm for Prl-induced JAK/Stat signaling is that interaction of Prl with its receptor induces receptor dimerization, activation of the JAK2 protein-tyrosine kinase and Stat 5 tyrosine phosphorylation, followed by dimerization and obligatory nuclear translocation (32-33). Among several mammalian Stat proteins, Stat1, Stat3, and Stat5 are capable of activation by the Prl (34). Stat5, however, plays a key role in Prl-induced milk protein gene expression and mammary gland differentiation (35-36). Here we demonstrated that while the Stat5 was kept in the non-phosphorylated inactivated form in the mammary gland from virgin mice, expression of MRG resulted in the activation of Stat5 in the transgenic mice. Stat 5 was originally identified as a mammary gland factor that binds to promoter sequences of β-casein and activates its transcription (35,37). Gene targeting experiment indicates that Stat 5a is mandatory for mammary gland differentiation (36). While Stat5 activity has been linked to alveolar differentiation and function, Stat3 appears to have opposite functions in control developmental cycles of mammary tissue (38). Conditional deletion of Stat3 induced a decrease in mammary epithelial apoptosis and a dramatic delay of the involution (39), indicating its role on the loss of alveolar function, cell death, and the initiation of mammary tissue remodeling. Our data on induction of Stat5 phosphorylation and activation further support the role of MRG on mammary gland differentiation and suggest that MRG is a mediator in JAK-Stat5 pathway of mammary gland differentiation.

MRG shares the highest sequence homology with MDGI, which is initially identified and purified from Ehrlich ascites mammary carcinoma cells (4), and then from the lactating bovine mammary gland (5-6) and from cow’s milk (40). Comparison of the expression and biological functions of MRG and MDGI revealed a similar in vitro pattern. However, their in vivo effect on mammary gland development and differentiation are different. In vitro studies of mouse and bovine MDGI suggest several functions of MDGI on growth and differentiation of mammary gland. These include 1) MDGI specifically inhibit the growth of normal mouse mammary epithelial cells (MEC),
and promote morphological differentiation: the appearance of bulbous alveolar-like structure and formation of fully developed lobuloalveolar structures (16); 2) Selective inhibition of endogenous MDGI expression in mouse MEC by use of antisense oligonucleotides suppresses the formation of alveolar-like structure and impairs β-casein synthesis in organ cultures (16); 3) 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 (14); and 4) MDGI expression in mouse mammary epithelium cells is hormonally regulated (41,42). Although a large body of evidence has suggested that MDGI promotes differentiation of mammary epithelial cells \textit{in vitro}, there is a concern about its role as differentiating factor for mammary gland \textit{in vivo}. The \textit{in vitro} data contrast with \textit{in vivo} situation with MDGI where neither over-expression nor gene deletion yields an overt phenotype in the mammary gland development and differentiation. In MDGI transgenic mice, there was no correlation between MDGI expression, proliferation rate and differentiation (26). Likewise, for the knockout mice no morphological evidence and functional differentiation were observed (43). These data suggest that MDGI does not play any important functional role in development and differentiation of mammary gland. In contrast with MDGI, which has inconsistent \textit{in vitro} and \textit{in vivo} effect on mammary differentiation, MRG revealed a similar differentiating effect on mammary epithelial cells both \textit{in vitro} and \textit{in vivo}. The expression and the \textit{in vitro} function of MRG on mammary epithelial cells share similarity with MDGI, which include an induction of differentiated phenotypes in both cells and mammary organ culture and association with human mammary gland functional differentiation during the pregnancy (3,17). Consistent with these \textit{in vitro} cellular differentiating effects, glands from the virgin MMTV/MRG mice possess a significant increase in the appearance of lobular alveoli-like structure vs. the control virgin mice and express differentiation-related β-casein gene. These data indicated that although the two closely related FABPs have the similar cellular effect on mammary epithelial cells \textit{in vitro}, only MRG plays an important functional role in regulation of development and differentiation of the gland in the whole animal.

Epidemiological data and animal studies imply that an early first full-term pregnancy induces the functional differentiation of the gland, which results in a decreased risk for the subsequent development of breast cancer (44-45). 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 (28-29). 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 (46). 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.
Figure Legends

Fig. 1. Southern blot analysis of MRG fusion gene in transgenic mice. DNAs were extracted from 1.5 cm sections of tails and digested with BamH1. The presence of a 2.2 kb transgene was detected by Southern blot analysis with full-length MRG cDNA probe. Lane 3, MM3; lane 4, MM4; lane 16, MM16; and lane 31, MM31.

Fig. 2. MRG transgene expression in control and homozygous transgenic lines. Ten-week old virgin MM-H1 and MM-H2 mice, and age matched control virgin mice were sacrificed and the inguinal 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 coding sequence. 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 for 314-bp β-actin (B). Lane 1, MRG plasmid as a positive control; lane 2-3, control mice; lane 4, MM-H2; lane 5, MM-H1. (C-D). Western analysis of MRG protein and actin expression. Western blot using the specific anti-MRG antibody was carried out. Lane 1, 10 ng of purified recombinant MRG protein; lane 2-3, control mice; lane 4, MM-H1; lane 5, MM-H2. Lanes 2-5 contained 50 µg of cellular protein.

Fig. 3. Tissue expression of MRG transgene. Total RNA was isolated from different organs from a 10-week old virgin MM-H2 transgenic mouse. Expression of MRG was analyzed by Northern blot with full-length human MRG cDNA probe (A) and normalized by visualization of ribosomal bands (B). Lane 1, mammary gland; lane 2, heart; lane 3, lung; lane 4, liver; lane 5, kidney; lane 6, brain. A high abundance of 1.4-kb transgene was detected in the mammary gland. Expression of 1.1-kb endogenous mouse MRG was detected in heart and brain. Each lane contained 30 µg of total RNA.

Fig. 4. Expression of mouse MRG and β-casein in control non-transgenic mice. Inguinal mammary glands were isolated from 10-week old non-transgenic virgin and 8-day pregnant mice. Expression of mouse MRG protein (A) was analyzed by Western blot and normalized for β-actin expression (B). Total protein was isolated and normalized, and 25 µg of total protein were subjected to Western analyses with either MRG antibody or anti-actin antibody. Lane 1 and 4, virgin mice; lane 2 and 3, pregnant mice. Densitometric scan indicates that MRG expression is increased 5-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). Lane 1, virgin mouse; lane 2, pregnant mouse.
Fig. 5. Whole mount histological analysis of mammary gland from female MM-H2 transgenic mouse and wild-type littermate. A 4-week old (A-B) and 10-week old (C-F) virgin MM-H2 mouse and an age-matched virgin wild-type littermate mouse were sacrificed, the right inguinal gland was removed and subjected to whole mount gland fix, defat, and staining. A, C, and E, wild-type control mouse. B, D, and F, 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, lower magnification (1x10). E & F, higher magnification pictures from (10X10). An open arrow indicates budding.

Fig. 6. Histological analysis of mammary gland. Whole inguinal mammary glands were isolated from 10-week old female virgin mice. All the sections were stained with H&E for histological analysis. A&B, control mouse mammary gland. A, 10x10; B, 40x10. C&D, mammary gland from MM-H2 transgenic mouse. E&F, mammary gland from a 6-day pregnant mouse. G&H, mammary gland from a 15-day pregnant mouse.

Fig. 7. Histological comparison of the glands from transgenic mouse to the gland from hormone-treated mouse. Whole inguinal mammary glands were isolated from 10-week old control virgin mouse, transgenic mouse, and hormone hCG-treated control mouse. All the sections were stained with H&E for histological analysis. A&B, control mouse mammary gland. A, 10x10, an arrow indicates inguinal lymph node. B, 40x10, an open arrow indicates a ductal structure. C&D, mammary gland from MM-H2 transgenic mouse. C, 10x10. D, 40x10, an open arrow indicates an alveoli-like structure. E&F, mammary gland from hCG-treated mouse. Nine-week old mice were treated with hCG 20 U/day for 8 days and then the glands were isolated for histological analysis. E, 10x10. F, 40x10, an open arrow indicates an alveoli-like structure.

Fig. 8. RT-PCR analysis of MRG transgene and β-casein expression. Ten-week old fourth generation virgin MM-H1 and MM-H2 mice, and age matched control virgin mice and control pregnant mouse were sacrificed and the inguinal 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). The 393-bp of the human MRG and the 480-bp of the mouse β-casein gene were amplified by PCR with sets of primer as described in Materials and Methods. Lanes 1-4, control mice; lane 4, control pregnant mouse.
mouse; lane 5, T47D breast cancer cell was used as a positive control for MRG expression; lane 6, MM-H1 mouse; lane 7, MM-H1 mouse; lane 8, MM-H2 mouse, lane 9, MM-H2 mouse.

Fig. 9. 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 µg 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).

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Fig. 1

![2.2 kb](image)

Fig. 2.

A. MRG mRNA

B. Actin mRNA

C. MRG Protein

D. Actin Protein
Fig. 3

A

B

1.4 kb
1.1 kb

28S
18S
Fig. 4.

A 1 2 3 4
B
MRG
Actin
C 1 2
Beta casein
D 18S 28S
Fig. 6.
Fig. 8.

![Image of gel electrophoresis showing bands for MRG, β-Casein, and Actin in lanes 1 to 9.]

Fig. 9.

![Image of gel electrophoresis showing bands for P-STAT5 and STAT5 in control and transgenic conditions.]

Pregnancy - - - - + +
Control Transgenic Control

P-STAT5

STAT5