Manic Fringe Promotes a Claudin-Low Breast Cancer Phenotype through Notch-Mediated PIK3CG Induction

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Abstract

Claudin-low breast cancer (CLBC) is a poor prognosis disease biologically characterized by stemness and mesenchymal features. These tumors disproportionately affect younger patients and women with African ancestry, causing significant morbidity and mortality, and no effective targeted therapy exists at present. CLBC is thought to originate from mammary stem cells, but little is known on how or why these tumors express a stable epithelial-to-mesenchymal transition phenotype, or what are the driving forces of this disease. Here, we report that Manic Fringe (Mfng), which encodes an O-fucosylpeptide 3-β-N-acetylgalcosaminyltransferase known to modify EGF repeats in the Notch extracellular domain, is highly expressed in CLBC and functions as an oncogene in this context. We show that Mfng modulates Notch activation in human and mouse CLBC cell lines, as well as in mouse mammary gland. Mfng silencing in CLBC cell lines reduced cell migration, tumorsphere formation, and in vivo tumorigenicity associated with a decrease in the stem-like cell population. Mfng deletion in the Lfngfl/fl;MMTV-Cre mouse model, in which one-third of mammary tumors resemble human CLBC, caused a tumor subtype shift away from CLBC. We identified the phosphoinositide kinase Pik3cg as a direct transcriptional target of Mfng-facilitated RBPIx-dependent Notch signaling. Indeed, pharmacologic inhibition of PI3K in CLBC cell lines blocked migration and tumorsphere formation. Taken together, our results define Mfng as an oncogene acting through Notch-mediated induction of Pik3cg. Furthermore, they suggest that targeting PI3K may prove beneficial for the treatment of CLBC subtype.

Introduction

Gene expression profiling has been used to subclassify human breast cancer into at least six molecular subtypes, including basal-like, claudin-low, HER2-enriched, luminal A, luminal B, and normal-like. Claudin-low breast cancer (CLBC) shares features with mammary stem cells and CLBC cells have undergone epithelial-to-mesenchymal transition (EMT; refs. 1, 2). It is widely believed that cancer stem-like cells play a key role in tumor recurrence and metastasis. Also, accumulating evidence suggest that EMT promotes dissemination, and may itself promote "stemness" (3–5).

Notch signaling controls mammary stem cell self-renewal and differentiation, and may regulate EMT in breast cancer cells (6–9). Indeed, dysregulated Notch activation has been implicated in breast cancer, especially in subtypes showing features of mammary stem cells and EMT (10). As a result, Notch has emerged as a potential drug target for poor prognosis breast cancer. However, the role of Notch in different subtypes is not clear, nor is the specific Notch receptor(s), ligand(s), and the modulator(s) involved.

Lunatic Fringe (Lfng), Manic Fringe (Mfng), and Radical Fringe (Rfng) encode a family of β3N-acetylgalcosaminyl-transferases that are known to modify EGF repeats in the extracellular domains of Notch receptors, thereby modulating ligand-mediated Notch activation (11). We recently reported that Lfng controls self-renewal and differentiation of mammary stem/progenitor cells by restricting Notch activation, and Lfng deficiency cooperates with the Met/Caveolin gene amplification to induce basal-like breast cancer (BLBC) and, less frequently, CLBC (12). Analysis of human breast cancer data showed significantly reduced levels of LFNG expression in BLBC and in a subset of CLBC as compared with other subtypes. In contrast, expression of Mfng was significantly higher in CLBC. In this study, we performed loss-of-function analysis for Mfng in CLBC cell lines as well as mouse models to determine roles for Mfng in CLBC. We also identified a critical downstream effector of Mfng-modulated Notch signaling in this context.
Materials and Methods

Cells
MDA-MB-231 was obtained from ATCC. Mouse cell line C0321 was established and maintained as previously described (13). Cells were resusculated from early passage liquid nitrogen stocks and cultured less than 3 months before reinitiating cultures. Cells were tested negative for mycoplasma contamination.

Cell proliferation, migration, and tumorsphere assays, drug treatment, and xenograft experiment
Cell proliferation was assessed using CellTiter96 AQueous One Solution Kit (Promega). Collective cell migration was measured in a wound-healing assay. Tumorspheres were cultured as previously described (13), and quantified using a cytometer (Celigo). For drug treatment, cells were incubated with AS-605240 (Selleck, ab23943) or normal IgG. DNA from immunoprecipitates was recovered by reversing the cross-linking and digestion with proteinase K, and then PCR amplified (see Supplementary Methods for primer sequences).

Histology, IHC, and X-Gal staining
Formalin-fixed paraffin-embedded tissues were processed for histologic and IHC analysis by standard procedures (see Supplementary Methods for primary antibodies used for IHC). Representative images were acquired with an Nikon Eclipse 80i microscope. X-Gal staining in the mammary gland was performed as previously described (12).

Western blot analysis and flow cytometry

Mice
Mouse experiments were performed in accordance with a protocol approved by University of Mississippi Medical Center (UMMC; Jackson, MS) Institutional Animal Care and Use Committee. Wild-type, Mfng<sup>−/−</sup>, Lfng<sup>−/−</sup>;MMTV-Cre, and Mfng<sup>−/−</sup>, Lfng<sup>−/−</sup>;MMTV-Cre cohorts were maintained on the FVB background.

Statistical analysis
Unpaired two-tailed t tests were performed for two group comparisons, and P value of 0.05 or less was considered statistically significant.

Accession codes
The microarray data have been deposited in the NCBI GEO under accession number GSE62481.

Results

Microarray gene expression analysis in xenografts
Total RNA was extracted from xenografts using RNeasy Mini Kit (Qiagen), and processed using the Ambion WT Expression Kit (Life Technologies) according to the manufacturer's instruction. The resultant biotinylated cRNA was fragmented and then hybridized to the GeneChip Human Gene 1.0 ST Array (Affymetrix). The arrays were processed and scanned using the Affymetrix Model 450 Fluidics Station and Affymetrix Model 3000 7G scanner (Affymetrix). *.cel files generated by Affymetrix Expression Console Software were used for further analysis.

Gene expression analysis of human dataset
Human breast cancer gene expression dataset GSE18229 was downloaded from Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18229). Expressions of Mfng, NOTCH4, and PIK3CG and clinical information of patient samples were extracted by an in-house Java parser. Averaged values were used if more than one probe was mapped to same genes. All plots and analyses were performed using R (http://www.r-project.org). P value was calculated by comparing expression means across all subtypes.

Chromatin immunoprecipitation and luciferase reporter assays
Chromatin immunoprecipitation (ChIP) assay was performed using EZ-ChIP kit (Millipore). Briefly, MDA-MB-231 cells were treated with 1% formaldehyde, neutralized, and resuspended in SDS lysis buffer for chromatin fragmentation with sonication (ultrasonic processor GE130). Sheared chromatin was diluted and then immunoprecipitated with anti-RBPJk (Abcam, ab23943) or normal IgG. DNA from immunoprecipitats was recovered by reversing the cross-linking and digestion with proteinase K, and then PCR amplified (see Supplementary Methods for primer sequences).

The PIK3CG promoter reporter was prepared by cloning the upstream region of PIK3CG gene (−1131 to −1) into the pGL3 vector (Promega). The mutagenesis of RBPJk-binding sites was introduced by replacing the sequence with an Nhel restriction site. All the clones are confirmed by sequencing. MDA-MB-231 cells were cotransfected with PIK3CG promoter reporter and Renilla luciferase plasmids, and harvested 48 hours posttransfection. Luciferase activities were measured with Dual-Luciferase Reporter Assay System (Promega) using GloMax 96 Microplate Luminometer (Promega). Each reporter activity was normalized with corresponding Renilla activity.

shRNA and qRT-PCR
The Mfng-shRNA construct targeting 5'-GTGCTGAGCTCT-GGATCATCGGAAAACGT-3’ sequences of Mfng gene was purchased from Origene. MDA-MB-231 and C0321 cells were transfected with the shRNA or scrambled control plasmid using FuGENE 6 Transfection Reagent (Promega). Stable cell lines expressing shRNA were generated by selection with Puromycin. For qRT-PCR, total RNA was extracted using RNeasy Mini Kit and reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad). PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) and quantitated with Bio-Rad CFX36 qPCR System (see Supplementary Methods for primer sequences).

Expression of Mfng, NOTCH4, and PIK3CG and clinical information of patient samples were extracted by an in-house Java parser. Averaged values were used if more than one probe was mapped to same genes. All plots and analyses were performed using R (http://www.r-project.org). P value was calculated by comparing expression means across all subtypes.

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Statistical analysis
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Results

Modulation of Notch activation by Mfng in CLBC cell lines and in the mouse mammary gland
In a survey for Notch pathway gene expression in a human breast cancer dataset, GSE18229 (1), we found that Mfng expression is significantly higher in CLBC as compared with other subtypes (P < 2e-16 from ANOVA; Fig. 1A). Interestingly, there is a
positive correlation between MFNG and NOTCH4 expressions across all subtypes (Fig. 1B). Claudin-low tumors from Lfngfl/fl; MMTV-Cre mice also showed elevated Mfng expression (12). Moreover, using SAM analysis on 385 mouse mammary tumor samples from 27 models, Mfng and Notch4 are found to be expressed at significantly higher levels in claudin-low subtype tumors than in tumors from all other subtypes (14). Thus, Mfng is upregulated in CLBC in both humans and mice.

To determine the function of Mfng in CLBC, we performed MFNG-knockdown in MDA-MB-231, a human breast cancer cell line with a claudin-low gene expression signature (1, 2). To this end, we selected a polyclonal cell population transfected with a MFNG shRNA expression vector. This population showed a 40% decrease in MFNG mRNA expression as well as a decrease in Notch1 and Notch4 protein accumulation as compared with control cells (Fig. 1C and D). Importantly, the size of Notch1 and Notch4 fragments detected is consistent with Notch intracellular domains (N-ICD), suggesting reduced Notch1 and Notch4 activation in these cells. Indeed, MFNG-knockdown cells expressed lower levels of a canonical Notch target gene, HEY1 (Fig. 1C). We also knocked down Mfng in a murine claudin-low cell line, C0321 (13). In this case, shRNA-expressing cells showed a 60% reduction in Mfng mRNA and decreased N4-ICD accumulation, but increased level of N1-ICD (Fig. 1C and D). shMfng C0321 cells showed decreased mRNA levels of Hes1 and Hes5, but increased Hey1 expression (Fig. 1C).

We next examined Mfng expression in the mouse mammary gland using a Mfngfl/fl reporter strain (Supplementary Fig. S1). Weak Mfng expression is noted at the branching sites of epithelial ducts in the pubescent gland. Interestingly, Mfng expression is upregulated dramatically during post-weaning involution. Next, we analyzed Notch protein levels in mammary tissue from different genotypes and stages. Mfng expression is highly expressed in human CLBC and modulates Notch activation in CLBC cell lines and in the mouse mammary gland. A, mean expression values of MFNG from the human breast cancer dataset GSE18229. Basal (basal-like), Claudin (claudin-low), Her2 (Her2-enriched), LumA (luminal A), LumB (luminal B), and Normal (normal breast-like) are six molecular subtypes. B, scatterplot for MFNG and NOTCH4 expressions in human breast cancers from GSE18229. C, qRT-PCR for Mfng and Notch target genes Hes1, Hes5, and Hey1 in MDA-MB-231 and C0321 cell lines stably expressing Mfng-shRNA or scrambled control. *P < 0.05. D, Western blot analysis for Notch receptors in the control and shMfng cell lines, and in mouse mammary tissues of indicated genotypes and stages.
null mice (Mfng<sup>-/-</sup>;Cre<sup>lox/lox</sup> referred to as Mfng<sup>−/−</sup>). A dramatic decrease in N4-ICD and increase in N1- and N3-ICD were noted in the Mfng<sup>−/−</sup> mammary gland during involution, but not during puberty. Interestingly, deletion of Mfng in the Lfng<sup>lox/lox</sup>;MMTV-Cre mice caused a decrease in N4-ICD accumulation during puberty (Fig. 1D). Thus, modulation of Notch1 activation by Mfng is context dependent: enhanced in MDA-MB-231, but inhibited in C0321 and in mouse mammary gland during involution. Mfng appears to enhance Notch4 activation in both human and mouse CLBC cell lines, as well as in the mouse mammary gland.

**Mfng regulates self-renewal of cancer stem-like cells and cell migration in CLBC cell lines**

Knockdown of Mfng had no effect on the growth of MDA-MB-231 or C0321 cells cultured on plastic (Supplementary Fig. S2); however, it dramatically decreased tumorsphere-forming capacity of both lines (Fig. 2A). Flow-cytometric analysis revealed a diminished population of CD44<sup>−</sup>CD24<sup>low</sup> cells (from 9.18% to 3.33%) in the Mfng-knockdown MDA-MB-231 cells (Fig. 2C). This population was shown to have enriched breast cancer stem-like cells and to express N1-ICD as well as Notch target genes (15). Knockdown of Mfng in C0321 caused a dramatic decrease in CD44<sup>−</sup>CD24<sup>−</sup> and CD44<sup>−</sup>CD24<sup>low</sup> populations, again, both of which are enriched for cancer stem-like cells (Fig. 2C; ref. 16). Mfng-knockdown cells showed a lower migration rate in the "wound-healing" assay (Fig. 2D). Finally, xenograft of the Mfng-knockdown MDA-MB-231 cells resulted in diminished tumor growth compared with the control line (n = 9, P = 0.0002; Fig. 2B). Taken together, these results indicate that Mfng promotes accumulation of stem-like tumor-initiating cells and cell migration, as well as enhancement of tumor growth in vivo.

**Mfng influences breast cancer subtypes in the Lfng<sup>lox/lox</sup>;MMTV-Cre mouse model**

Mfng may regulate mammary epithelial differentiation through modulation of Notch. Indeed, the mature Mfng<sup>−/−</sup> mammary gland showed increased levels of the luminal cell marker cytokeratin 8 (CK8), as well as basal cell marker CK14, associated with a modest decrease in ERα level and a dramatic decrease in PRβ (Fig. 3A). Despite this, Mfng<sup>−/−</sup> mice showed normal lactation and post-weaning involution, and no mammary tumor formation. To test for a role of Mfng in CLBC pathogenesis, we crossed Mfng null mice to the Lfng<sup>lox/lox</sup>;MMTV-cre model, which develop basal-like (~2/3) and claudin-low/spindle cell (~1/3) mammary tumors (12). Deletion of Mfng in this model caused increased CK8 expression as well as decreased levels of a stem cell marker, Aldh1 (Fig. 3A). Unexpectedly, some of the Mfng<sup>−/−</sup>;Lfng<sup>lox/lox</sup>;MMTV-cre mice succumbed to lymphoma before the onset of mammary tumor formation, likely due to the loss of both Lfng and Mfng in B- and T cells. Nevertheless, we were able to harvest mammary tumors from some of these mice. Interestingly, two of three were adenosquamous carcinomas and one showed histology resembling the basal-like tumor from the Lfng<sup>lox/lox</sup>;MMTV-cre model. The claudin-low subtype, which...
often displays spindloid histology (12), was not found (Fig. 3B). Unlike basal-like and claudin-low tumors, the adenosquamous carcinoma showed nuclear staining of ERα and PR in some cells (Fig. 3C). Adenosquamous carcinoma was never found in more than 50 tumors collected from Lfngfl/fl;MMTV-cre mice during our previous and present studies. Thus, deletion of Mfng caused squamous differentiation at the expense of claudin-low subtype. Compared with basal-like tumors, claudin-low tumors and cell lines from the Lfngfl/fl;MMTV-cre mice express more Notch4 and less Notch1 (12, 13). Consistent with a subtype shift away from claudin-low, we observed a decrease in Notch4 and increase in Notch1 protein accumulation in Mfng-knockdown C0321 cells (Fig. 1D).

Pik3cg is a direct target of Mfng-enhanced Notch signaling in CLBC

To identify downstream mediators of Mfng, we performed microarray gene expression analysis on six xenograft tumors, three each from control and Mfng shRNA-expressing MDA-MB231 cells. One of the most downregulated genes in Mfng knockdown tumors is Pik3cg, which appears to be a hub in the reactome functional interaction network of differentially expressed genes (Fig. 4A; Supplementary Table S1 and Supplementary Fig. S3). Encoding the γ catalytic subunit of phosphatidylinositol-4,5-bisphosphate 3-kinase, Pik3cg has recently been shown to promote breast cancer growth and metastasis (17, 18). We verified that knockdown of Mfng caused a dramatic decrease in Pik3cg level, as well as reduced Akt phosphorylation in CLBC cells (Fig. 4B). The PIK3CG gene promoter harbors multiple consensus Rbpj-binding sites, including two in a region less than 1 Kb upstream of the start codon. To test for Rbpj binding to these sequences, we performed ChIP. Sequences surrounding both sites were amplified from the Rbpj–chromatin complex. Luciferase reporter assay using constructs from the PIK3CG promoter, including one or both Rbpj-binding sites revealed that both sites are required for the Notch-ICD–dependent transcriptional activation (Fig. 4B).
Thus, Pik3cg is a direct target of Rbpjk-dependent, Notch signaling in CLBC. Moreover, PIK3CG expression positively correlates with MFNG expression in human breast tumors ($P < 2.2e^{-16}$; Fig. 4C and Supplementary Table S2). Also, PIK3CG, but not PIK3CA, is highly expressed in CLBC subtype (Fig. 4C, and data not shown). Interestingly, Notch pathway activation conferred resistance to PI3K inhibitors in a chemical genetic screen on human cancer cell lines (19), consistent with our finding that Notch activation upregulates Pik3cg expression.

Next, we tested for the importance of Pik3cg in CLBC cells using a selective PI3K inhibitor, AS-605240. "Wound-healing" assays showed that inhibition of PI3K activity almost completely blocked the migration of C0321 cells, and significantly attenuated that of MDA-MB-231 (Fig. 4D). The same concentration of AS-605240 had no effect on growth of MDA-MB-231, and did not impede C0321 proliferation until 2 days after treatment (Supplementary Fig. S4), indicating that failed "wound healing" is not due to inhibition of cell growth. Interestingly, treatment of MDA-MB-231 cell line with AS-605240 for 3 days caused a decrease of 17.2 ± 1.8% in the CD44+CD24low cells. To avoid complication of the inhibitory effect of AS-605240 on cell proliferation, we performed flow cytometry in C0321 cells after treatment for only 2 days. Again, AS-605240 treated C0321 line showed modest but consistent reduction (13.6 ± 1.9%, $P < 0.05$) in the CD44+CD24low cancer stem cell-enriched population (Fig. 4D). Indeed, treatment with AS-605240 resulted in a dramatically decreased capacity of...
tumorsphere formation in both cell lines (Fig. 4D). Taken together, PI3Kγ activity is not only required for cell migration, but also contributes to maintenance of claudin-low cancer stem-like cells.

**Discussion**

Multiple Notch receptors are expressed in the developing mammary gland and in breast cancer, and individual Notch receptors may exert distinct roles in different mammary cell types. For instance, Notch4 has been shown to regulate self-renewal of stem or bipotential progenitor cells (8, 20), whereas Notch3 is reported to function in luminal progenitor cells (8, 21). In this study, we found that MFNG expression in breast cancer is highly correlated with expression of NOTCH4, but not with expression of other Notch receptors. In addition, knockdown of MFNG in human and mouse CLBC cell lines consistently decreased Notch4 activation, whereas deletion of MFNG in the mouse mammary gland resulted in decreased Notch4 activation during involution. Thus, it appears that MFNG primarily controls Notch4-mediated signaling in mammary stem cells, which is thought to be the cell-of-origin for CLBC.

MFNG expression in the mammary gland is dramatically upregulated during post-weaning involution. Interestingly, deletion of MFNG caused increased activation of Notch1 and Notch3, and decreased Notch4 expression/activation during involution. In this context, MFNG may regulate parity-induced stem cell activity through differential regulation of Notch receptor activation. The involuting mammary gland microenvironment can promote tumor progression in some cases (22). To some extent, the microenvironment of claudin-low tumors is reminiscent of that of postpartum mammary glands, where MFNG may enhance self-renewal of stem-like cells and promote EMT.

Loss of MFNG on the Lfngfl/fl;MMTV-cre background induced formation of adenocarcinoma in the expense of claudin-low mammary tumors. In this case, deletion of both Lfng and MFNG in the mammary gland caused dramatically decreased activation of multiple Notch receptors (Fig. 1D), suggesting that overall reduction in Notch signaling may lead to squamous differentiation of the mammary epithelium. Interestingly, inactivating mutations in Notch pathway genes have been identified in squamous cell carcinoma of other tissues, including head and neck, lung, and bladder (23).

Pik3cg is aberrantly expressed in many invasive human breast tumors and its expression level correlates with metastatic potential of breast cancer cell lines (18). Here, we demonstrate that Pik3cg is a downstream effector of MFNG-facilitated Notch activation. Previous studies indicate that Pik3cg may promote migration and invasion of breast cancer cells, while inhibiting anoikis (17, 18). Our results suggest that Pik3cg contributes to breast cancer aggressiveness not only by promoting cell migration, but also by maintaining cancer stemness.

In conclusion, this study revealed a novel oncogenic role for MFNG in CLBC. Our discovery of a specific role of MFNG-controlled Notch signaling in CLBC provides new insights into the regulation of cancer stem cells in specific breast cancer subtypes. Finally, identification of Pik3cg as a Notch target prompts a PI3Kγ-targeting strategy for treatment of CLBC and perhaps other poor prognosis breast cancers, and opens a new avenue to search for prognostic biomarkers for breast cancers based on expression of MFNG, NOTCH1, and PIK3CG.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: S. Zhang, L. Miele, K. Xu

Development of methodology: S. Zhang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Zhang, W.-C. Chung, S.E. Egan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Zhang, W.-C. Chung, G. Wu, L. Miele, K. Xu

Writing, review, and/or revision of the manuscript: S. Zhang, G. Wu, S.E. Egan, L. Miele, K. Xu

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Zhang

Study supervision: K. Xu

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