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AWARD NUMBER: W81XWH-07-1-0375

TITLE: Induction of a Pregnancy-Like Mammary Gland Differentiation by Docosapentaenoic Omega-3 Fatty Acid

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REPORT DATE: September 2008

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 September 2008	Annual	1 Sep 2007 – 31 Aug 2008

4. TITLE AND SUBTITLE

Induction of a Pregnancy-Like Mammary Gland Differentiation by Docosapentaenoic Omega-3 Fatty Acid

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0375

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

North Shore-Long Island Jewish Health System
Lake Success, NY 11042

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. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

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

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The protective effect of early pregnancy against breast cancer can be attributed to the transition from undifferentiated cells in the nulliparous to the differentiated mature cells during pregnancy. Considerable evidences suggest strongly that the n-3 polyunsaturated fatty acid (PUFA) content of adipose breast tissue is inversely associated with an increased risk of breast cancer. Here we report that there was a decrease in n-6/n-3 PUFA ratio and a significant increase in concentration of n-3 PUFA DPA and EPA in the pregnant gland. The functional role of n-3 PUFAs on differentiation was supported by the studies in fat-1 transgenic mouse, which converts endogenous n-6 to n-3 PUFAs. Thus, alternation of n-6/n-3 fatty acid compositional ratio in favor of n-3 PUFA and particularly DPA and EPA is one of the underlying mechanisms of pregnancy-induced mammary differentiation.

15. SUBJECT TERMS
No Subject Terms provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	28	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

A1. Controversy over the association between omega-3 fatty acids (n-3 PUFA) and lower risk of breast cancer. Although experimental animal studies indicate that including n-3 PUFAs in the diet has cancer preventive benefits, findings from case-control and cohort studies have **inconsistently** reported an association between questionnaire-based assessment of n-3 PUFA intake and breast cancer risk (1-2). In a recent meta-analysis, Saadatian-Elahi (2) analyzed 11 case-control and 3 cohort studies published in 1966-2002 and reported an inconsistent finding from case-control studies but suggested that data from cohort studies support a significant inverse association between n-3 PUFA and breast cancer risk. The most recent systematic review of 20 cohorts with different demographic characteristics by MacLean also suggests that there is **no significant association** between n-3 PUFA and cancer incidence (1). However, results from studies of n-3 PUFA concentrations in **adipose tissue** (3-7) and **erythrocytes** (8-9) were more consistent. In **mammary** tissue, a variety of evidences suggest strongly that the content of n-3 PUFA in adipose breast tissue is **inversely** associated with increased risk of breast cancer incidence and progression. The most comprehensive study came from the European Community Multicenter, in which the fatty acid contents of adipose tissue in postmenopausal breast cancer cases and controls were analyzed in five European countries (3). The study showed a significantly lower ratio of n-3 to n-6 PUFA in breast cancers vs. controls. Recent study by Bougnoux et al (7) also found that a composite measure of a low ratio of n-3 to n-6 fatty acid in adipose tissue was associated with increased risk of breast cancer. Two most recent large scale case-control studies in China and Japan clearly demonstrated that level of n-3 PUFA, **measured in erythrocytes**, is significantly associated with **lower risk** of breast cancer (8-9).

We believe that studies from questionnaire-based assessment of fatty acid intake should be interpreted with caution. First, studies on n-3 PUFA consumption varied a great deal across study cohorts. Second, interpretation of the data is limited by significant differences in the methods used to ascertain exposure to n-3 PUFA. Third, of particular note is the fact that n-3 PUFA consumption generally consists of varying the ratio of n-3 to n-6 PUFA without consideration of n-6 fatty acid consumption. Very importantly, when calculating n-3 PUFA consumption, the background n-6 PUFA consumption has to be considered. It is becoming more accepted that most of the beneficial effects including cancer prevention is mediated by alternation of the n-6/n-3 compositional ratio but not the exact amount of n-3 PUFA. It is important to emphasize that all studies in these systematic analysis were prospective in design and no randomized clinical trial data exist.

A2. Two unique transgenic models to study the effect of n-6/n-3 PUFA compositional ratio on mammary gland differention (see Appendix 1-2).

A2-1. N-6/n-3 fatty acid ratio in Fat-1 transgenic mice. Mammals are unable to produce n-3 PUFAs from the more abundant n-6 type, so have to rely on their diet for these nutrients. A unique **fat-1** gene was identified in the roundworm *Caenorhabditis elegans*, which can catalyze the n-6 to n-3 conversion. A **fat-1 transgenic** mouse model capable of converting n-6 fatty acids to n-3 fatty acids was recently established (32). When fed with a diet high in n-6 and low in n-3 fatty acids, the transgenic animals are characterized by an abundance of n-3 fatty acid and a balanced n-6/n-3 fatty acid ratio of **2-1:1** in their tissues and organs, whereas wild type mice have a ratio of **> 30** (29-30). This model allows one to produce two different fatty acid profiles (high vs. low n-6/n-3 ratios) in the animals by using just a single diet, which avoids the potential problems associated with dietary supplement of fish oil including various amount of different n-3 PUFAs and contaminants. Using this transgenic model, we demonstrated that the ratio n-6/n-3 PUFA in mammary gland was **dropped 12-fold** from 25 in wild type mice to 2 in transgenic mice (**Appendix 1**).

A2-2. MRG transgenic mice: capable of preferential accumulation of n-3 PUFAs into mammary gland (**Appendix 1 and 2**). Mammary derived growth inhibitor Related Gene (**MRG**). We have previously identified, cloned, and characterized a differentiation factor and a fatty acid binding protein MRG in human mammary gland (34-36). Interestingly, MRG revealed no homology to any other known growth

inhibitors; rather, they revealed extensive sequence homology to **fatty acid binding protein** (**FABP**) (37). The sequence of **MRG** was found to be **identical** to the later deposited sequences of human brain type **(B-) FABP** in GenBank (accession #AJ002962) (37). Cellular FABP comprise a well-established family of cytoplasmic hydrophobic ligand binding proteins and are involved in binding and intracellular transport of PUFAs (38). Brain has the highest content of n-3 PUFA or lowest n-6/n-3 ratio among all the tissues (39-40). Preferential accumulation of n-3 PUFA in brain is associated with abundant expression of MRG/B-FABP (39-40). Indeed, among many fatty acids, n-3 PUFAs and particularly DHA has highest ligand binding affinity for MRG (K_d 10 nM) (41). Using previously established MRG transgenic mouse (36), we demonstrated that expression of MRG in mammary gland results in significant alternation of n-6/n-3 PUFA compositional **ratio** in favor of n-3 PUFAs (**Appendix 2**).

BODY

A notable finding relevant to this study is that there is a decrease in n-6/n-3 ratio in the pregnant gland compared with the virgin gland. Of specific interest is the observation of a robust increase of a specific n-3 DPA in differentiated pregnant gland from non-detectable in virgin gland to an abundant accumulation in the pregnant gland. Alternation of n-6/n-3 ratio in favor of n-3 PUFA in the mammary gland of fat-1 transgenic mouse induces differentiation and also significantly increases the DPA accumulation. Expression of MRG, a previously identified mammary differentiation factor and also a brain type fatty acid binding protein (B-FABP), in the transgenic mouse results in mammary differentiation and a robust DPA accumulation. We **hypotheses** that n-3 DPA is one of the mediators in the differentiation effect of pregnancy on breast epithelial cells; and thus application of n-3 DPA to mammary gland can lower breast cancer risk by making the mammary epithelial cells behave like the glands during pregnancy

SA1. Determine molecular mechanism for induction of mammary differentiation by n-3 PUFA DPA. One of the hallmarks for functional mammary differentiation is the expression of milk protein β-casein, which is mediated by phosphorylation of Stat5 (36-37). Consistent with induction of mammary differentiation by n-3 PUFAs, DPA activates Stat5 and induces β-casein. We will determine if induction of mammary differentiation by individual n-3 PUFAs, primarily DPA that preferentially accumulated in the differentiated gland during pregnancy, is mediated by activation of Jak2-Stat5 signaling pathway.

SA1-1. Induction of differentiated mammary morphology by alternation of n-6/n-3 ratio. We investigated whether an alternation of n-6/n-3 compositional ratio in favor of n-3 PUFAs will affect mammary development and differentiation. The effect of n-6/n-3 ratio change on mammary gland development and differentiation was assayed by morphological analyses of ductal elongation and appearance of a differentiated alveolar-like branching morphogenesis. Whereas 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 differentiation starts at the onset of pregnancy with the expansion of secretory lobular-alveolar architecture. Whole mount preparations of the mammary glands from 6-week to 14-week of virgin wild type and virgin fat-1 transgenic mice were examined to determine the effect of the different n-6/n-3 ratios on mammary gland development. Whereas no effect on ductal outgrowth during the early mammary gland development was observed (data not shown), increasing n-3 PUFA composition in the transgenic mouse resulted in a significant alternation in the developmental pattern of the branching points of ducts. Fig. 1 shows a representative mammary gland analysis of virgin transgenic mice vs. virgin wild-type control and pregnant littermate. Whereas the limited budding was developed in the wild-type gland (Fig. 1A), a gland from a 10-week old transgenic mouse

exhibited multiplicity of budding (Fig. 1B) and a gland from a 14-week transgenic mouse showed a robust budding morphology (Fig. 1C), a phenotype quite similar to the early pregnant mouse (Fig. 1D). A similar budding morphology was also observed in the transgenic mice at age of 8 and 12-week but not in the age-matched control mice. Transgenic mice at age 6-week did not show a significant budding morphology at the end bud region (data not shown).

SA1-2. Stimulation of β-casein expression and induction of Stat5 activation. In mammary gland development, the alveolar buds represent a developmental pathway that eventually leads to secretory alveoli during differentiation. To determine if the mammary epithelial cells were functionally as well as morphologically differentiated, the expression of the early differentiation marker milk protein β-casein was analyzed by real time RT-PCR. Fig. 1E shows representatives of β-casein expression in two virgin control mice and two age-matched virgin fat-1 mice. Whereas minimal levels of β-casein were detectable in non-differentiated virgin mice, increasing n-3 PUFAs composition in the fat-1 mammary gland significantly enhanced β-casein expression, resulting in an average 6.5-fold increase over control mice. These results indicate that the mammary glands of the fat-1 mice have 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.

The transcriptional activation of β-casein gene expression in mammary gland is mediated at least in part by Jak2 and Stat5 pathway. Phosphorylation on tyrosine is essential for Stat 5 binding and its transcriptional activity. We examined tyrosine phosphorylation of Stat5 in mammary glands of virgin control mice and virgin transgenic mice (Fig. 1F). Whereas undetectable or very limited phosphorylated Stat5 protein was observed in the gland from the non-differentiated virgin control mice, Stat5 phosphorylation was significantly increased in the mammary gland from virgin fat-1 mouse. These data demonstrated that alternation of n-6/n-3 compositional ratio in favor of n-3 fatty acid results in a phosphorylation of Stat5, indicating a potential role of n-3 fatty acid in activating of Stat5 in the mammary gland and induction of mammary gland differentiation.

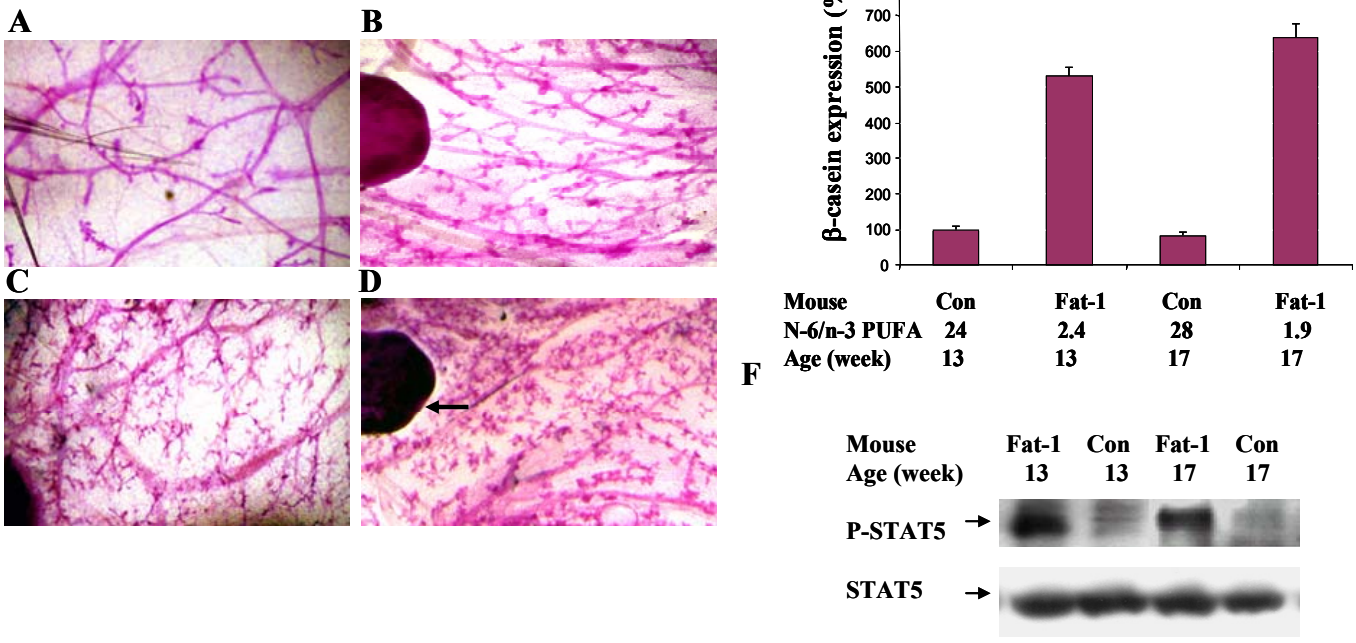

Fig. 1. Histological and molecular analysis of mammary gland differentiation in fat-1 mice. **A-D**. Whole mount histological analysis of mammary glands of fat-1 transgenic mice and wild type littermates. Two transgenic as well as two age-matched non-pregnant control mice were sacrificed at age 6, 8, 10, 12, and 14-week and subjected to whole mount morphological analysis. The right inguinal gland was removed and subjected to whole mount gland fix, defat, and staining. Representative virgin fat-1 mice, an virgin control mouse, and an early pregnant (8-day pregnant) wild-type littermate mouse were presented. A, a 14-week old wild type virgin mouse. B, a 14-week old fat-1 virgin mouse. C, a 14-week old fat-1 virgin mouse. D, a 14-week old wild type early pregnant mouse. An arrow indicates the inguinal lymph node. **E**. Quantitative RT-PCR analysis of β-casein expression. Inguinal mammary glands were isolated from age-matched virgin control and fat-1 mice. RNA was isolated and subjected to real time PCR analysis. Relative expressions of mouse β-casein gene in the mammary glands from fat-1 mice were calculated in comparison to that from control mouse. The β-casein gene expression in the 13-week old control mouse was taken as 100% and regarded as control. All the other values were expressed as a percentage of the control. The mouse beta actin gene was used as endogenous control. The numbers represent the means ± SD of duplicate samples. Statistical comparisons for both fat-1 mice relative to control mice indicate $p < 0.001$ for the relative β-casein expression. **F**. Induction of Stat5 phosphorylation in the mammary glands of fat-1 transgenic mice. Thirteen-and 17-week old virgin control mice and age-matched transgenic mice 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 Star5 antibody and normalized for total Stat5 expression.

SA1-3. Induction of Stat5 activation and mammary differentiation by DPA and EPA. Although we demonstrated that a decrease in n-6/n-3 ratio in mammary gland of fat-1 mouse resulted in a differentiated phenotype, it is not clear whether DPA and EPA, which were preferentially accumulated in the gland during pregnancy, play a role in the induction of mammary differentiation. Using MCF-10 mammary epithelial cells, we analyzed the effect of DPA, EPA, and DHA on activation of Jak2 and Stat5. Whereas DPA and EPA activated Jak2 and Stat5, DHA did not induce Jak2 and Stat5 phosphorylation (Fig. 2A). We also analyzed the effect of DPA on induction of Srat5 phosphorylation in mammary organ culture. Whereas limited phosphorylated Stat5 protein was detectable in the non-treated gland, treatment of glands with DPA significantly stimulated Stat5 phosphorylation, resulting in a 5.6-fold and 7.8-fold increase over the control glands, respectively (Fig. 2B).

We then used an *ex vivo* model involving mouse whole-organ culture of mammary gland to study whether n-3 PUFAs DPA, EPA, and DHA can regulate milk protein β-casein. Inguinal mammary glands from virgin mice were cultured for 6 days with or without 30 μM DPA, or EPA, or DHA. Consistent with the observed differentiated phenotype in the transgenic gland, a differentiation with stimulation of β-casein was observed in the glands treated with DPA. Expression of β-casein mRNA was significantly increased in DPA treated glands with an average 6.4-fold increase over the control non-treated glands (Fig. 2C). A similar significant stimulation of β-casein expression was also observed in EPA-treated glands, resulting in a 5.7-fold increase over controls (Fig. 2D). Treatments of glands with DHA resulted in a slight increase (2.4-fold) in β-casein expression over controls (Fig. 2E).

To functionally validate the role of Start5 on n-3 PUFA-induced mammary differentiation, we examined the effect of DPA on induction of β-casein expression on *ex vivo* model using mammary glands from Stat5a-deficient Stat5a^{tm1Mam} mice (29). In Stat5a^{tm1Mam} mice, mammary ductal development through pregnancy is normal, but lobuloalveolar development is severely reduced and there is no milk secretion even after prolonged suckling. Whereas DPA induced a significant stimulation of β-casein expression in the glands from wild type mice (Fig. 2C), there were only a slight increase but not significant in DPA-treated Stat5 knock out glands (Fig. 2F). These data indicate that the preferential accumulation of n-3 PUFAs, such as DPA and EPA, in the differentiated mammary

gland during pregnancy m ay act as a factor i nducing f unctional mammary gland differentiation mediated by activation of Jak2 and Stat5.

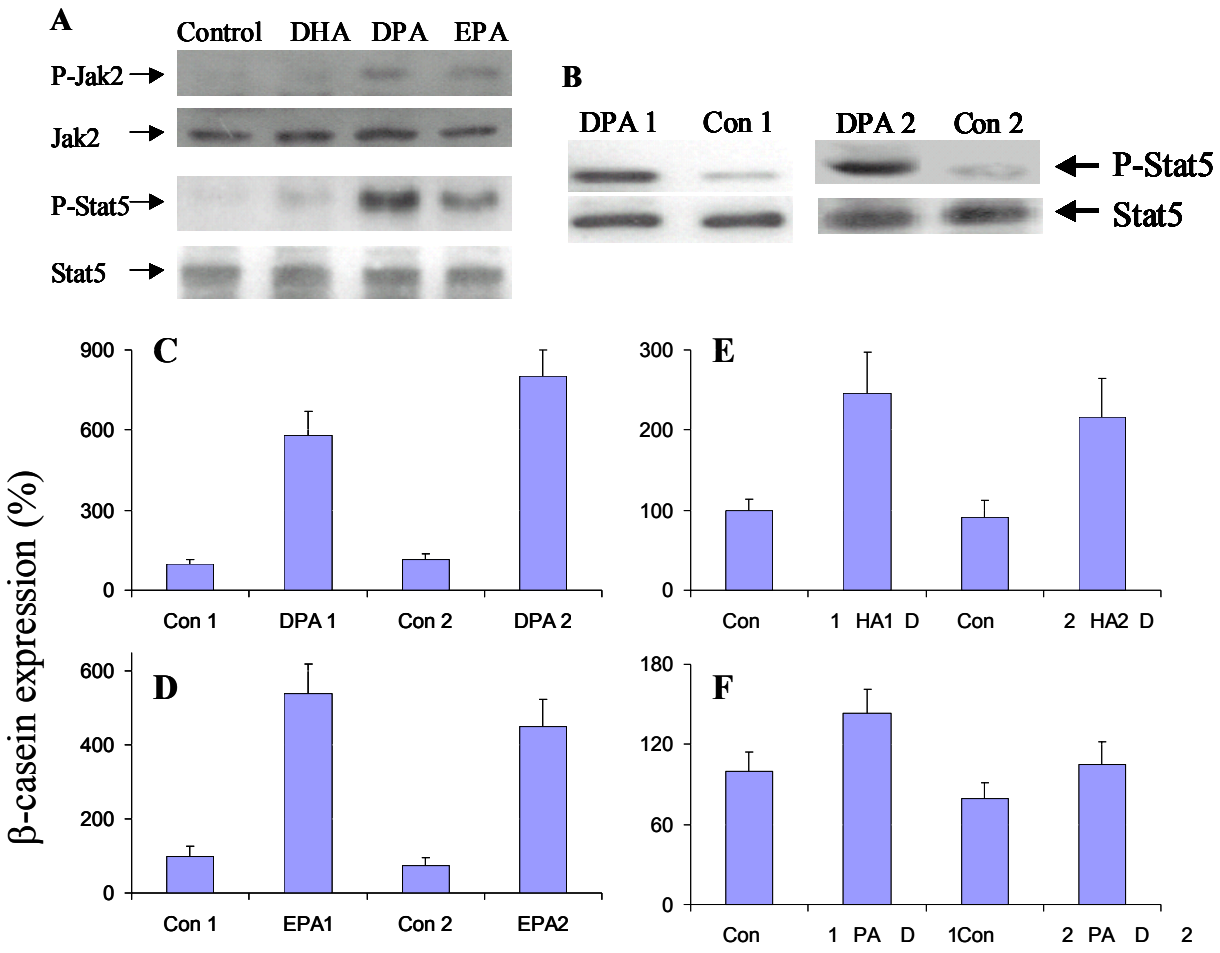

Fig. 2. Induction of Jak 2 and Stat5 activation and β-casein expression by DPA and EPA. **A**. MCF-10 cells. Cells were treated with 10 μM of DHA, DPA, and EPA for 36 hours. Total cellular protein was isolated, subjected to Western analysis with antibodies against phosphorylated Jak2 and Stat5, and normalized with total Jak2 and Stat5 expression. **B**. Mammary organ culture. Two pairs of inguinal mammary glands from two 14-week virgin mice were cultured in the medium supplemented with bovine pituitary extract, insulin, EGF, and hydrocortisone as described in Methods for 2 days with or without 30 μM DPA. Total protein was isolated, normalized, and 400 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 Star5 antibody and normalized for total Stat5 expression. **C-F**. Stimulation of β-casein expression by n-3 PUFAs. Two pairs of inguinal mammary glands from two 14-week wild type virgin non-transgenic control mice (C-E) and Stat5a knockout mice ***Stat5a***^{tm1Mam} (F) were cultured for 6 days with or without 30 μM DPA (C and F), or EPA (D), or DHA (E) in the organ culture medium. Fresh media containing n-3 PUFAs were added every two days. At the end of 6-day treatment, the gland was subjected to RNA extraction for RT-PCR analysis of β-casein expression. Relative expressions of mouse β-casein gene in the mammary glands treated with n-3 PUFAs were calculated in comparison with that from Con 1 mouse, which was taken as 100% and regarded as control. All the other values were expressed as a percentage of the control. The mouse beta actin gene was used as endogenous control. The numbers represent the means ± SD of duplicate samples.

SA2. Does DPA prevent development of preneoplastic lesions and mammary tumorigenesis? For cancer prevention studies using n-3 PUFA, most of the experiments use crude marine oil with a various different classes of n-3 PUFAs. Although EPA, DPA, linolenic acid, and DPA are considered as a group of n-3 PUFA, there is an urgent need for further investigation of the precise activities of each individual n-3 PUFAs on mammary differentiation and breast cancer prevention. Unlike DHA and EPA, which are widely available and abundant in fish oil and extensively studied, there are much less functional studies for DPA. We will test the hypothesis that n-3 DPA, an under-explored n-3 PUFA preferentially accumulated in the pregnant gland, mimics the pregnancy effect on differentiation and prevention of mammary tumor.

Not finished yet

KEY RESEARCH ACCOMPLISHMENTS AND REPORTABLE OUTCOMES

1. There is a change in n-3 to n-6 PUFA composition, favoring a lower n-6/n-3 ratio in mammary gland following pregnancy and more interestingly, there is a significant increase in n-3 PUFA DPA and EPA in the pregnant mammary.
2. The functional role of n-3 PUFAs on differentiation was supported by the studies in fat-1 transgenic mouse, which converts endogenous n-6 to n-3 PUFAs. Alternation of n-6/n-3 ratio in favor of n-3 PUFA and particularly DPA in the mammary gland of fat-1 mouse resulted in development of lobuloalveolar-like structure and milk protein β-casein expression, mimicking differentiated state of the pregnant gland.
3. DPA and EPA activated Jak2-Stat5 pathway and induced a functional differentiation with production of β-casein

CONCLUSIONS

The possibility of preventing breast cancer with dietary factors that induce mammary differentiation is of practical interest to the high-risk women. We studied if pregnancy-mediated breast cancer prevention is associated with an alternation of n-6/n-3 ratio in favor of n-3 PUFA. Notable findings to this study are there is a change in n-3 to n-6 PUFA composition, favoring a lower n-6/n-3 ratio in mammary gland following pregnancy and more interestingly, there is a significant increase in n-3 PUFA DPA and EPA in the pregnant mammary. Our data suggest alternation of n-6/n-3 ratio in favor of n-3 PUFA and particularly DPA and EPA may be one of the underlying mechanisms for pregnancy-mediated mammary differentiation. In supporting this novel notion, we demonstrated a similar n-6/n-3 ratio change and a differentiated phenotype in the mammary gland from the transgenic mouse expressing fat-1 gene that converts endogenous n-6 to n-3 PUFAs. In addition, the differentiation effect of DPA and EPA on mammary gland was also demonstrated in the mouse mammary organ culture. Our studies have taken two well-established epidemiological observations and animal studies of the decreased risk of breast cancer in association with pregnancy-induced differentiation and n-3 PUFA, to point to an under-explored area mechanistically linking an alternation of n-6/n-3 ratio and particularly DPA and EPA to pregnancy-induced differentiation and potential breast cancer prevention. It is noteworthy to emphasize here that because the degree of mammary gland differentiation induced by n-3 PUFAs is not likely to be compatible to the differentiation that occurs during full term pregnancy, we are not sure whether the induced gland differentiation is one of the major contributing factors for n-3 PUFA-mediated breast cancer prevention.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 47, Issue of November 21, pp. 47319–47325, 2003
Printed in U.S.A.

Induction of Mammary Gland Differentiation in Transgenic Mice by the Fatty Acid-binding Protein MRG*

Received for publication, July 25, 2003, and in revised form, August 25, 2003
Published, JBC Papers in Press, September 15, 2003, DOI 10.1074/jbc.M308131200

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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 mouse mammary tumor virus 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 lobuloalveolar 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 *versus* 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 a mediator of the differentiating effects of pregnancy on breast epithelium, and overexpression of MRG in young nulliparous mice can induce differentiation.

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[1] (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 number 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 β-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 fibers, 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

* This work was supported in part by Grant CA93542-2 from the National Institutes of Health and Grant DAMD17-01-1-0353 (Postdoctoral Fellowship Award) from the United State Army Breast Cancer Research Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MRG, mammary-derived growth inhibitor-related gene; FABP, fatty acid-binding protein; B-FABP, brain type FABP; H-FABP, heart-derived FABP; hCG, human chorionic gonadotropin; MDGI, mammary-derived growth inhibitor; MMTV, mouse mammary tumor virus; RT, reversed transcription; Prl, prolactin; JAK, Janus kinase.

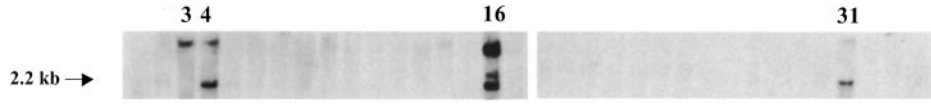

FIG. 1. **Southern blot analysis of MRG fusion gene in transgenic mice.** DNAs were extracted from 1.5-cm sections of tails and digested with BamHI. The presence of a 2.2-kb transgene was detected by Southern blot analysis with full-length MRG cDNA probe. *Lane 3*, MM3; *lane 4*, MM4; *lane 16*, MM16;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

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 BamHI and ApaI 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 first-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 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. 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 described previously (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 RNeasy MINI Kit (Qiagen). One μg of total RNAs were used for RT reaction using oligo$(dT)_{15}$ primers (Roche Applied Science), and one-fifth of this reaction was amplified by PCR using Roche Applied Science PCR kit. Each reaction consisted of 30 cycles in the GeneAmp PCR System 2400 (PerkinElmer Life Sciences). The parameters for PCR were: denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 90 s. One-third of the PCR products were electrophoresed through 1.2% agarose gel. The 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 TCC TCA GTC CAA AGT-3′ and 5′-TTG AAA TGA CTG GAA AGG AAA TAG-3′; and 3) human *MRG*, 5′-GTGGAGGCTTTCTGTGCTACCTGG-3′ and 5′-TGCCTTCTCATAGTGGCGAACAG-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 described previously (17). The removed gland was subjected to whole mount fix, defat, and staining as described previously (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 and 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 Hormone Chorionic Gonadotropin (hCG)—Treatment of virgin mouse with hCG was performed as described previously by Russo *et al*. (27–29) with some modifications. Briefly, virgin mice were injected intraperitoneal with hCG, 20 units/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 Figs. 1 and 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 first-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,

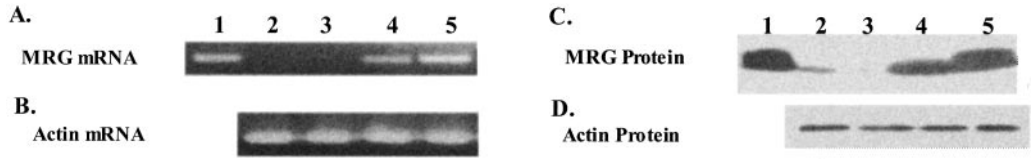

FIG. 2. ***MRG* transgene expression in control and homozygous transgenic lines.** Ten-week-old virgin MM-H1 and MM-H2 mice and age-matched control virgin mice were sacrificed, and the inguinal mammary glands were removed. The left gland was subjected to RNA isolation and RT-PCR analysis, and the right gland was subjected protein isolation and Western analysis. *A*, RT-PCR analysis of *MRG* using primers within *MRG* cording 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; *lanes 2* and *3*, control mice; *lane 4*, MM-H2; *lane 5*, MM-H1. *C* and *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; *lanes 2* and *3*, control mice; *lane 4*, MM-H1; *lane 5*, MM-H2. *Lanes 2–5* contained 50 μg of cellular protein.

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 non-transgenic 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. 2*C*). 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 *versus* control pregnant mouse by Western blot (Fig. 4*A*). 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. 4*C*).

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 weeks old in wild-type mice with ductal elongation

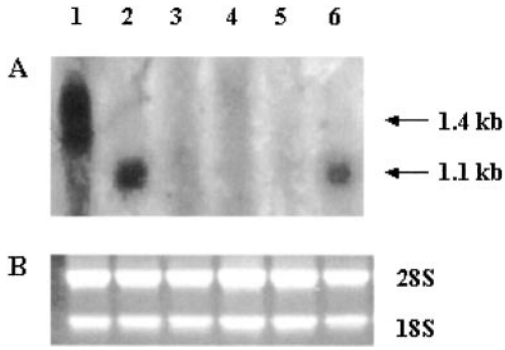

FIG. 3. **Tissue expression of *MRG* transgene.** Total RNA was isolated from different organs from a 10-week-old virgin MM-H2 transgenic mouse. Expression of *MRG* was analyzed by Northern blot with full-length human *MRG* cDNA probe (*A*) and normalized by visualization of ribosomal bands (*B*). *Lane 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.

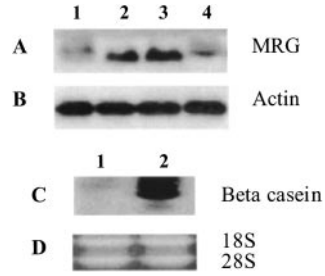

FIG. 4. **Expression of mouse MRG and β-casein in control non-transgenic mice.** Inguinal mammary glands were isolated from 10-week old non-transgenic virgin and 8-day pregnant mice. Expression of mouse MRG protein (*A*) was analyzed by Western blot and normalized for β-actin expression (*B*). Total protein was isolated and normalized, and 25 μg of total protein were subjected to Western analyses with either MRG antibody or anti-actin antibody. *Lanes 1* and *4*, virgin mice; *lanes 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.

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 to 12 weeks 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- and 10-week-old transgenic mouse *versus* wild-type control littermate. Mammary ducts in the transgenic virgin (Fig. 5*B*) as well as in the control virgin littermate (Fig. 5*A*) filled the typical half-length of the inguinal gland and appeared

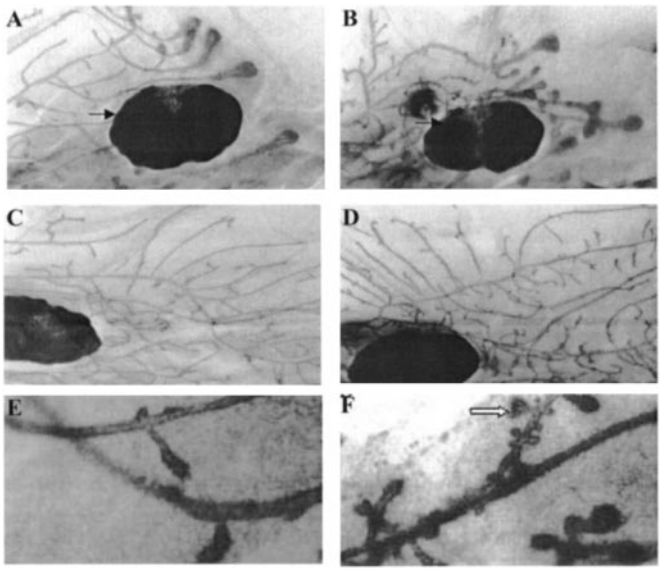

FIG. 5. **Whole mount histological analysis of mammary gland from female MM-H2 transgenic mouse and wild-type littermate.** A 4-week-old (*A* and *B*) and 10-week-old (*C–F*) virgin MM-H2 mouse and an age-matched virgin wild-type littermate mouse were sacrificed, and the right inguinal gland was removed 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* and *B*, lower magnification images from (Nikon, 2 × 10). *Arrows* indicate the inguinal lymph node and the direction for duct extension (from *left* to *right*). *C* and *D*, lower magnification (1 × 10). *E* and *F*, higher magnification pictures from (10 × 10). An *open arrow* indicates budding.

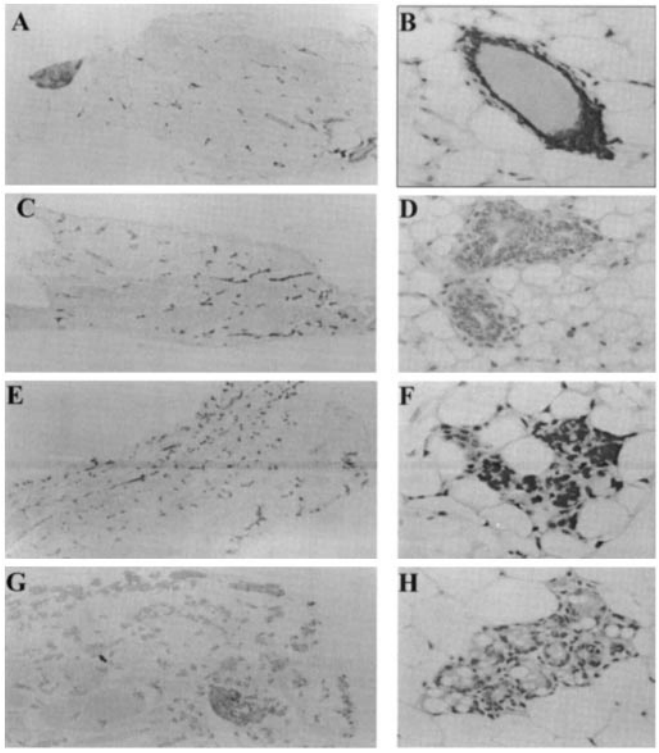

FIG. 6. **Histological analysis of mammary gland.** Whole inguinal mammary glands were isolated from 10-week-old female virgin mice. All the sections were stained with hematoxylin and eosin for histological analysis. *A* and *B*, control mouse mammary gland. *A*, 10 × 10; *B*, 40 × 10. *C* and *D*, mammary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

normal. At the 10 weeks old, the mammary gland was completely filled with the ducts in both control (Fig. 5*C*) and transgenic mouse (Fig. 5*D*). 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. 5*E*), transgenic gland exhibited multiplicity of budding (Fig. 5*F*).

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 hematoxylin and eosin-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 weeks old) transgenic mice compared with the age-matched control mice. However, starting at 8 weeks old, a significantly different morphology was observed in the transgenic mice *versus* 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* and *B*), formation of the alveolar-like structure were observed in the gland from a transgenic mouse (*C* and *D*). Since the observed morphology did not look like the fully differentiated phenotype with expanded alveolar lumina containing lipid droplets, we further investigated whether 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. 6*F*). 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. 6*H*).

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 *et al.* (27–29) that treatment of rat with human placental hCG resulted in a similar effect on mammary differentiation as pregnancy. 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 units/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* and *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. 7, *C* and *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

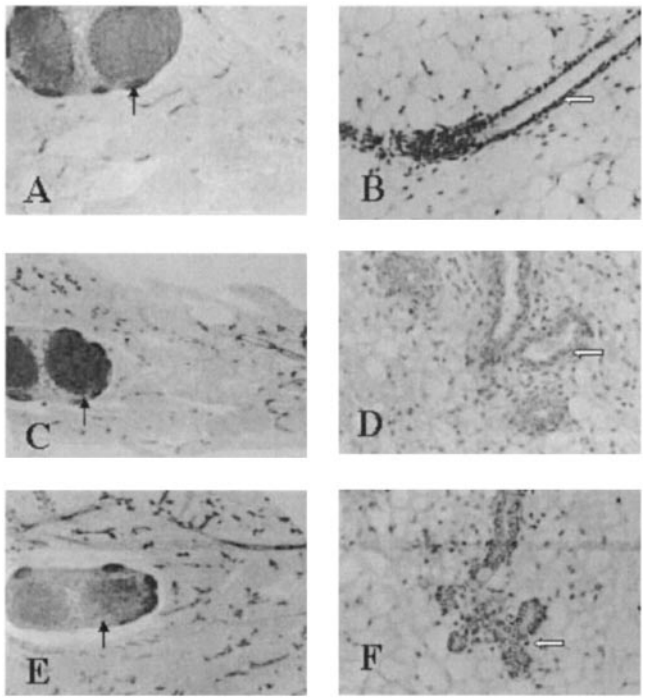

FIG. 7. **Histological comparison of the glands from transgenic mouse to the gland from hormone-treated mouse.** Whole inguinal mammary glands were isolated from 10-week-old control virgin mouse, transgenic mouse, and hormone hCG-treated control mouse. All the sections were stained with hematoxylin and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

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 non-transgenic 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 whether 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, *lanes 6–9*). However, no detectable β-casein transit was observed in age-matched control virgin mice (Fig. 8, *lanes 1–3*). As expected, expression of β-casein was detected in an 8-day pregnant of normal mouse (Fig. 8, *lane 4*). We also investigated the

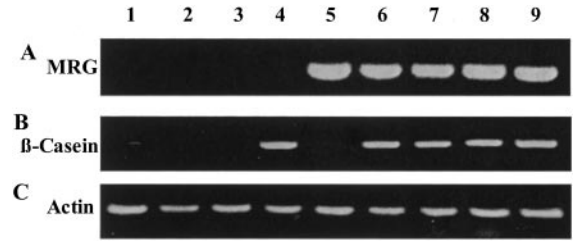

FIG. 8. **RT-PCR analysis of *MRG* transgene and *β-casein* expression.** Ten-week-old fourth generation virgin MM-H1 and MM-H2 mice, age-matched control virgin mice, and control pregnant mouse were sacrificed, and the inguinal mammary glands were removed. Expression of *MRG* transgene (*A*) and *β-casein* 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 under "Materials and Methods." *Lanes 1–4*, control mice; *lane 4*, control pregnant 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.

late marker whey acidic protein expression in the transgenic mouse. No whey acidic protein 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- 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. 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 dem-

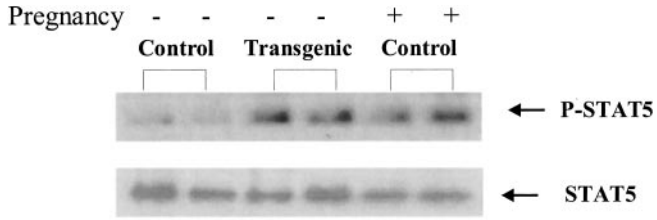

FIG. 9. **Induction of Stat5 phosphorylation in the mammary glands by pregnancy and MRG transgene expression.** Ten-week-old virgin control mice (*lanes 1* and *2*), age-matched transgenic MM-H1 mice (*lanes 3* and *4*), and age-matched control 4-day pregnant mice (*lanes 5* and *6*) were sacrificed, and 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*).

onstrated that 1) exogenous expression of MRG resulted in differentiated gland morphology with increased formation of lobuloalveoli-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. Stat5 was originally identified as a mammary gland factor that binds to promoter sequences of β-casein and activates its transcription (35, 37). Gene targeting experiments indicate that Stat5a 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 controlled 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 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 mammary epithelial cells by use of antisense oligonucleotides suppresses the formation of alveolar-liken 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 *in vitro*, there is a concern about its role as differentiating factor for mammary gland *in vivo*. The *in vitro* data contrast with *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 knock-out 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 *in vitro* and *in vivo* effect on mammary differentiation, MRG revealed a similar differentiating effect on mammary epithelial cells both *in vitro* and *in vivo*. The expression and the *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 *in vitro* cellular differentiating effects, glands from the virgin MMTV/MRG mice possess a significant increase in the appearance of lobular alveolilike structure *versus* 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 *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 is 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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the FEBS Journal

Activation of Stat5 and induction of a pregnancy-like mammary gland differentiation by eicosapentaenoic and docosapentaenoic omega-3 fatty acids

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Keywords
breast cancer prevention; DPA; EPA; n-3 fatty acid; pregnancy

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(Received 7 February 2007, revised 23 March 2007, accepted 7 May 2007)

doi:10.1111/j.1742-4658.2007.05869.x

The protective effect of early pregnancy against breast cancer can be attributed to the transition from undifferentiated cells in the nulliparous to the differentiated mature cells during pregnancy. Considerable evidence suggests strongly that the n-3 polyunsaturated fatty acid (PUFA) content of adipose breast tissue is inversely associated with an increased risk of breast cancer. Here, we report that there was a decrease in the n-6/n-3 PUFA ratio and a significant increase in concentration of n-3 PUFA docosapentaenoic acid and eicosapentaenoic acid in the pregnant gland. The functional role of n-3 PUFAs on differentiation was supported by the studies in the fat-1 transgenic mouse, which converts endogenous n-6 to n-3 PUFAs. Alternation of the n-6/n-3 ratio in favor of n-3 PUFA, and particularly docosapentaenoic acid, in the mammary gland of fat-1 mouse resulted in development of lobulo-alveolar-like structure and milk protein β-casein expression, mimicking the differentiated state of the pregnant gland. Docosapentaenoic acid and eicosapentaenoic acid activated the Jak2/Stat5 signaling pathway and induced a functional differentiation with production of β-casein. Expression of brain type fatty acid binding protein brain type fatty acid binding protein in virgin transgenic mice also resulted in a reduced ratio of n-6/n-3 PUFA, a robust increase in docosapentaenoic acid accumulation, and mammary differentiation. These data indicate a role of mammary derived growth inhibitor related gene for preferential accumulation of n-3 docosapentaenoic acid and eicosapentaenoic acid in the differentiated gland during pregnancy. Thus, alternation of n-6/n-3 fatty acid compositional ratio in favor of n-3 PUFA, and particularly docosapentaenoic acid and eicosapentaenoic acid, is one of the underlying mechanisms of pregnancy-induced mammary differentiation.

Studies have consistently shown that women who have undergone an early full-term pregnancy have a significantly reduced lifetime risk of breast cancer [1–5]. This protective effect can also be demonstrated in animal models. The highly proliferating and undifferentiated gland of the virgin rat exhibits maximal susceptibility to neoplastic transformation, whereas the fully differentiated gland of parous rats or virgin rats treated with placental hormone human chorionic gonadotropin (hCG) is protected from tumor development [6–8].

Abbreviations
AA, arachidonic acid; COX, cyclo-oxygenase; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FABP, fatty acid binding protein; B-FABP, brain type FABP; hCG, human chorionic gonadotropin; MMTV, mouse mammary tumor virus; MRG, mammary derived growth inhibitor related gene; PUFA, polyunsaturated fatty acid; RXR, retinoid X receptor.

Because both pregnancy and hCG treatment induce differentiation of the mammary gland, the protective effect of pregnancy against breast cancer can be attributed to the transition from undifferentiated mammary cells in the nulliparous to the differentiated mature cells during pregnancy.

Whereas most of the studies indicate that n-6 polyunsaturated fatty acid (PUFA) promotes tumorigenesis, n-3 PUFA prevents and suppresses tumorigenesis [9–11]. Altered composition levels of n-3 and n-6 PUFA have been observed in tumor cells as compared to their normal counterparts, consistent with their opposite effects on tumorigenesis [12–19]. When prostatic levels of PUFA in relation to the histopathological stages were analyzed, it was found that the n-3 to n-6 PUFA ratio in prostate tumor was three-fold lower in controls [12]. Similar PUFA composition profiles were also demonstrated in serum in normal controls, patients with benign prostatic hyperplasia, and patients with prostate cancer [13]. The ratio of n-3 to n-6 PUFA decreased in the following order of normal, hyperplasia, and prostate cancer. The PUFA composition of human gliomas was found to be different from nonmalignant brain tissue. Levels of n-3 PUFA docosahexaenoic acid (DHA) were significantly reduced in the glioma samples compared with normal brain samples; in contrast, the glioma content of the n-6 PUFA linoleic acid was significantly greater than that observed in the control samples [14,15]. As for mammary tissue, a variety of evidence suggests strongly that the n-3 PUFA content of adipose breast tissue is inversely associated with increased risk of breast cancer incidence and progression. The most comprehensive study came from the European Community Multicenter, in which the fatty acid contents of adipose tissue in postmenopausal breast cancer cases and controls were analyzed in five European countries [16]. The study showed a significantly lower ratio of n-3 to n-6 PUFA in breast cancer cases versus controls. Similar studies with a smaller sample size also support this inverse association between the ratio of n-3 to n-6 PUFA and breast cancer risk [17], breast cancer metastasis [18], and sensitivity of mammary tumors to cytotoxic drugs [19].

The preventative effect of a dietary supplement of n-3 PUFAs to the mother on the risk of breast cancer risk for offspring has been reported. Offspring of the rat fed an n-6 PUFA diet during pregnancy developed significantly more mammary tumors and had a shorter tumor latency than the offspring of the rat fed an n-3 PUFA diet [20]. Similarly, early exposure to an n-3 PUFA diet in the prepubertal stage also reduced mammary tumorigenesis in the experimental rats [21]. These data suggest that consumption of n-3 PUFAs at prenatal or prepubertal stage will affect mammary gland development (e.g. induction of differentiation) and thus reduce the risk of breast cancer. The molecular basis underlying the opposing effects of n-3 and n-6 PUFAs is still not fully understood. It is believed that much of the preventing effects are attribute to their anti-inflammation activity, mediated by alternation of cyclo-oxygenase (COX) metabolism [22].

Although studies in laboratory animal and *in vitro* models report significant suppressive effects of dietary n-3 PUFA on the incidence, growth rate, or proliferation of mammary and many other different tumors, the most recent systematic review of 20 cohorts suggest that there is no significant association between n-3 PUFA and the incidence of cancer [23]. However, this statement of lack of association between n-3 PUFA and cancer risk should be interpreted with caution. First, studies on n-3 PUFA consumption varied a great deal across study cohorts. Second, interpretation of the data is limited by significant differences in the methods used to ascertain exposure to n-3 PUFA. Third, of particular note is the fact that n-3 PUFA consumption generally consists of varying the ratio of n-3 to n-6 PUFA without consideration of n-6 fatty acid consumption. Very importantly, when calculating n-3 PUFA consumption, the background n-6 PUFA consumption has to be considered. It is assumed that most of the beneficial effects including cancer prevention is mediated by alternation of the n-3/n-6 compositional ratio but not the exact amount of n-3 PUFA [24–27]. Because a higher n-6/n-3 PUFA ratio is considered to be a risk factor for breast cancer, we are interested in testing the hypothesis that pregnancy-induced mammary gland differentiation and breast cancer prevention is mediated in part by a PUFA composition change in mammary gland. We demonstrated that there is a change in the ratio of n-3 to n-6 PUFA composition with a significant increase in n-3 docosapentaenoic acid (DPA) and eicosapentaenoic acid (EPA) in the mammary gland following pregnancy. Alternation of the n-6/n-3 ratio in favor n-3 fatty acid in mammary gland mimics the effect of pregnancy on mammary differentiation.

Results

Alternation of n-6/n-3 fatty acid compositional ratio in the mammary gland of the pregnant mouse

We have examined the mammary PUFA profiles in virgin, pregnant, and postpregnant mice. As summarized in Table 1, two fatty acid ratios are expressed:

Table 1. Analyses of fatty acid ratio and relative contents of n-3 PUFAs EPA, DPA, and DHA in mammary glands. Whole inguinal mammary fat pads were isolated and contents of fatty acids were analyzed by gas chromatography. The n-6/n-3 ratio is given by (Linoleic acid 18:2 n-6 + AA 20:4 n-6):(Linolenic acid 18:3 n-3 + EPA 20:5 n-3 + DPA 22:5 n-3 + DHA 22:6 n-3). Relative concentrations of individual n-3 PUFA were expressed as the percentage in a comparison of total PUFA contents of a combination of EPA, DHA, and DPA. For comparison of the fatty acid concentration profile in nontransgenic control virgin versus pregnant mice, a total of eight mice were killed, including four 18-week-old virgin and four age-matched late pregnant (18-day-old) mice. Data represent the means ± SD of two separate experiments with four mammary gland samples. Statistical comparisons for both ratio 1 and ratio 2 in pregnant glands relative to the virgin glands indicate $P < 0.01$ for the n-6/n-3 fatty acid ratio; relative concentration of n-3 PUFAs in pregnant glands versus virgin glands indicates $P < 0.02$ for EPA and $P < 0.009$ for DHA. For comparison, virgin fat-1 mice versus nontransgenic virgin controls, we fed the mice with a diet high in n-6 and low in n-3 fatty acids, as described in Experimental procedures. A total of three 12-week-old virgin controls and three age-matched fat-1 transgenic mice were killed and subjected to fatty acid analysis. Data represent the means ± SD of three mammary gland samples. Statistical comparisons for ratio 1 and ratio 2 in the fat-1 transgenic glands relative to the control glands indicate $P < 0.01$ and $P < 0.001$, respectively. Statistical comparisons for relative concentration of n-3 PUFAs in fat-1 glands versus control glands indicates $P < 0.001$ for EPA and $P < 0.01$ for DHA.

	Ratio 1 (%) n-6/n-3	Ratio 2 (%) AA/EPA + DPA + DHA	Relative expression (%)		
			EPA/total	DPA/total	DHA/total
Virgin	21.0 ± 1.2	2.1 ± 0.2	8.0 ± 2.1	0	92.0 ± 8.1
Pregnant	7.2 ± 1.4	1.0 ± 0.2	19.2 ± 3.9	18.2 ± 3.5	62.6 ± 7.8
Control	445.8 ± 89.1	25.2 ± 3.1	3.9 ± 0.2	0	96.1 ± 8.1
Fat-1	116.5 ± 10	2.1 ± 0.2	22.5 ± 3.1	21.2 ± 3.2	56.3 ± 6.8

ratio 1 includes a wider range of n-6 and n-3 fatty acids and ratio 2 only reflects the polyunsaturated (more than four double bonds) fatty acids. It is noteworthy that since we exclude C18:2 n-6 and C18:3 n-3, which are in very high abundance in the gland, ratio 2 represents the status of the most studied polyunsaturated n-6 arachidonic acid (AA) and n-3 EPA, DPA, and DHA. There is a 2.9-fold and 2.1-fold decrease in the n-6/n-3 ratio 1 and ratio 2 in the pregnant gland compared with the virgin gland, respectively, suggesting a preferential accumulation of n-3 over n-6 PUFA in the gland during pregnancy.

Preferential accumulation of n-3 PUFA DPA and EPA in the pregnant mammary gland

Although there is a minor change in the n-6/n-3 PUFA ratio 2 from 2.1 in virgin gland to 1 in pregnant gland, when individual PUFA content was analyzed in the mammary gland, a significant increase in n-3 DPA and EPA in pregnant glands versus control glands is observed (Table 1). Whereas there was an abundant DHA in the virgin control gland, the amount of DPA and EPA was either undetectable or very limited. There was a robust increase in n-3 DPA during pregnancy from a nondetectable amount in virgin gland to an abundant accumulation in the pregnant gland. The relative concentration of EPA was increased more than two-fold from the pregnant gland versus virgin gland. The relative DHA concentration was decreased from 92% in the control gland to 62% in the pregnant gland. There are two types of DPA: n-6 and n-3 fatty acid. Omega 3 DPA (22:5 n-3) is the elongation product of EPA (20:5 n-3) or the precursor for DHA (22:6 n-3) by addition of one more double bond. Whereas most studies on n-3 PUFA use EPA and DHA, which are widely available and also abundant in fish oil, few biological studies specifically using n-3 DPA have ever been reported. The preferential accumulation of n-3 DPA and EPA in the mammary gland during pregnancy may indicate their specific function in mammary differentiation.

Alternation of the n-6/n-3 ratio in mammary gland of the fat-1 transgenic mouse

The alternation of n-6/n-3 compositional ratio in favor of n-3 fatty acid and a robust increase of n-3 DPA and EPA in the mammary gland following pregnancy indicate the potential role of the n-6/n-3 ratio on mammary gland differentiation during pregnancy. However, we are unsure whether an alternation of the n-6/n-3 ratio is one of the instigators of mammary gland differentiation or merely a correlative product during pregnancy. It is quite likely that the observed alternation of the n-6/n-3 compositional ratio might be one of the many changes in the gland in preparation for breast nursing, but not the contributory factor. To determine whether n-3 PUFA and particularly DPA and EPA induce mammary gland differentiation, we investigated the role of the n-6/n-3 compositional ratio on mammary differentiation using our recently developed fat-1 transgenic model [28]. In the transgenic mouse, fat-1 can convert n-6 to n-3 fatty acids and

result in an abundance of n-3 and a reduction in n-6 fatty acids in the organs and tissues of these mice, in the absence of dietary n-3 fatty acids. When transgenic and wild-type mice were maintained on an identical diet that was high in n-6 but very low in n-3 fatty acids, the tissue fatty acid profiles of the two groups turned out to be quite different. Previously, n-6/n-3 ratios were determined in several organ samples, including muscle, heat, brain, liver, kidney, lung, and spleen. Whereas the n-6/n-3 ratio was in the range 20–50 in most organs in control mouse, it dropped almost to 1 in the transgenic mouse. We determined the n-6/n-3 ratio in the mammary gland in control versus transgenic mice. Whereas there is a 3.8-fold decrease in the ratio reflecting a wider range of n-6 and n-3 fatty acids from 446 in wild-type mice to 117 in transgenic mice, the ratio 2 of n-6/n-3 PUFA in mammary gland dropped 12-fold from 25 in wild-type mice to 2 in transgenic mice (Table 1). When individual PUFA content was analyzed, we also observed a robust increase of n-3 DPA, from being nondetectable (0%) in the gland from a control mouse to an abundant amount (20% of total PUFAs) in the gland from the fat-1 mouse.

Induction of differentiated mammary morphology by alternation of the n-6/n-3 ratio

We next investigated whether an alternation of the n-6/n-3 compositional ratio in favor of n-3 PUFAs affects mammary development and differentiation. The effect of an n-6/n-3 ratio change on mammary gland development and differentiation was assayed by morphological analyses of ductal elongation and appearance of a differentiated alveolar-like branching morphogenesis. Whereas the mammary gland development starts at approximately 3 weeks old in wild-type mice with ductal elongation and development of the initial branching structure, the differentiation starts at the onset of pregnancy with the expansion of secretory lobulo-alveolar architecture. Whole mount preparations of the mammary glands from 6-week to 14-week-old virgin wild-type and virgin fat 1 transgenic mice were examined to determine the effect of the different n-6/n-3 ratios on mammary gland development. Whereas no effect on ductal outgrowth during the early mammary gland development was observed (data not shown), increasing n-3 PUFA composition in the transgenic mouse resulted in a significant alternation in the developmental pattern of the branching points of ducts. Figure 1 shows a representative mammary gland analysis of virgin transgenic mice versus a virgin wild-type control and pregnant littermate. Whereas the limited budding was developed in the wild-type gland (Fig. 1A), a gland from a 10-week-old transgenic mouse exhibited multiplicity of budding (Fig. 1B) and a gland from a 14-week-old transgenic mouse showed a robust budding morphology (Fig. 1C), a phenotype quite similar to the early pregnant mouse (Fig. 1D). A similar budding morphology was also observed in the transgenic mice at 8 and 12 weeks but not in the age-matched control mice. Transgenic mice at age 6 weeks did not show a significant budding morphology at the end bud region (data not shown).

Stimulation of β-casein expression and induction of Stat5 activation

In mammary gland development, the alveolar buds represent a developmental pathway that eventually leads to secretory alveoli during differentiation. To determine whether the mammary epithelial cells were functionally as well as morphologically differentiated, the expression of the early differentiation marker milk protein β-casein was analyzed by real time RT-PCR. Figure 1E shows β-casein expression in two virgin control mice and two age-matched virgin fat-1 mice. Whereas minimal levels of β-casein were detectable in nondifferentiated virgin mice, increasing n-3 PUFA composition in the fat-1 mammary gland significantly enhanced β-casein expression, resulting in an average 6.5-fold increase over control mice. These results indicate that the mammary glands of the fat-1 mice have the morphological formation of an alveolar-like structure and functional expression of the early differentiation marker, β-casein. The histological as well as molecular changes observed in the gland from the transgenic mice resemble the differentiated phenotype in the gland from the early pregnant mice.

The transcriptional activation of β-casein gene expression in mammary gland is mediated at least in part by the Jak2/Stat5 signaling pathway. Phosphorylation on tyrosine is essential for Stat5 binding and its transcriptional activity. We examined tyrosine phosphorylation of Stat5 in the mammary glands of virgin control mice and virgin transgenic mice (Fig. 1F). Whereas undetectable or very limited phosphorylated Stat5 protein was observed in the gland from the nondifferentiated virgin control mice, Stat5 phosphorylation was significantly increased in the mammary gland from the virgin fat-1 mouse. These data demonstrated that alternation of the n-6/n-3 compositional ratio in favor of n-3 fatty acid results in a phosphorylation of Stat5, indicating a potential role of n-3 fatty acid in activating of Stat5 in the mammary gland and induction of mammary gland differentiation.

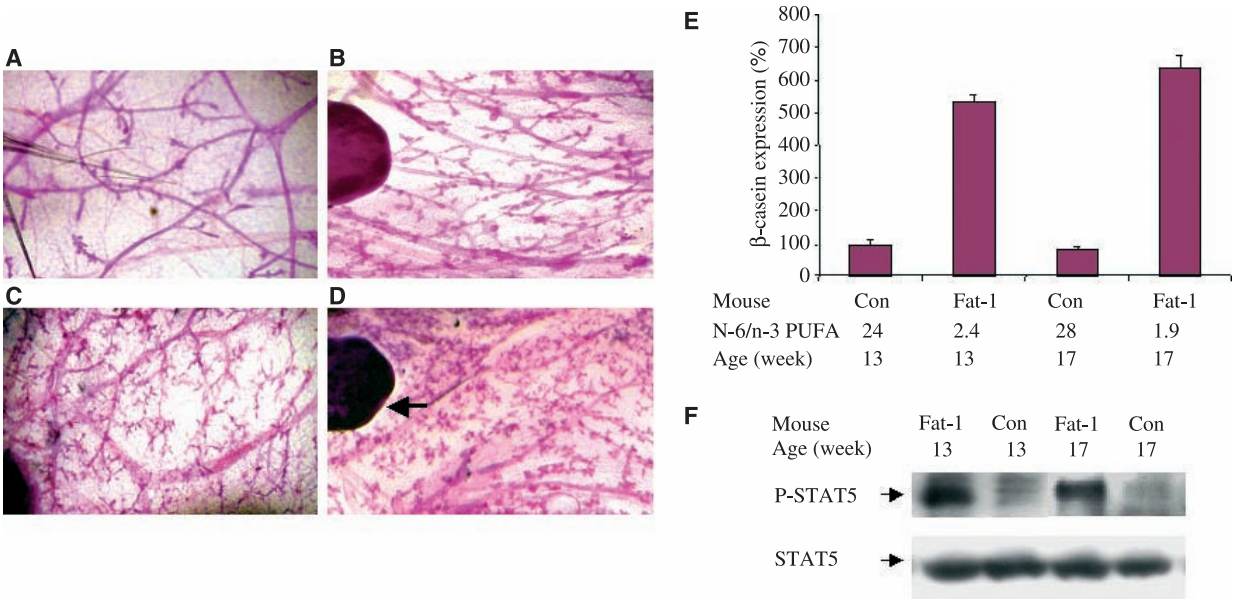

Fig. 1. Histological and molecular analysis of mammary gland differentiation in fat-1 mice. (A–D) Whole mount histological analysis of mammary glands of fat-1 transgenic mice and wild-type littermates. Two transgenic as well as two age-matched nonpregnant control mice were killed at 6, 8, 10, 12 and 14 weeks and subjected to whole mount morphological analysis. The right inguinal gland was removed and subjected to whole mount gland fix, defat, and staining. Representative virgin fat-1 mice, an virgin control mouse, and an early pregnant (8 days pregnant) wild-type littermate mouse were presented. (A) A 14-week-old wild-type virgin mouse. (B) A 14-week-old fat-1 virgin mouse. (C) A 14-week-old fat-1 virgin mouse. (D) A 14-week-old wild-type early pregnant mouse. An arrow indicates the inguinal lymph node. (E) Quantitative RT-PCR analysis of β-casein expression. Inguinal mammary glands were isolated from age-matched virgin control and fat-1 mice. RNA was isolated and subjected to real time PCR analysis. Relative expressions of mouse β-casein gene in the mammary glands from fat-1 mice were calculated compared to that from control mouse. The β-casein gene expression in the 13-week-old control mouse was taken as 100% and regarded as the control. All the other values were expressed as a percentage of the control. The mouse β-actin gene was used as endogenous control. Data represent the mean ± SD of duplicate samples. Statistical comparisons for both fat-1 mice relative to control mice indicate $P < 0.001$ for the relative β-casein expression. (F) Induction of Stat5 phosphorylation in the mammary glands of fat-1 transgenic mice. Thirteen- and 17-week-old virgin control mice and age-matched transgenic mice were killed, and inguinal mammary glands were removed. Total protein was isolated, normalized, and 300 μg of total protein was subjected to immunoprecipitation with Stat5 antibody followed by western analysis. The expression of phosphorylated Stat5 was determined by using a specific antiphosphorylated Stat5 antibody and normalized for total Stat5 expression.

Induction of Stat5 activation and mammary differentiation by DPA and EPA

Although we demonstrated that a decrease in the n-6/n-3 ratio in the mammary gland of the fat 1 mouse resulted in a differentiated phenotype, it is not clear whether DPA and EPA, which were preferentially accumulated in the gland during pregnancy, play a role in the induction of mammary differentiation. Using MCF-10 mammary epithelial cells, we analyzed the effect of DPA, EPA, and DHA on activation of Jak2 and Stat5. Whereas DPA and EPA activated Jak2 and Stat5, DHA did not induce Jak2 and Stat5 phosphorylation (Fig. 2A). We also analyzed the effect of DPA on induction of Stat5 phosphorylation in a mammary organ culture. Whereas limited phosphorylated Stat5 protein was detectable in the nontreated gland, treatment of glands with DPA significantly stimulated Stat5 phosphorylation, resulting in a 5.6-fold and 7.8-fold increase over the control glands, respectively (Fig. 2B).

We then used an *ex vivo* model involving mouse whole-organ culture of the mammary gland to study whether n-3 PUFAs DPA, EPA, and DHA can regulate milk protein β-casein. Inguinal mammary glands from virgin mice were cultured for 6 days with or without 30 μM DPA, or EPA, or DHA. Consistent with the observed differentiated phenotype in the transgenic gland, a differentiation with stimulation of β-casein was observed in the glands treated with DPA. Expression of β-casein mRNA was significantly increased in DPA treated glands with an average 6.4-fold increase over the control nontreated glands (Fig. 2C). A similar significant stimulation of β-casein expression was also observed in EPA-treated glands, resulting in a 5.7-fold increase over controls (Fig. 2D). Treatment of glands

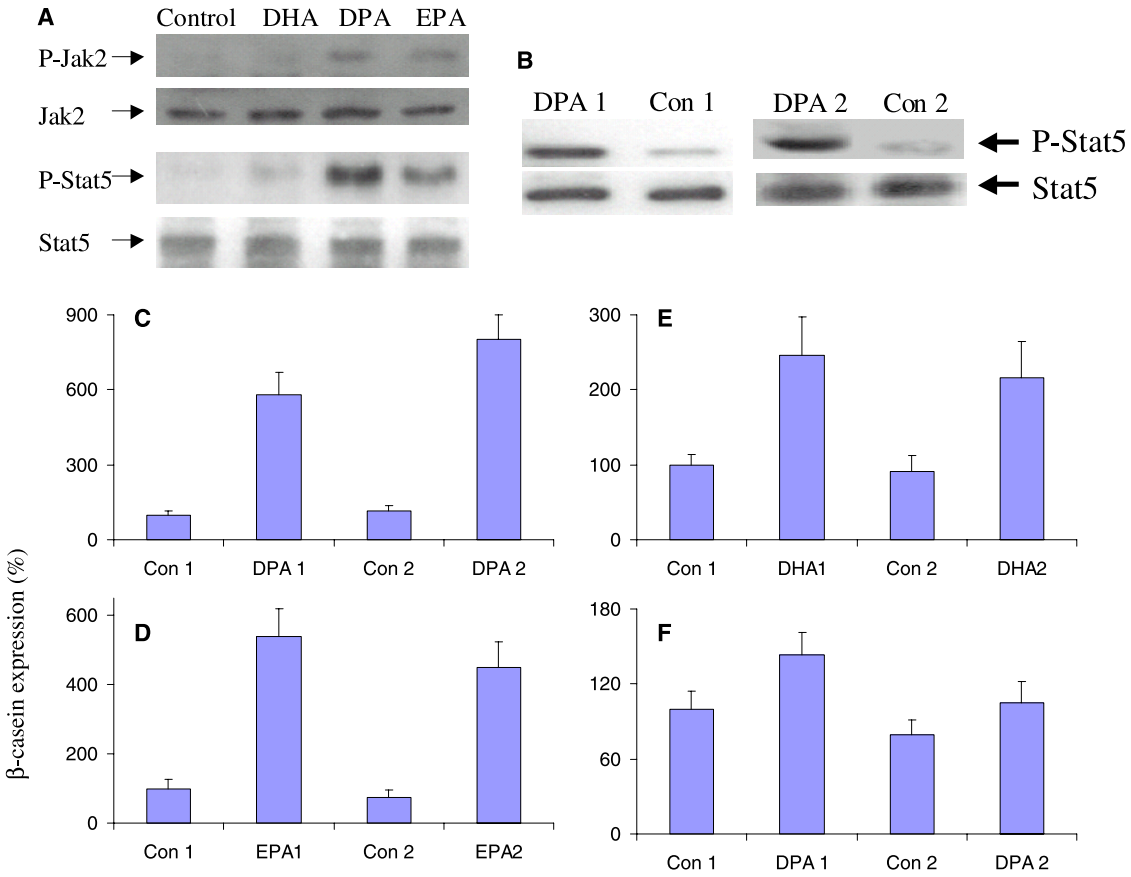

Fig. 2. Induction of Jak 2 and Stat5 activation and β-casein expression by DPA and EPA. (A) MCF-10 cells. Cells were treated with 10 μM of DHA, DPA, and EPA for 36 h. Total cellular protein was isolated, subjected to western analysis with antibodies against phosphorylated Jak2 and Stat5, and normalized with total Jak2 and Stat5 expression. (B) Mammary organ culture. Two pairs of inguinal mammary glands from two 14-week-old virgin mice were cultured in the medium supplemented with bovine pituitary extract, insulin, epidermal growth factor, and hydrocortisone as described in Experimental procedures for 2 days with or without 30 μM DPA. Total protein was isolated, normalized, and 400 μg of total protein was subjected to immunoprecipitation with Stat5 antibody followed by western analysis. The expression of phosphorylated Stat5 was determined by using a specific antiphosphorylated Stat5 antibody and normalized for total Stat5 expression. (C–F) Stimulation of β-casein expression by n-3 PUFAs. Two pairs of inguinal mammary glands from two 14-week-old wild-type virgin nontransgenic control mice (C–E) and Stat5a knockout mice *Stat5a*^{tm1Mam} (F) were cultured for 6 days with or without 30 μM DPA (C,F), EPA (D), or DHA (E) in the organ culture medium. Fresh media containing n-3 PUFAs were added every 2 days. At the end of 6-day treatment, the gland was subjected to RNA extraction for RT-PCR analysis of β-casein expression. The relative expressions of mouse β-casein gene in the mammary glands treated with n-3 PUFAs were calculated in comparison with that from Con 1 mouse, which was taken as 100% and regarded as the control. All the other values were expressed as a percentage of the control. The mouse β-actin gene was used as endogenous control. Data represent the means ± SD of duplicate samples.

with DHA resulted in a slight increase (2.4-fold) in β-casein expression over controls (Fig. 2E).

To functionally validate the role of Stat5 on n-3 PUFA-induced mammary differentiation, we examined the effect of DPA on induction of β-casein expression on an *ex vivo* model using mammary glands from Stat5a-deficient Stat5a^{tm1Mam} mice [29]. In Stat5a^{tm1Mam} mice, mammary ductal development through pregnancy is normal, but lobulo-alveolar development is severely reduced and there is no milk secretion even after prolonged suckling. Whereas DPA induced a significant stimulation of β-casein expression in the glands from wild-type mice (Fig. 2C), there was only a slight increase but not significant in DPA-treated Stat5 knockout glands (Fig. 2F). These data indicate that the preferential accumulation of n-3 PUFAs, such as DPA and EPA, in the differentiated mammary gland during pregnancy may act as a factor inducing functional mammary gland differentiation mediated by activation of Jak2 and Stat5.

Induction of accumulation of DPA and EPA to mammary gland by mammary derived growth inhibitor related gene (MRG), a brain type fatty acid binding protein (B-FABP)

The increased concentration of n-3 DPA and EPA in the pregnant gland indicates a potential specific mechanism for preferential accumulation of DPA and EPA to the gland during pregnancy. Cellular FABP comprise a well-established family of cytoplasmic hydrophobic ligand binding proteins and are involved in binding and intracellular transport of PUFAs. Human B-FABP, initially identified as a mammary gland differentiation factor MRG [30,31], has a preferential binding to n-3 PUFAs [32] and induces mammary differentiation [33,34]. As shown in Fig. 3A, expression of MRG protein was significantly increased in the pregnant glands. Expression of MRG in virgin mammary gland (Fig. 3B) in previously established MRG transgenic mice [34] induced gland differentiation with increased milk protein β-casein (Fig. 3C). When the n-6/n-3 PUFA compositional ratio was analyzed in the glands from MRG versus control mice (Table 2), we found a significant decrease in the n-6/n-3 compositional ratio in the MRG gland, which was similar to that observed in the pregnant gland. Interestingly, MRG expression also resulted in a robust increase in DPA accumulation, from being nondetectable in the control gland to a high abundance in the MRG transgenic gland, whereas the relative DHA concentration was decreased, from 80% in the control gland to 60% in the MRG gland. The relative concentration of EPA was slightly increased, but not statistically significant, in the MRG gland versus the control gland. Our data not only confirm the role of MRG in mimicking the pregnancy effect on mammary differentiation, but also indicate its role as a mediator for specific accumulation of n-3 DPA to mammary glands during pregnancy.

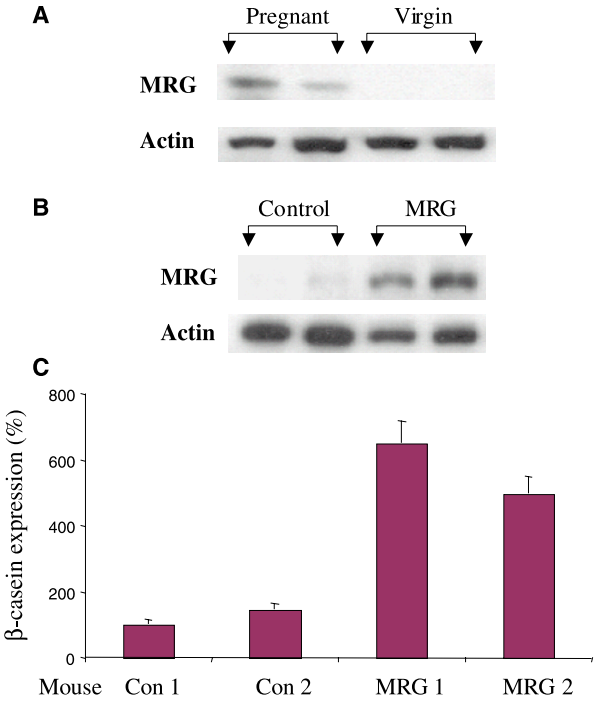

Fig. 3. Expression of MRG on mammary glands and induction of mammary differentiation. (A) Expression of mouse MRG in pregnant mammary glands from nontransgenic control mice. Inguinal mammary glands were isolated from 14-week-old pregnant (15-day-old) and age-matched virgin mice. Expression of mouse MRG protein was analyzed by western blot and normalized for β-actin expression. (B) Western analysis of MRG transgene expression in virgin mammary glands of two 12-week-old MRG transgenic and two age-matched nontransgenic control mice. (C) Expression of β-casein gene in two MRG transgenic mice (MRG 1 and MRG 2) and two nontransgenic littermates (Con 1 and Con 2) was determined by quantitative RT-PCR analysis. β-casein gene expression in control 1 mouse was taken as 100% and regarded as the control. All the other values were expressed as a percentage of the control. The mouse β-actin gene was used as endogenous control. Data represent the means ± SD of duplicate samples.

Table 2. Alternation of n-6/n-3 compositional ratio by MRG. Fatty acid compositional ratio was analyzed in three 15-week-old virgin control and three age-matched virgin MRG transgenic mice. The n-6/n-3 ratio is given by (Linoleic acid 18:2 n-6 + AA 20:4 n-6):(Linolenic acid 18:3 n-3 + EPA 20:5 n-3 + DPA 22:5 n-3 + DHA 22:6 n-3). Relative concentrations of individual n-3 PUFA were expressed as the percentage in comparison of total PUFA contents of combination of EPA, DHA, and DPA. Data represent the means ± SD of three mammary gland samples. Statistical comparisons for both ratio 1 and ratio 2 in MRG glands relative to control glands indicate $P < 0.03$ for the n-6/n-3 fatty acid ratio. Statistical comparison of relative concentration of n-3 PUFAs in MRG glands versus control glands indicates $P < 0.02$ for DHA. The slight increase in relative concentration of EPA in MRG glands versus virgin glands is not statistically different.

	Ratio 1 (%) n-6/n-3	Ratio 2 (%) AA/EPA + DPA + DHA	Relative expression (%)		
			EPA/total	DPA/total	DHA/total
Control	18.0 ± 2.7	2.0 ± 0.2	21.2 ± 4.1	0	79.8 ± 7.2
MRG	12.6 ± 2.5	1.4 ± 0.2	25.4 ± 5.6	14.3 ± 5.1	60.3 ± 5.8

Discussion

The possibility of preventing breast cancer with dietary factors that induce mammary differentiation is of practical interest for women at high risk. We investigated whether pregnancy-mediated breast cancer prevention is associated with an alternation of the n-6/n-3 ratio in favor of n-3 PUFA. A notable finding of this study is that there is a change in n-3 to n-6 PUFA composition, favoring a lower n-6/n-3 ratio in the mammary gland following pregnancy and, more interestingly, there is a significant increase in n-3 PUFA DPA and EPA in the pregnant mammary. Our data suggest that an alternation of the n-6/n-3 ratio in favor of n-3 PUFA, and particularly DPA and EPA, may be one of the underlying mechanisms for pregnancy-mediated mammary differentiation. To support this novel notion, we demonstrated a similar n-6/n-3 ratio change and a differentiated phenotype in the mammary gland from the transgenic mouse expressing the fat-1 gene that converts endogenous n-6 to n-3 PUFAs. In addition, the differentiation effect of DPA and EPA on the mammary gland was also demonstrated in the mouse mammary organ culture. Our studies, comprising two well-established epidemiological observations, as well as animal studies, of the decreased risk of breast cancer in association with pregnancy-induced differentiation and n-3 PUFA, highlight an under-explored area mechanistically linking an alternation of the n-6/n-3 ratio, and particularly DPA and EPA, to pregnancy-induced differentiation and potential breast cancer prevention. It is noteworthy that because the degree of mammary gland differentiation induced by n-3 PUFAs is not likely to be compatible with the differentiation that occurs during full term pregnancy, we are unsure whether the induced gland differentiation is one of the major contributing factors for n-3 PUFA-mediated breast cancer prevention.

Very importantly, although EPA, DAH, and DPA are considered as a group of n-3 PUFA, each n-3 PUFA may have unique functions. EPA is thought to be a better substrate for COX-2 than AA and thus can effectively compete with AA for COX, resulting in reduced production of inflammatory prostaglandin E_2 [35]. In this regard, EPA is an anti-inflammatory agent. Indeed, it has been reported that EPA, but not DHA, decreases mean platelet volume; the first indication of platelet activation, in normal subjects [36]. DHA, which is preferentially accumulated in the brain, particularly in fetal brain, may play a major role during the early postnatal brain development when cellular differentiation and active synaptogenesis take place [37,38]. Compared to DHA and EPA, there are much less functional studies available for DPA. A differential anti-angiogenic effect has been reported for DPA compared to DHA and EPA, in that the effect of DPA was stronger than those of EPA and DHA in suppressing tube-forming activity in endothelial cells induced by vascular endothelial growth factor [39]. In the present study, we report a preferential accumulation of DPA in the differentiated mammary gland during the pregnancy. Furthermore, when comparing the differentiating effects of DPA, DHA, and EPA on mammary organ culture, DPA and EPA had a much stronger effect in the induction of β-casein than that of DHA. Our data suggest a potential specific function of DPA and EPA on mammary gland differentiation during pregnancy. It has been reported that dietary n-3 fatty acid intake at the prepubertal stage induces mammary differentiation by reducing the number of terminal end buds and increasing the presence of lobulo-alveolar structures [21].

Omega-3 PUFAs EPA, DPA, and DHA in mammal tissues derive both from endogenous synthesis from desaturation and elongation of 18:3 n-3 and/or from dietary origin, primarily marine products and fish oils. The pathway leading to the conversion of EPA into DHA involves an elongation step, catalyzed by an elongating enzyme complex, leading to the conversion of EPA into DPA (22:5 n-3); followed by a desaturation step, which results in the conversion of DPA into DHA. Because liver is the principal site of desaturation and elongation [40], a robust increase of DPA in the differentiated mammary gland is likely mediated mainly by preferential uptake of DPA presumably through its FABP, but not by elongation of EPA in the mammary gland. Among the many cellular FABPs, B-FABP is the potential candidate for intracellular DPA binding protein. Previously, we identified and characterized MRG in the human mammary gland [30]. MRG was identified initially as a differentiating factor for mammary gland and was found to be identical to the later identified human B-FABP [31]. Compared with all other tissues, the brain, a terminally differentiated state, has the highest content of n-3 PUFA or the lowest n-6/n-3 ratio [28,41]. Preferential accumulation of n-3 PUFA in the brain is associated with abundant expression of MRG/B-FABP [37,38]. Because MRG induces mammary gland differentiation [34] and its protein expression is associated strongly with human mammary gland differentiation, with the highest expression observed in the differentiated alveolar mammary epithelial cells from the lactating gland [33], it is quite likely that MRG is a mediator for intracellular accumulation of n-3 fatty acid, and particularly DPA, in the differentiated mammary glands during pregnancy. In fact, we demonstrated that forced expression of MRG in virgin gland from mouse mammary tumor virus (MMTV)/MRG

transgenic mice reduced the n-6/n-3 compositional ratio and resulted in a robust increase in the relative concentration of n-3 DPA.

Whereas 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 present study, we demonstrated that an alternation of the endogenous n-6/n-3 ratio induced a significant alveoli-like budding morphology in the end bud region of the virgin gland, a phenotype resembling a differentiated alveoli structure in the pregnant gland. Although the underlying mechanism for n-3 PUFA-induced differentiation is not completely understood, the data clearly indicate the role of n-3 fatty acid on the Jak2/Stat5 signaling pathway. One of the hallmarks for functional mammary differentiation is the expression of milk protein β-casein, which is mediated by phosphorylation of Stat5 [29,42]. The general paradigm for Jak2/Stat5 signaling is that the interaction of prolactin with its receptor induces receptor dimerization, activation of the Jak2 protein-tyrosine kinase and Stat5 tyrosine phosphorylation, followed by dimerization and obligatory nuclear translocation [43]. Because n-3 PUFA failed to induce β-casein expression in the Stat5 knockout glands, we present here a working model for the role of n-3 PUFA on mammary gland differentiation during pregnancy (Fig. 4). In this model, the pregnant mammary gland, with an increased expression of B-FABP MRG, undergoes an n-6/n-3 PUFA compositional ratio change in favor of n-3 PUFA and particularly an n-3 DPA and EPA. An increase in n-3 DPA and EPA, and perhaps other n-3 PUFAs, stimulates Jak2 and Stat5 activation, and induces β-casein expression and gland differentiation. This model indicates that induction of mammary differentiation by alternation of the n-6/n-3 ratio is mediated in part by activation of Jak2/Stat5 signaling pathway. Another potential mechanism underlying the n-3 fatty acid-induced mammary differentiation is the activation of nuclear receptor retinoid X receptor (RXR), which has served as a target for the development of RXR-selective retinoids for chemoprevention [44,45]. Recent studies indicate that dietary fatty acids are ligands for nuclear receptors and therefore could act as agonists and induce receptor transactivation [46]. In an extensive effort to search for endogenous ligands for RXR, a factor in brain tissue from adult mice was identified that activates RXR. Interestingly, one such RXR ligand was identified as n-3 fatty acid DHA [47]. Thus, an intriguing possibility is that n-3 PUFAs such as DPA function as endogenous ligands for RXR in the mammary gland during differentiation.

Consistent with rat mammary tumors developing from an undifferentiated gland, human breast cancer initiates in the terminal ductal lobular, the most undifferentiated structures frequently found in the breast of young nulliparous women [5]. The realization that specific reproductive-related differentiating events alter the risk of breast cancer in a predictable fashion raises the possibility that events known to decrease the risk of breast cancer might be mimicked pharmacologically or by dietary factors. We provide here a new concept: n-3 PUFA, and particularly DPA, as being one of the mediators in the differentiation effect of pregnancy on breast epithelial cells; thus, the application of n-3 DPA to the mammary gland may lower the risk of breast cancer by making the mammary epithelial cells behave like the glands during pregnancy.

Experimental procedures

Fatty acid analysis

Lipid extraction, methylation, and fatty acid analysis were performed as previously described [28,48]. Briefly, an aliquot of mammary tissue homogenate in a glass methylation tube was mixed with 1 mL of hexane and 1 mL of 14% BF_3/MeOH reagent. After being blanketed with nitrogen,

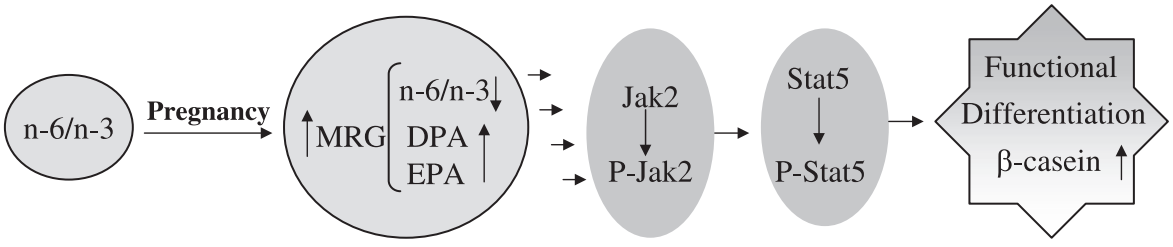

Fig. 4. A model for mammary gland differentiation during pregnancy. According the model, pregnancy triggers a decrease in the n-6/n-3 compositional ratio with more n-3 PUFA and particularly DPA and EPA accumulated in the mammary gland, which is mediated by B-FABP MRG. Increased n-3 PUFAs activates Stat5 by induction of Stat5 tyrosine phosphorylation, stimulates milk protein β-casein expression, and induces mammary gland differentiation.

the mixture was heated at 100 °C for 1 h, cooled to room temperature and methyl esters were extracted in the hexane phase following addition of 1 mL of H_2O. The samples were centrifuged at 3000 ***g*** for 1 min, and then the upper hexane layer was removed and concentrated under nitrogen. Fatty acid methyl esters were analyzed by gas chromatography using a fully automated HP5890 system equipped with a flame-ionization detector (Hewlett-Packard, Palo Alto, CA, USA). The chromatography utilized an Omegawax 250 capillary column (30 m × 0.25 mm inner diameter). The oven program is initially maintained at 180 °C for 5 min, then increased to 200 °C at 2 °C·min^{-1} and held for 48 min. Peaks were identified by comparison with fatty acid standards (Nu-chek-Prep, Elysian, MN, USA), and the area percentage for all resolved peaks was analyzed using a Perkin-Elmer M1 integrator (Perkin Elmer; Foster City, CA, USA). Fatty acid mass was determined by comparing areas of various analyzed fatty acids to that of a fixed concentration of external standard when added.

Fat-1 transgenic mice

We recently developed a fat-1 transgenic mouse model capable of converting n-6 fatty acids to n-3 fatty acids [28]. When fed with a diet high in n-6 and low in n-3 fatty acids (10% safflower oil from ResearchDiets Inc., New Brunswick, NJ, USA), the transgenic animals are characterized by an abundance of n-3 fatty acid and a balanced n-6/n-3 fatty acid ratio of 1 : 1 in their tissues and organs, whereas wild-type mice have a ratio of > 30. This model allows one to produce two different fatty acid profiles (high versus low n-6/n-3 ratios) in the animals by using just a single diet, which avoids the potential problems associated with dietary supplement of fish oil including various amount of different n-3 PUFAs and contaminants.

MRG transgenic mice

The MRG transgenic model under the control of MMTV regulatory promoter was previously established in the FVB/N mouse [33].

Stat5 knockout mice

Mice homozygous for the *Stat5a*^{tm1Mam} targeted mutation were purchased from Jackson Laboratory.

Mammary gland organ culture

A pair of inguinal whole mammary gland was removed from 14-week-old virgin female mice (FVB/n background) as previously described [32]. The glands were cultured in medium 199 containing 5% fetal bovine serum, with medium changed every 2 days. The medium was supplemented with following components from Clonetics (Cambrex, San Diego, CA, USA): bovine pituitary extract (52 μg·mL^{-1}), insulin (5 μg·mL^{-1}), epidermal growth factor (10 ng·mL^{-1}), and hydrocortisone (1 μg·mL^{-1}). The glands were cultured in the organ culture for 4 days before addition of fatty acid. DPA was dissolved in ethanol. The final concentration of ethanol in the organ culture medium was 0.1%. At termination, the glands were subjected to RNA and protein extraction for real time PCR and western analysis.

Whole mount histological analysis of mammary gland

Whole inguinal mammary glands were removed from virgin control as well as virgin transgenic mice. The removed gland was subjected to whole mount fix, defat, and staining as previously described [33]. 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.

Quantitative RT-PCR analyses

RNA was isolated and subjected to real time PCR analysis using the TaqMan PCR core reagent kit (Applied Biosystems, Foster City, CA, USA) and ABI Prism 7700 Sequence Detection System (Applied Biosystems). Data were analyzed using Sequence Detection System (SDS) software, version 1.6.3. Results were obtained as *Ct* (threshold cycle) values. *Ct* is inversely proportional to the starting template copy number. Relative expressions of mouse β-casein gene in the mammary glands from fat-1 mice or the gland treated with DPA were calculated compared to that from control mouse or a nontreated gland using the Δ*Ct* method (User Bulletin #2, Applied Biosystems). Sequences for mouse β-casein primers and probe are: forward primer: 5′-TTCTTAACCC CACCGTCCAA-3′; reverse primer: 5′-GAAAATAACCT GGAAATCCTCTTAGACA-3′; probe: 5′-TCCCTGCCA CTCCACAACATTCCG-3′.

Statistical analysis

Statistical analyses were performed by using the chi-square test implemented in SPSS, version 11.0 (SPSS Inc., Chicago, IL, USA). All statistical analyses were two-sided, and $P < 0.05$ was considered statistically significant for all comparisons.

Animals

All experiments involving animals were approved by the institutional IACUC committee.

Acknowledgements

Supported in part by a grant CA93542-2 from NIH, a grant DAMD17-01-1-0353 (Postdoctoral Fellowship Award) from the United State Army Breast cancer Research Program, and a research grant from the American Cancer Society RSG-03-140-01-CNE (JXK).

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