Cancer Research

Pivotal Role of Reduced *let-7g* Expression in Breast Cancer Invasion and Metastasis

Pengxu Qian[1,3], Zehua Zuo[1,4], Zhengsheng Wu[1,2], Xianyi Meng[3], Gaopeng Li[1], Zhengzhou Wu[3], Weijie Zhang[1], Sheng Tan[1], Vijay Pandey[6], Yandan Yao[7], Puyue Wang[3], Liqing Zhao[3], Jun Wang[1], Qiang Wu[2], Erwei Song[7], Peter E. Lobie[5,6], Zhinan Yin[3,5], and Tao Zhu[1,5]

Abstract

Screening of the entire *let-7* family of microRNAs (miRNA) by *in situ* hybridization identified *let-7g* as the only member, the diminished expression of which was significantly associated with lymph node metastasis and poor survival in breast cancer patients. Abrogation of *let-7g* expression in otherwise nonmetastatic mammary carcinoma cells elicited rapid metastasis from the orthotopic location, through preferential targets, Grb2-associated binding protein 2 (GAB2) and fibronectin 1 (FN1), and consequent activation of p44/42 mitogen-activated protein kinase (MAPK) and specific matrix metalloproteinases. Treatment with estrogen or epidermal growth factor specifically reduced the expression of mature *let-7g* through activation of p44/42 MAPK and subsequently stimulated expression of GAB2 and FN1, which, in turn, promoted tumor invasion. We thus identify *let-7g* as a unique member of the *let-7* miRNA family that can serve as a prognostic biomarker in breast cancer and also propose a paradigm used by specific signaling molecules via *let-7g* to cooperatively promote breast cancer invasion and metastasis. Thus, *let-7* family members neither possess equivalent clinicopathologic correlation nor function in breast cancer. *Cancer Res; 71(20); 6463–74. ©2011 AACR.*

Introduction

microRNAs (miRNA) are small noncoding RNAs ranging in size by 20 to 25 nucleotides. miRNAs posttranscriptionally repress gene expression mostly by recognizing complementary target sites in the 3′-untranslated region (3′-UTR) of target mRNAs (1–3). miRNAs are involved in the regulation of a continuous biological processes leading to the acquisition of metastatic potential, such as aberrant adhesion, migration, and invasion, and neoangiogenesis (2–4). A limited number of miRNAs, either upregulated (such as miR-10b, miR-21, miR-373, and miR-520c) or downregulated (such as miR-98, *let-7a*, miR-31, and miR-146), have thus far been identified to play a role in cancer metastasis (5).

The human *let-7* miRNA family consists of 13 members located in 8 genomic locations frequently deleted in human cancers (6). Nine distinct mature *let-7* miRNAs with identical seed sequences are produced from 12 precursor sequences from miRBase (7). Aberrant expression of *let-7* miRNA has been associated with poor prognosis of several types of cancer (8–11). For example, expression of *let-7* miRNAs is reduced in non–small-cell lung cancer patients and associated with poor prognosis (12), and increased expression of *let-7a* substantially reduces tumor burden in a *K-Ras* murine lung cancer model (13). While *let-7* is widely viewed as a tumor suppressor miRNA, upregulation of certain *let-7* family members has also been observed (6), although less frequently. Although *let-7* family members were generally believed to have overlapping targets and therefore redundant roles, whether individual members of the *let-7* family possess specific roles in cancer development and metastasis is largely undefined (6, 14).

In this report, amongst all mature *let-7* miRNAs in the *let-7* family, *let-7g* was identified as the only one, reduced expression of which correlates with worse survival outcome in breast cancer patients. Grb2-associated binding protein 2 (GAB2) and fibronectin 1 (FN1) were identified as the novel targets of *let-7g* that increased activity of p44/42 mitogen-activated protein kinase (MAPK) and matrix metalloproteinase (MMP)-2/MMP-9. Estrogen and epidermal growth factor (EGF) specifically reduced *let-7g* expression and increased expression of GAB2 and FN1. We therefore propose a model whereby estrogen and EGF promote breast cancer metastasis through several feedback loops via the specific interplays between *let-7g* and its downstream cascades.

Authors' Affiliations: [1]Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China; [2]Department of Pathology, Anhui Medical University, Hefei, Anhui; [3]State Key Laboratory of Medicinal Chemical Biology, College of Life Sciences, Nankai University, Tianjin; [4]Division of Basic Medicine, Wuhan University College of Medicine; [5]Overseas Opening Laboratory of Wuhan University College of Medicine, Wuhan, Hubei, PR China; [6]Cancer Sciences Institute of Singapore, Center for Life Sciences, Singapore; and [7]Department of Breast Surgery, No. 2 Affiliated Hospital, Sun-Yat-Sen University, Guangzhou, PR China

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P. Qian and Z. Zuo contributed equally to this work.

Corresponding Authors: Tao Zhu, Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, PR China. Phone: 86-551-3602461; Fax: 86-551-3601505; E-mail: zhut@ustc.edu.cn or Zhinan Yin, College of Life Sciences, Nankai University, Tianjin 300071, PR China. Phone: 86-22-2249-4623; Fax: 86-22-2350-3270; E-mail: yinzn@nankai.edu.cn

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Materials and Methods

Cell lines and cell culture

All human breast cancer cell lines used in this study were obtained from the American Type Culture Collection and cultured in conditions as recommended. All cells were maintained in a humidified incubator at 37°C and 5% CO_2.

Detection of miRNA expression by *in situ* hybridization in formalin-fixed paraffin-embedded tissues

For detecting expression of *let-7g* family miRNAs in invasive mammary carcinoma and normal mammary tissues, *in situ* hybridization was carried out as previously described (15) with modifications (16). Briefly, 3 μm thick tissue microarray sections were deparaffinized, rehydrated, and then digested and refixed in 4% paraformaldehyde. Sections were then replaced with hybridization solution and incubated with locked nucleic acid (LNA)-modified probes (Exiqon) for *let-7* miRNAs, *U6* (positive control), and scrambled RNA (negative control) at 60°C for 20 hours. The slides were then incubated with mouse anti-digoxin antibody followed by binding to streptavidin-biotin-peroxidase complex solution, and the sections were stained with 3,3′-diaminobenzidine solution and counterstained with hematoxylin solution. The stained sections were reviewed and scored for expression of *let-7* miRNAs under the microscopy (Olympus). The sections were scored on the basis of the intensity and the percentage of stained cells.

RNA analysis

Cultured cell lines were used for miRNA extraction using the mirVana miRNA Isolation Kit (Ambion). miRNA fraction was then converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen). All available TaqMan miRNA assays were used for expression analysis as indicated. For mRNA analysis, cultured cells were used for extraction of mRNA using TRIzol Plus RNA Purification system. SYBR Premix Ex Taq Kit (Takara) was used to determine the expression levels of *GAB2*, *FN1*, and other analyzed genes (Supplementary Table S5). The relative amount of gene transcripts was normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

Gelatin zymography

Activity of MMP-2 and MMP-9 was measured by the method of gelatin zymography as previously described (17) with several modifications. Conditioned media were obtained by incubation of MCF cells with serum-free medium for 24 hours and were then concentrated 80-fold using Amicon Ultra Centrifugal Filter Units (Millipore) and normalized by protein concentrations. Samples were loaded on 10% SDS-PAGE gels containing 0.1% gelatin. Electrophoresis was carried out under nonreducing conditions at 100 V and 4°C. Gels were washed in 2.5% Triton X-100, incubated in substrate buffer (50 mmol/L Tris-HCl, pH 8.0, 50 mmol/L NaCl, 10 mmol/L $CaCl_2$, and 0.05% Brij 35) for 40 hours at 37°C, stained with Coomassie stain solution (Bio-Rad), and destained in 20% methanol and 10% acetic acid. Gelatinolytic activity was identified as a clear band on a blue background.

Results

Reduced *let-7g* expression is prognostic for poor survival of breast cancer patients

To specifically define *let-7* miRNA species involved in mammary carcinoma progression, we used digoxigenin-labeled LNA–miRNA probes to detect abundance of 9 mature *let-7* miRNAs in archived breast cancer specimens ($n = 86$) and breast tissue specimens from patients with benign breast diseases ($n = 21$) using *in situ* hybridization (15, 16). The expression of *let-7* miRNAs were detected predominantly within the cytoplasm of the benign luminal epithelial cells or carcinoma cells (Fig. 1A, Supplementary Fig. S1A). Of all the *let-7* miRNAs examined and graded double blindly, we observed significantly diminished expression of only *let-7a*, *let-7c*, and *let-7g* (Fig. 1A) in breast cancer compared with normal specimens ($P = 0.031$, Supplementary Table S1). For direct comparison, detection of the different *let-7* miRNAs on consecutive sections of one sample of invasive ductal carcinoma is provided in Supplementary Fig. S1A. A heat map displaying the expression of all *let-7* miRNAs in normal and cancer tissues is provided in Supplementary Fig. S1G. Heat maps displaying expression of *let-7* miRNAs according to estrogen receptor, progesterone receptor, or HER2 status are also provided in Supplementary Fig. S1G. The association between the expression of all *let-7* family members and clinicopathologic characteristics of breast cancer and these data are summarized in Supplementary Table S2. A statistically significant association was observed between lower expression of *let-7a* and tumor size ($P = 0.041$) and Ki-67 labeling index ($P = 0.036$), between lower expression of *let-7b* and Ki-67 labeling index ($P = 0.036$), between lower expression of *let-7c* and progesterone receptor-negative status ($P = 0.011$) and HER2-negative status ($P = 0.040$), between lower expression of *let-7g* and clinical stage ($P = 0.017$), lymph node metastasis ($P < 0.0001$), and Ki-67 labeling index ($P = 0.001$), and between lower expression of *let-7i* with lymph node metastasis ($P = 0.040$), progesterone receptor-negative status ($P = 0.021$), and HER2-negative status ($P = 0.030$).

We next examined the correlation between the expression of *let-7* miRNAs and survival in the breast cancer patient cohort with 5-year follow-up ($n = 80$), using Kaplan–Meier survival analyses. Patients with lower expression levels of *let-7g* exhibited a significantly worse survival outcome than those with higher expression level of *let-7g* (Fig. 1B, Supplementary Table S5; Fig. 1C, Supplementary Table S5). Recurrence-free survival and overall survival outcomes according to estrogen receptor, progesterone receptor, or HER2 status are provided in Supplementary Tables S3 and S4. Again, *let-7g* was the only *let-7* family member whose reduced expression was associated with decreased survival in the patient subgroups. In contrast, the expression levels of none of the remaining 8 miRNAs could predict survival outcome in breast cancer patients (Supplementary Table S5). We further showed that the reduced expression level of *let-7g* was a significant predicator for patient survival independent of clinical stage and lymph node metastasis in breast cancer by multivariate analyses. We therefore further concentrated on *let-7g* to gain insight on its functional role and mechanism. For comparison of

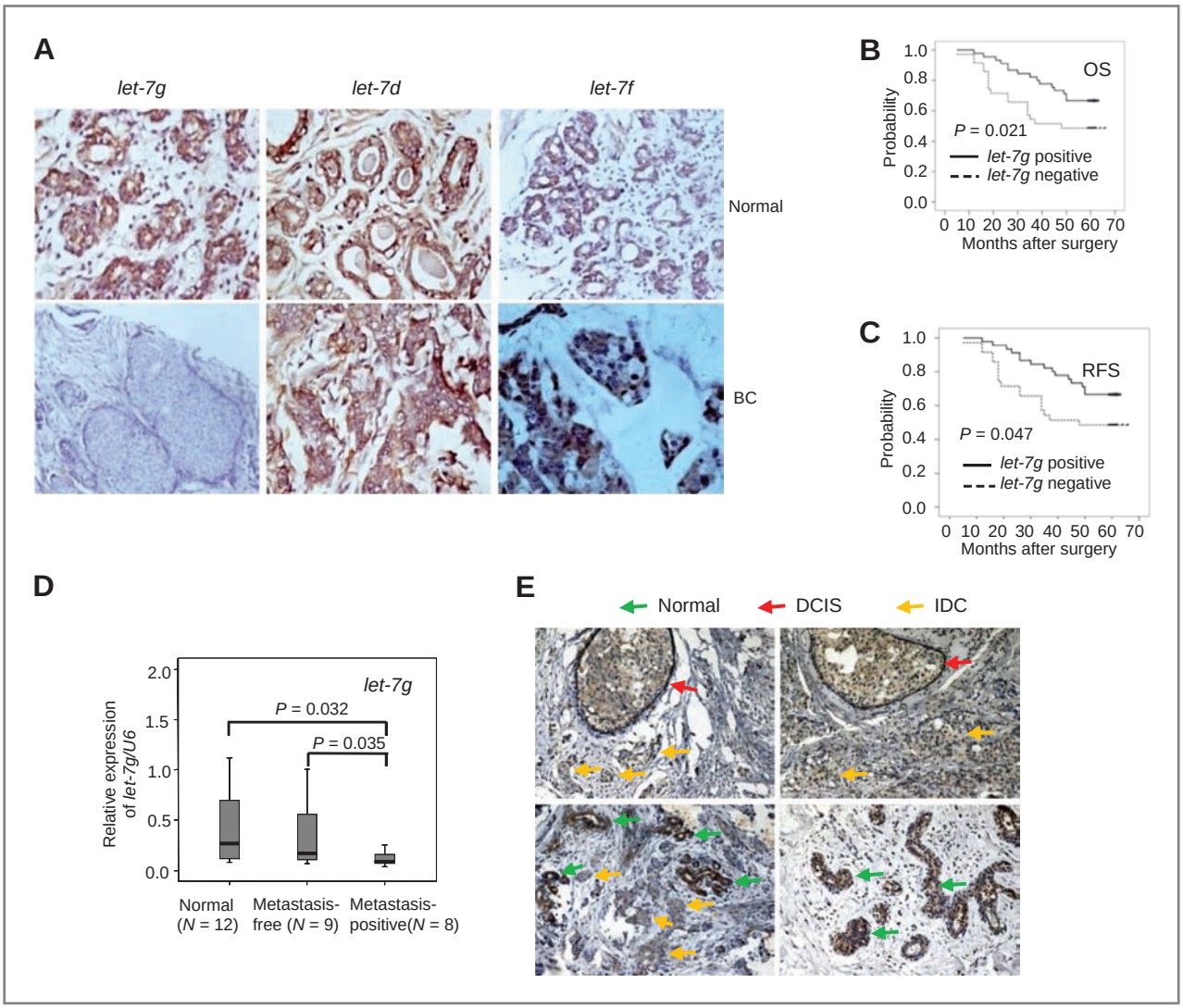


Figure 1. *let-7g* expression is inversely correlated with tumor metastasis and poor prognosis. A, expression levels of *let-7d*, *let-7f*,and *let-7g* were examined in mammary ductal carcinoma specimens and normal mammary specimens. One representative example of tissue microarray sections analyzed by *in situ* hybridization. B and C, correlation between expression level of *let-7g* and 5-year patient survival analysis. D, expression levels of *let-7g* in primary mammary tumors and benign breast disease tissues were analyzed by quantitative real-time PCR. E, graded decreased expression of *let-7g* from normal breast epithelium to premalignant lesion [low-grade of ductal carcinoma *in situ* (DCIS)] to invasive breast cancer. IDS, invasive ductal carcinoma; OS, overall survival; RFS, recurrence-free survival; BC, breast cancer.

speci-ficity, *let-7d* and *let-7f* were included in parallel in further studies.

We next examined *let-7g* expression in fresh mammary carcinoma specimens by quantitative real-time PCR analysis using stem-looped miRNA-specific reverse transcription primers and TaqMan probes. The expression level of *let-7g* was significantly lower in breast cancer patients with lymph node metastases than those from patients with no detectable lymph node metastases (Fig. 1D). However, the expression level of neither *let-7d* nor *let-7f* miRNA exhibited significant differences between the breast cancer patient cohorts with or without lymph node metastases (Supplementary Fig. S1B and C).

We also examined the relative expression of *let-7g* in benign breast tissue, premalignant proliferative lesions, and mammary carcinoma specimens by *in situ* hybridization (Fig. 1E). Interestingly, progressively reduced expression of *let-7g* was observed from normal breast epithelium to ductal carcinoma *in situ* to invasive ductal carcinoma, supporting a role of reduced expression of *let-7g* in both the initiation and progression of breast cancer. Finally, we examined the relative expression of *let-7g* in an array of mammary epithelial and mammary carcinoma cell lines using quantitative real-time PCR (Supplementary Fig. S1D). The highest expression levels of *let-7g* were observed in 2 immortalized but otherwise normal human mammary epithelial cells, whereas low or undetectable levels of *let-7g* expression were observed in highly invasive mammary carcinoma cell lines (such as MDA-MB-231 and MDA-MB-468). In comparison,

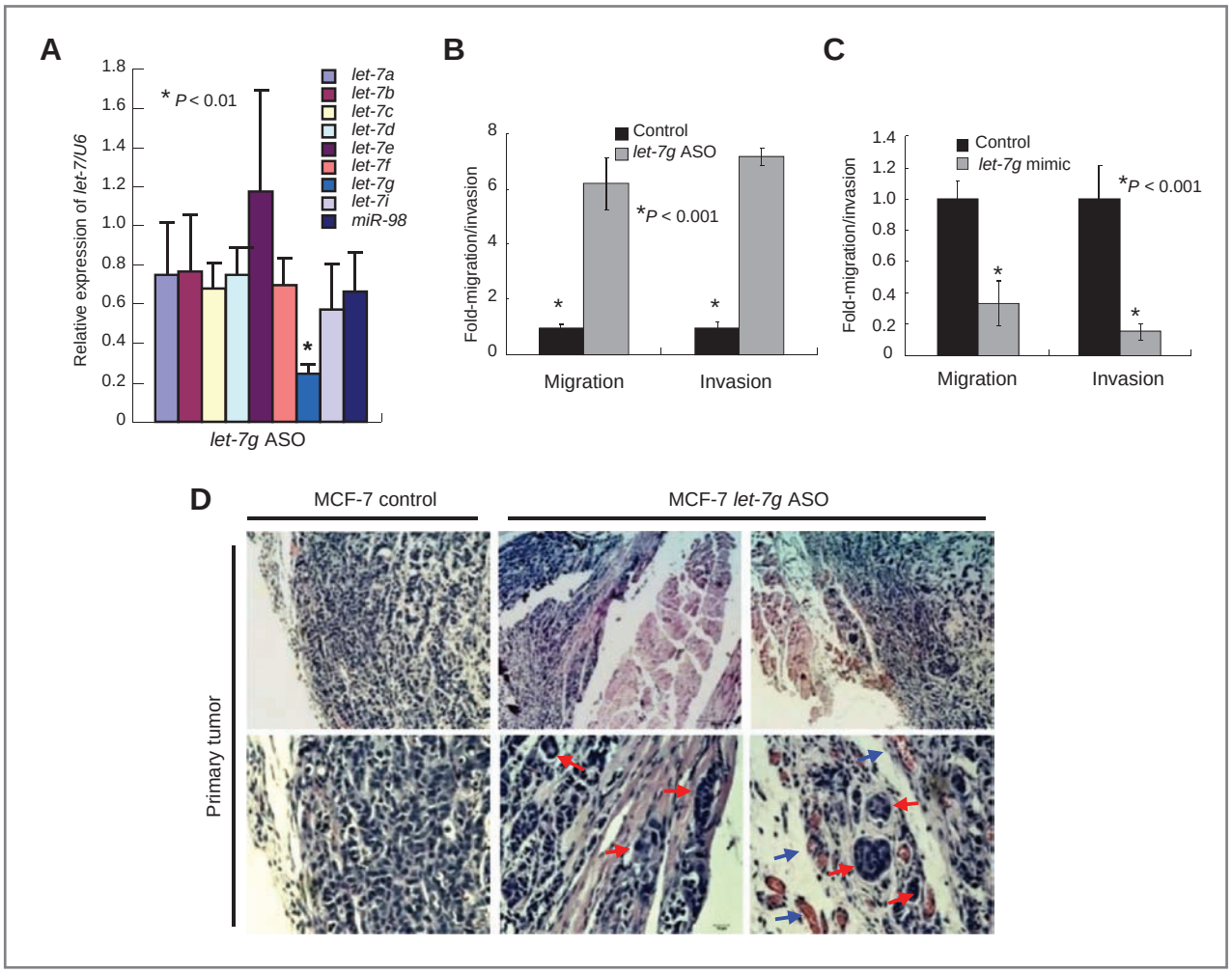


Figure 2. *let-7g* depletion converted noninvasive MCF-7 cells to a highly invasive and metastatic phenotype. A, verification of the specificity of *let-7g* ASO by quantitative real-time PCR. B, Transwell migration and invasion assays of MCF-7 cells transfected with *let-7g* ASO or scrambled sequence oligonucleotide. C, Transwell migration and invasion assays of MDA-MB-231 cells transfected with *let-7g* mimic or scrambled sequence oligonucleotide. D, *let-7g* depletion rendered mammary carcinoma cells more aggressive. Primary mammary tumors formed by MCF-7-luc cells infected with either lentivirus-expressing *let-7g* ASO or scrambled sequence oligonucleotide at week 5 after orthotopic transplantation were stained with hematoxylin and eosin. Abundant tumor angiogenesis (blue arrow) and lymphangiogenesis were visualized in the margins of primary tumor nodule. Tumor emboli (red arrow) were observed in lymphatic vessels.

expression levels of *let-7d* (Supplementary Fig. S1E) and *let-7f* (Supplementary Fig. S1F) were not significantly different between the low-invasive and high-invasive mammary carcinoma cell lines.

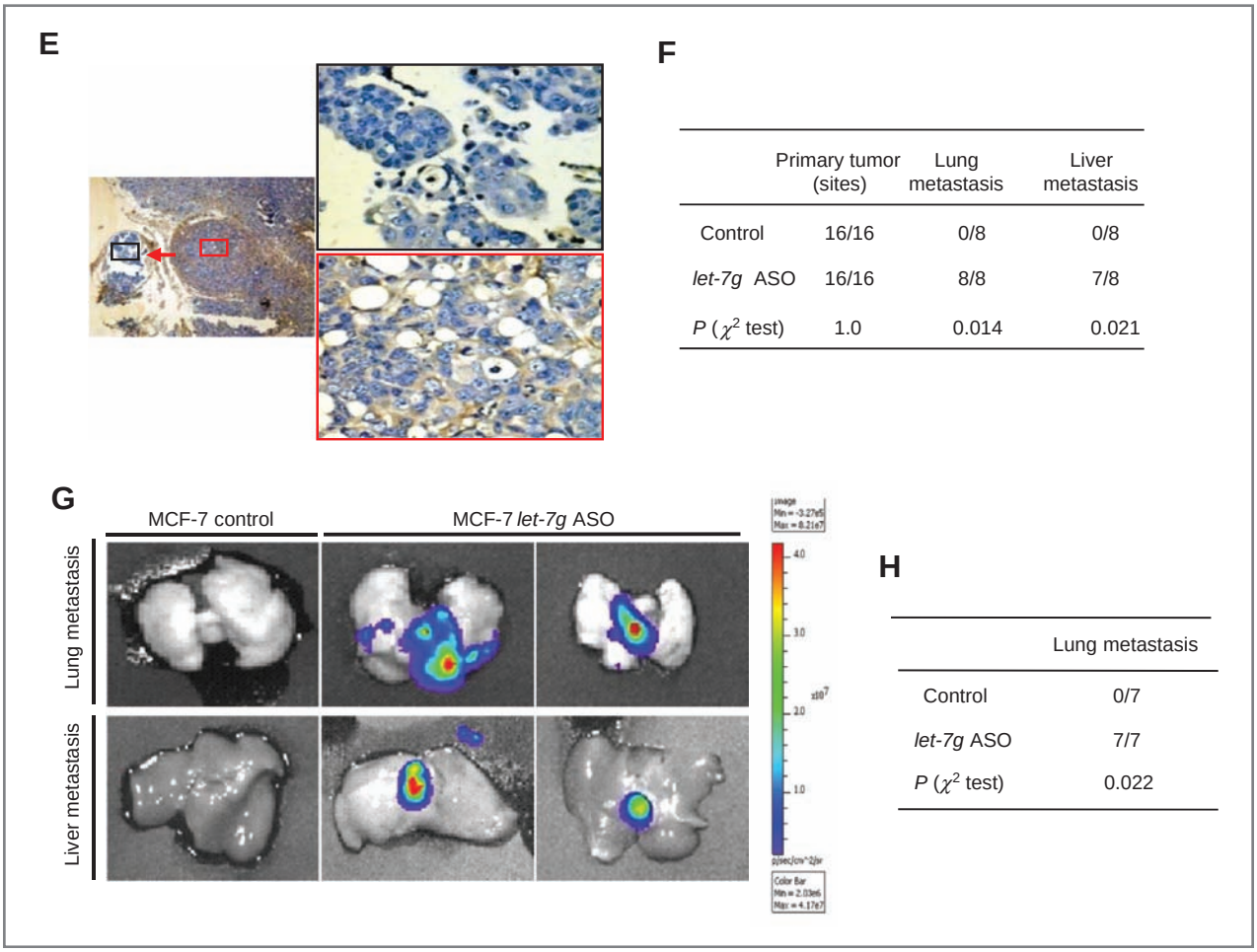
let-7g depletion promotes mammary carcinoma cell migration and invasion *in vitro*

Given the inverse correlation between *let-7g* levels and invasiveness in mammary carcinoma cells, we next assessed the potential role of *let-7g* in mammary carcinoma cell motility through manipulation of the expression level of *let-7g* by transfection of either antisense oligonucleotide (ASO) or synthetic miRNA mimic (Supplementary Fig. S2A and B). *let-7g* ASO exhibited a high specificity to deplete its own target sequence but did not appreciably deplete other *let-7* miRNAs by using quantitative real-time PCR analysis (Fig. 2A).

let-7g depletion in low-invasive MCF-7 cells led to a more rapid closing of the wound (Supplementary Fig. S2C) and a potent increase in cell migration and invasion than the control cells (Fig. 2B). Conversely, forced expression of *let-7g* in the highly invasive MDA-MB-231 cells resulted in retarded wound closing (Supplementary Fig. S2D) and significant reduction in cell migration and invasion (Fig. 2C). The same experimental approach was adopted in 2 other mammary carcinoma cell lines (low-invasive T47D and highly invasive MDA-MB-468; Supplementary Fig. S2E–H).

let-7g depletion initiates distant metastasis *in vivo*

We further determined whether *let-7g* expression depletion would initiate invasion and metastasis of otherwise nonmetastatic MCF-7 cells *in vivo*. To this end, MCF-7-luc cells were infected with either lentivirus-expressing *let-7g* ASOs or



	Primary tumor (sites)	Lung metastasis	Liver metastasis
Control	16/16	0/8	0/8
let-7g ASO	16/16	8/8	7/8
P (χ^2 test)	1.0	0.014	0.021

	Lung metastasis
Control	0/7
let-7g ASO	7/7
P (χ^2 test)	0.022

Figure 2. (*Continued*) E, examination of *let-7g* expression in tumor emboli within vasculature as well as primary tumors by *in situ* hybridization. F, incidence of primary tumor, lung metastasis, and liver metastasis in mice with orthotopic injection of MCF-7-luc cells infected with either lentivirus-expressing *let-7g* ASO ($n = 8$) or scrambled sequence oligonucleotide ($n = 8$). G, MCF-7-luc cells infected with either lentivirus-expressing *let-7g* ASO ($n = 8$) or scrambled sequence oligonucleotide ($n = 8$) were orthotopically transplanted and representative bioluminescent imaging of lungs and livers of mice was shown. H, incidence of lung metastases in mice receiving tail vein injection of MCF-7-luc cells infected with either lentivirus-expressing *let-7g* ASO ($n = 7$) or scrambled sequence oligonucleotide ($n = 7$).

control oligonucleotides (Supplementary Fig. S2I) and injected orthotopically into the mammary fat pad of female BALB/c nude mice. Exposed bioluminescent imaging of lung and liver was used to prevent anatomical superimposition of the primary tumor sites. Primary tumors derived from *let-7g*–depleted cells were poorly encapsulated and highly invasive (Fig. 2D). Tumor emboli (red arrow) were observed in lymphatic vessels. In contrast, tumors derived from control cells remained well-confined and noninvasive. Interestingly, in the primary tumors, cells of tumor emboli within tumor vasculature exhibited no expression of *let-7g* (Fig. 2E, red arrow), further indicative that *let-7g* depletion potently promotes breast cancer invasion and metastasis.

Orthotopic injection of *let-7g*–depleted MCF-7-luc cells resulted in the formation of pulmonary (8 of 8, $P = 0.014$) and hepatic (7 of 8, $P = 0.021$) metastases in host mice observed by bioluminescent imaging 5 weeks after injection (Fig. 2F and G, Supplementary Fig. S2K). No metastases were detected in the mice with orthotopic transplanted with control cells. In addition, metastatic tumors were readily detectable in the lungs of all mice (7 of 7) injected with *let-7g*–depleted MCF-7-luc cells by bioluminescent imaging 4 weeks post–tail vein injection, whereas no metastases were detectable in the lungs of host mice (0 of 7) injected with the control cells (Fig. 2H and I, Supplementary Fig. S2L). It was interesting to note that bone metastases were only observed in the mice injected with *let-7g*–depleted cells (Supplementary Fig. S2L).

Concordantly, forced expression of *let-7g* by infection with lentivirus-expressing *let-7g* was sufficient to prevent the pulmonary macrometastases of highly invasive MDA-MB-231 cells (0 of 8, $P = 0.015$) in a xenograft model by tail vein injection (Supplementary Fig. S2M–O).

GAB2 and FN1 are targeted by *let-7g* to regulate mammary carcinoma cell migration and invasion

To identify downstream targets of *let-7g*, 2 sets of paired cells were used to examine the mRNA expression profile of an

array of genes that have been reported to be involved in cancer invasion and metastasis (18, 19). MDA-MB-231 cells transfected with either *let-7g* mimic or control and MCF-7 cells transfected with either *let-7g* ASO or scrambled sequence control were used. The data derived from the 2 complementary pairs of screening systems were largely concordant (Supplementary Table S6).

To further identify the putative direct targets of *let-7g*, we used 3 algorithms for mRNA target prediction—MiRanda, RNAhybrid, and TargetScan (20–22). *GAB2* and *FN1* were of particular interest among the candidate genes, as both have been implicated in breast cancer metastasis in animal models and the clinical setting (23–29). Both the *GAB2*-encoded mRNA and *FN1*-encoded mRNA contain a 3′-UTR element that is partially complementary to *let-7g* and carries the identical sequence in the multiple mammalian mRNA orthologues (Supplementary Fig. S3A–C).

Forced expression of *let-7g* reduced the activity of a luciferase reporter gene containing the 3′-UTR of *GAB2* (Fig. 3A), indicating that *let-7g* directly targets *GAB2*. We further identified that a single *let-7g* cognate binding site, LCS2, within the *GAB2* 3′-UTR, was the major target site for *let-7g* and thereby

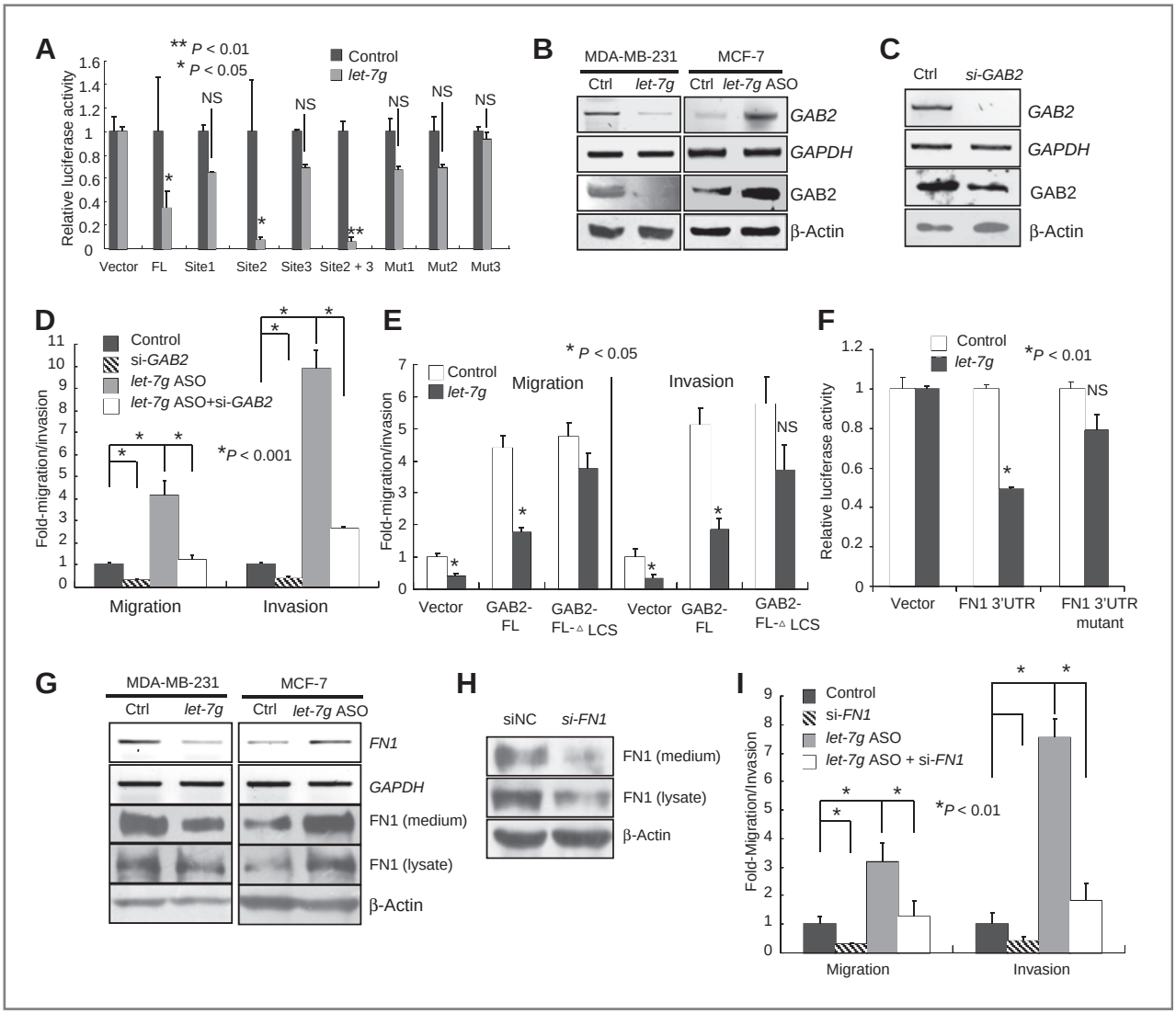


Figure 3. Identification of GAB2 and FN1 as mediators of *let-7g* depletion promoted cell migration and invasion. A, *let-7g* targeted GAB2 through its 3′-UTR. MCF-7 cells were transfected with plasmid reporter encompassing *GAB2* 3′-UTR at different length. Luciferase assay was conducted and normalized to internal firefly luciferase activity. B, *let-7g*–regulated GAB2 expression. MDA-MB-231 or MCF-7 cells were transfected with *let-7g* mimic or *let-7g* ASO or negative control (Ctrl). *GAB2* mRNA (top 2 panels) and protein (bottom 2 panels) were detected. C, the efficacy of *GAB2* siRNA was verified by either reverse transcriptase PCR (RT-PCR; top 2 panels) or Western blot (bottom 2 panels). D, *let-7g* depletion promoted cell migration and invasion were diminished by GAB2 silencing. E, *let-7g* depletion promoted cell migration and invasion were mediated by its interaction with the 3′-UTR of GAB2. F, *let-7g* targeted FN1 through its 3′-UTR. G, *let-7g*–regulated FN1 expression. MDA-MB-231 or MCF-7 cells were transfected with *let-7g* mimic or *let-7g* ASO or negative control. Transfected cells were used for analysis of *FN1* mRNA (top 2 panels) and protein (bottom 3 panels). H, the efficacy of FN1 siRNA was verified by either RT-PCR (top 2 panels) or Western blot (bottom 2 panels). I, *let-7g* depletion promoted cell migration and invasion were diminished by FN1 silencing. NS, nonsignificant.

sufficient to regulate GAB2 expression (Fig. 3A, Supplementary Fig. S3B). Consistently, forced expression of *let-7g* diminished GAB2 expression at both mRNA and protein levels (Fig. 3B), indicating GAB2 is a bona fide target of *let-7g*.

We further determined whether GAB2 was required for *let-7g*–mediated mammary carcinoma cell motility. Specific depletion of GAB2 by siRNAs significantly abrogated *let-7g* depletion–enhanced MCF-7 cell motility (Fig. 3C and D, Supplementary Fig. S3E and F). The functional importance of the interaction between *let-7g* and GAB2 3′-UTR was also determined by using either full-length GAB2 (open reading frame + 3′-UTR) with or without mutation at *LCS2*. Thus, GAB2 is a critical mediator for the *let-7g* depletion–enhanced cell migration and invasion (Fig. 3E).

Similarly, we identified FN1 as a direct target of *let-7g* and mapped the major *let-7g* binding site within the *FN1* 3′-UTR (Fig. 3F and G, Supplementary Fig. S3C and D). We showed that siRNA-mediated depletion of FN1 expression significantly abrogated the enhanced migration and invasion consequent to *let-7g* depletion (Fig. 3H and I). Thus, both GAB2 and FN1 play important roles downstream of *let-7g*.

p44/42 MAPK and MMPs are essential for *let-7g*–mediated mammary carcinoma cell migration and invasion via GAB2 and FN1

We further investigated the downstream signaling pathway used by GAB2 to mediate *let-7g* effects on cell motility. Increased expression of GAB2 has been shown to play an important role in promoting mammary carcinoma metastasis through activation of the p44/42 MAPK pathway (23, 25). Elevated GAB2 expression and p44/42 MAPK activity were observed in *let-7g*–depleted MCF-7 cells compared with the control oligonucleotide–transfected cells. Moreover, this effect was substantially abrogated by GAB2-specific siRNA (Fig. 4A), indicating that *let-7g* depletion directly increased GAB2 expression to promote p44/42 MAPK activation.

To define the p44/42 MAPK downstream molecules that mediate the effect of *let-7g*, we focused on the gelatinases MMP-2 and MMP-9, which are regulated by the p44/42 MAPK pathway (30–33), as well as possessing the capacity to degrade type IV collagen and mediate tumor cell invasion (34, 35). Indeed, *let-7g* depletion potently increased the expression and activity of MMP-2/MMP-9 (Fig. 4B and C). Furthermore, abrogation of GAB2 expression by specific siRNAs diminished the expression and enzymatic activity of MMP-2/MMP-9 consequent to *let-7g* depletion (Fig. 4B and C). Similarly, we showed that FN1 expression was required for the increased MMP-2/MMP-9 expression and invasion consequent to *let-7g* depletion (Supplementary Fig. S3G). To further show that MAPK activity was required for the increased MMP-2/MMP-9 activation, *let-7g*–depleted MCF-7 cells were treated with either the MAP/ERK kinase 1/2 (MEK1/2)-specific inhibitor U0126 or MMP-2/MMP-9–specific inhibitor SB-3CT. Inhibition of MEK1 activity was shown to be sufficient to diminish MMP-2/MMP-9 activity and *let-7g* depletion–promoted cell motility (Fig. 4D and E). It is apparent that the p44/42 MAPK–MMP-2/MMP-9 pathway was used to enhance cell migration and invasion consequent to *let-7g* depletion.

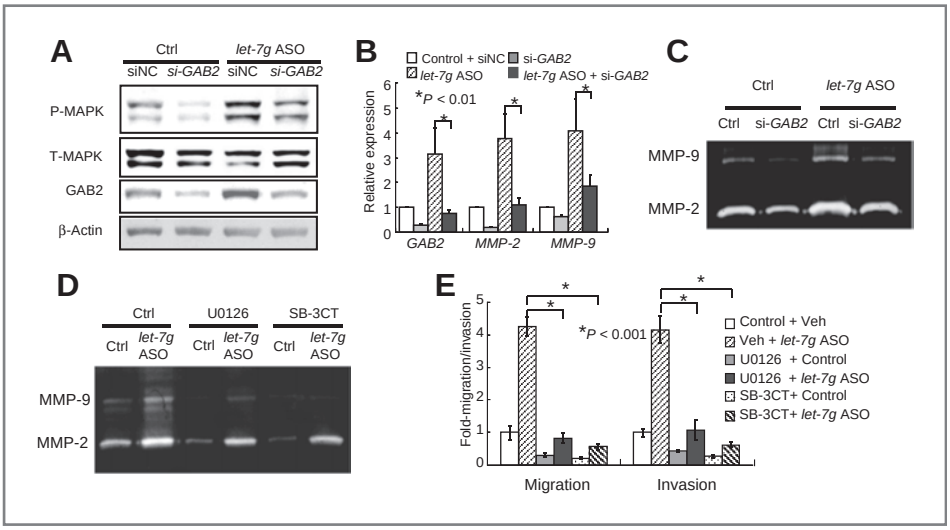


Figure 4. Identification of p44/42 MAPK and MMP-2/MMP-9 as mediators for *let-7g*–regulated mammary carcinoma cell migration and invasion. A, *let-7g* depletion increased p44/42 MAPK phosphorylation via elevated GAB2 expression. MCF-7 cells were transiently transfected with *let-7g* ASO and/or si-*GAB2* and their respective negative control. The expression of GAB2, phospho (P)-p44/42 MAPK, total (T)-p44/42 MAPK, and β-actin was detected by Western blotting. B, *let-7g* depletion increased *MMP-2*/*MMP-9* gene transcription. Transfected cells as described in (A) were used for transcription analysis of *GAB2*, *MMP-2*, and *MMP-9* by quantitative real-time PCR. C, *let-7g* regulated enzymatic activity of MMP-2/MMP-9 via GAB2. Transfected cells as described were used for measuring MMP-2 and MMP-9 activity by gelatin zymography. D, *let-7g* regulated enzymatic activity of MMP-2/MMP-9 via p44/42 MAPK. MCF-7 cells were transiently transfected with *let-7g* ASO or negative control. U0126 (10 μmol/L), SB-3CT (10 μmol/L), and dimethyl sulfoxide were respectively added to the medium. Supernatant was collected and concentrated for analysis of MMP-2 and MMP-9 activity by gelatin zymography. E, inhibition of p44/42 MAPK and MMP-2/MMP-9 activity abolished *let-7g*–mediated cancer cell migration and invasion.

GAB2 and FN1 are preferentially targeted by *let-7g* for p44/42 MAPK activation

We next sought to determine why *let-7g* depletion alone should possess such striking biological effects and its cellular context in breast cancer metastasis. To answer both questions, we sought to determine possible differential effects of *let-7g* depletion on cell behavior compared with the depletion of other *let-7* family members. Both *let-7d* and *let-7f* ASOs were included as controls, and the specificity of each ASO for the 3 *let-7* miRNAs was first determined by quantitative real-time PCR (Fig. 5A). Both *let-7d* and *let-7f* were chosen as controls for full functional analyses, as *let-7d* with a 4-nucleotide difference to *let-7g* would represent a distant structural control and *let-7f* with only a 2-nucleotide difference would represent a closer structural control. Functionally, *let-7g* depletion was more potent to promote the migration and invasion of MCF-7 cells than the depletion of either *let-7d* or *let-7f* (Fig. 5B). Thus, *let-7g* depletion exhibited significantly higher efficacy to promote cancer cell migration and invasion.

Mechanistically, *let-7g* more potently inhibited the activity of 3′-UTR–luciferase constructs for *GAB2* or *FN1* genes than *let-7d* or *let-7f* in 2 types of cells (Fig. 5C, Supplementary Fig. S4H). Consistently, *let-7g* ASO also more potently increased the activity of 3′-UTR–luciferase construct for *GAB2* or *FN1* genes than *let-7d* or *let-7f* (Fig. 5D). The effect of forced expression of all of the different *let-7* family members on the 3′-UTR of GAB2 and FN1 is presented in Supplementary Fig. S3H. Concordantly, we observed that the expression level of *let-7g* but not *let-7d* or *let-7f* negatively correlated with GAB2 or FN1 mRNA level in an array of breast epithelial cell lines (Supplementary Fig. S4B–G). GAB2 and FN1 were therefore preferentially targeted by *let-7g* in mammary carcinoma cells. Consistently, we showed that *let-7g* depletion, but not *let-7d* or *let-7f* depletion, potently increased the expression of GAB2 or

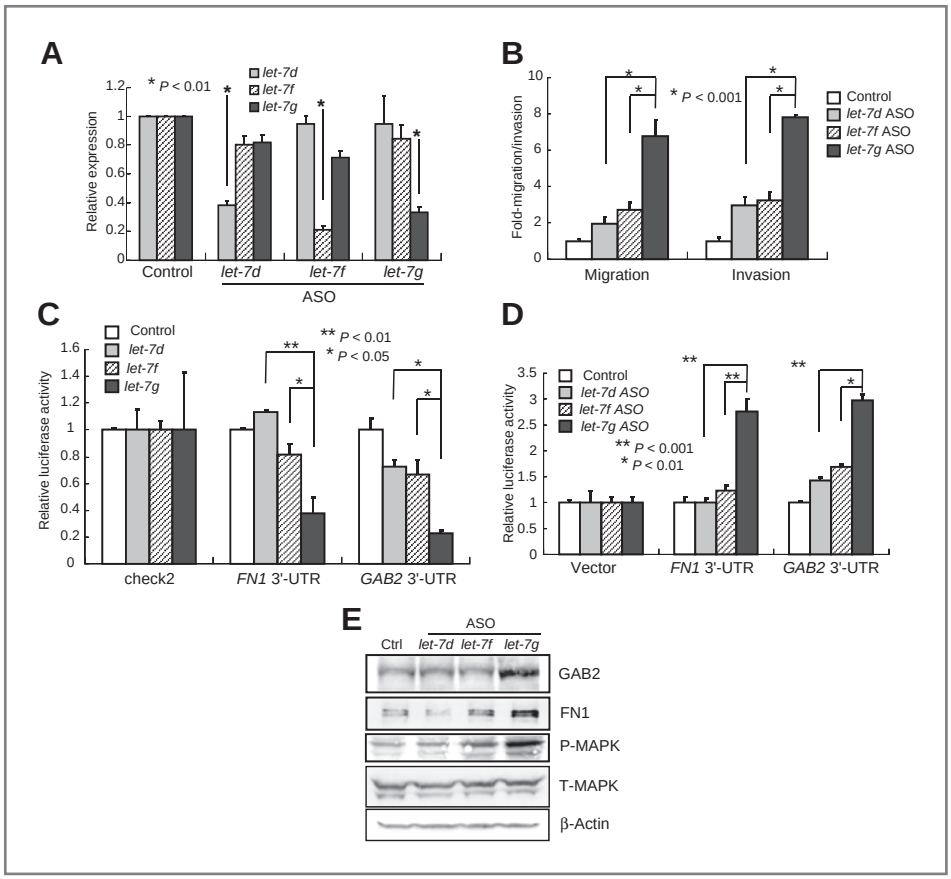


Figure 5. *let-7g* depletion increased the expression of GAB2 and FN1 for the selective activation of p44/42 MAPK. A, *let-7* ASOs specifically diminished the expression of their respective *let-7* miRNA by quantitative real-time PCR. B, *let-7g* ASO more potently promotes cell migration and invasion. Transwell migration assay and Matrigel invasion assay of MCF-7 cells transfected with either scrambled sequence oligonucleotide or ASO for *let-7d*, *let-7f*,or *let-7g* were conducted. C, GAB2 and FN1 were preferentially targeted by *let-7g* via binding to its respective 3′-UTR. MCF-7 cells transfected with plasmid reporter psiCHECK2-GAB2 3′-UTR or psiCHECK2-FN1 3′-UTR together with either scrambled sequence oligonucleotide or synthetic miRNA mimic for *let-7d*, *let-7f*,or *let-7g*. Luciferase assay was conducted and normalized to internal firefly luciferase activity. D, *let-7g* ASO preferentially promoted the expression of GAB2 and FN1 via their respective 3′-UTR. MCF-7 cells transfected with plasmid reporter psiCHECK2-GAB2 3′-UTR or psiCHECK2-FN1 3′-UTR together with either scrambled sequence oligonucleotide or antisense oligonucletotide for *let-7d*, *let-7f*,or *let-7g*. Luciferase assay was conducted and normalized to the internal firefly luciferase activity. E, *let-7g* depletion preferentially increased GAB2 and FN1 expression and p44/42 MAPK activation. MCF-7 cells were transiently transfected with ASO for *let-7d*, *let-7f*,or *let-7g* or scrambled sequence oligonucleotide. The expression of GAB2, FN1, phospho (P)-p44/42 MAPK, total (T)-p44/42 MAPK, and β-actin was detected by Western blotting.

FN1 and significantly activated p44/42 MAPK (Fig. 5E). We therefore propose that increased GAB2 and FN1 consequent to *let-7* depletion represents an integrated modulation of the p44/42 MAPK pathway specific for *let-7g* but not for the other *let-7* miRNAs examined. The functional specificity of *let-7g* shown herein was highly consistent with our clinical studies that only decreased expression of *let-7g* significantly correlated with proliferative status, lymph node metastasis, and prognosis of breast cancer patients (Supplementary Tables S2 and S5).

Estrogen and EGF modulate the expression of *let-7g* and GAB2

We further sought to determine whether etiologic factors stimulatory of breast cancer metastasis, such as estrogen and epidermal growth factor receptor (EGFR)/HER2 ligands, might contribute to promotion of mammary carcinoma cell invasion through regulation of *let-7g* expression.

Treatment of MCF-7 cells with 17β-estradiol (E2) resulted in rapid and specific reduced *let-7g* expression (Fig. 6A and B, Supplementary Fig. S5A and B). E2 increased GAB2 expression at both the mRNA and protein levels (Fig. 6B and C). The specificity of E2 treatment via ERα was verified by using fulvestrant (Fig. 6D). Transfection of the *let-7g* mimic substantially abrogated the effect of E2 on GAB2 expression, indicating that E2 stimulated GAB2 expression via the interaction between *let-7g* and GAB2 3′-UTR (Fig. 6E). We further showed that estrogen promoted migration and invasion of MCF-7 (Fig. 6F) and T47D cells (Supplementary Fig. S5C) via *let-7g* depletion and elevated GAB2 expression.

Similarly, the effects of EGF on *let-7g* and GAB2 expression and cell motility were also detected. EGF promoted mammary carcinoma cell migration and invasion (Fig. 6I, Supplementary Fig. S5D) through *let-7g* depletion and consequently increased GAB2 expression (Fig. 6G–L) via EGFR/HER2 (Fig. 6J). Thus, both E2 and EGF reduced *let-7g* expression and consequently increased GAB2 expression, leading to enhancement of cell migration and invasion.

Similarly, we showed that FN1 expression was increased by E2 and EGF via their repression of *let-7g* expression (Supplementary Fig. S5E–H). Increased FN1 expression was also required for E2 and EGF stimulation promoted mammary carcinoma cell migration and invasion via *let-7g* repression (Supplementary Fig. S5I and J).

We also determined what pathways might mediate E2- and EGF-promoted *let-7g* depletion. It has been recently reported that proliferative signals can promote *let-7* family downregulation via the p44/42 MAPK pathway (36). Serum-deprived MCF-7 cells were therefore pretreated with U0126 and followed by E2 treatment. Interestingly, neither estrogen nor EGF stimulation with or without U0126 affected the expression of primary *let-7g* (Supplementary Fig. S5K), despite that both ERα and c-Myc binding sites at the *let-7g* promoter have been either identified or predicted (Supplementary Fig. S5L), indicative that reduction of mature *let-7g* expression by estrogen or EGF stimulation may be achieved through a posttranscriptional mechanism. In contrast, U0126 significantly abrogated E2- or EGF-induced repression of mature *let-7g* (Fig. 6M). Thus, E2 or EGF stimulation specifically decreased functional *let-7g* expression via p44/42 MAPK pathway.

Discussion

The aberrant expression of miRNAs in cancer progression has previously been determined mostly by microarray profiling, bead-based technologies, and quantitative real-time PCR in either fresh or archived formalin-fixed paraffin-embedded tumor specimens or blood samples. However, these approaches are inevitably confounded by the heterogeneity of the biospecimens, especially the cell population of the tumor microenvironment including stromal and inflammatory cells. This is probably one explanation for the substantial conflicting data in the field (37). For example, miR-10b has been reported prometastatic (38, 39) or antimetastatic (40, 41) in breast cancer independently by multiple groups. In this study, the expression of the entire *let-7* family miRNAs was examined in clinical specimens by *in situ* hybridization with clinicopathologic analysis. Strikingly, reduced expression of *let-7g* in mammary carcinoma was uniquely linked with tumor metastasis and poor patient survival. The functional significance of this negative correlation was further supported by *in vitro* and *in vivo* studies (Fig. 2). We have therefore identified a unique member of the *let-7* family as an independent prognostic marker for breast cancer.

let7g seems to be specifically involved in breast cancer metastasis for 2 reasons. First, pathologic and prognostic analyses of survival outcome indicated that *let-7g* was the only *let-7* miRNA family member to exhibit a significant correlation of diminished expression with poor survival in breast cancer patients. Second, of the all the mature *let-7* miRNAs examined, *let-7g* showed the highest efficacy to diminish the reporter activities of the 3′-UTR of GAB2 and FN1. Although our data indicated that *let-7e*, *let-7i*, or miRNA-98 also significantly reduced the reporter activities of 3′-UTR of GAB2 and/or FN1, however, none of these miRNAs exhibited reduced expression in clinical specimens, indicating that these *in vitro* molecular events do not possess any pathophysiologic relevance. Moreover, even though reduced expression of *let-7a* and *let-7c* was observed in breast cancer tissue, neither of these miRNAs could efficiently target the 3′-UTR of either GAB2 or FN1. Therefore, our results show that amongst all *let-7* family members, only *let-7g* fulfilled 2 critical criteria at the same time: (i) reduced expression in breast cancer and an expression level that inversely correlated with metastasis and survival and (ii) efficacious targeting of the 3′-UTR of both GAB2 and FN1 to promote tumor invasion and metastasis. Amongst *let-7* family members, diminished expression of *let-7g* is therefore specifically involved in breast cancer metastasis. Our results do not exclude possible roles of other *let-7* family members in promoting tumor growth or proliferation. For example, reduced expression of *let-7a* in breast cancer was associated with larger tumor size and higher proliferative status, indicative that reduced *let-7a* expression may contribute to tumor growth. That *let-7a* does not influence survival outcome of breast cancer patients can easily be understood, in that death from breast cancer is primarily

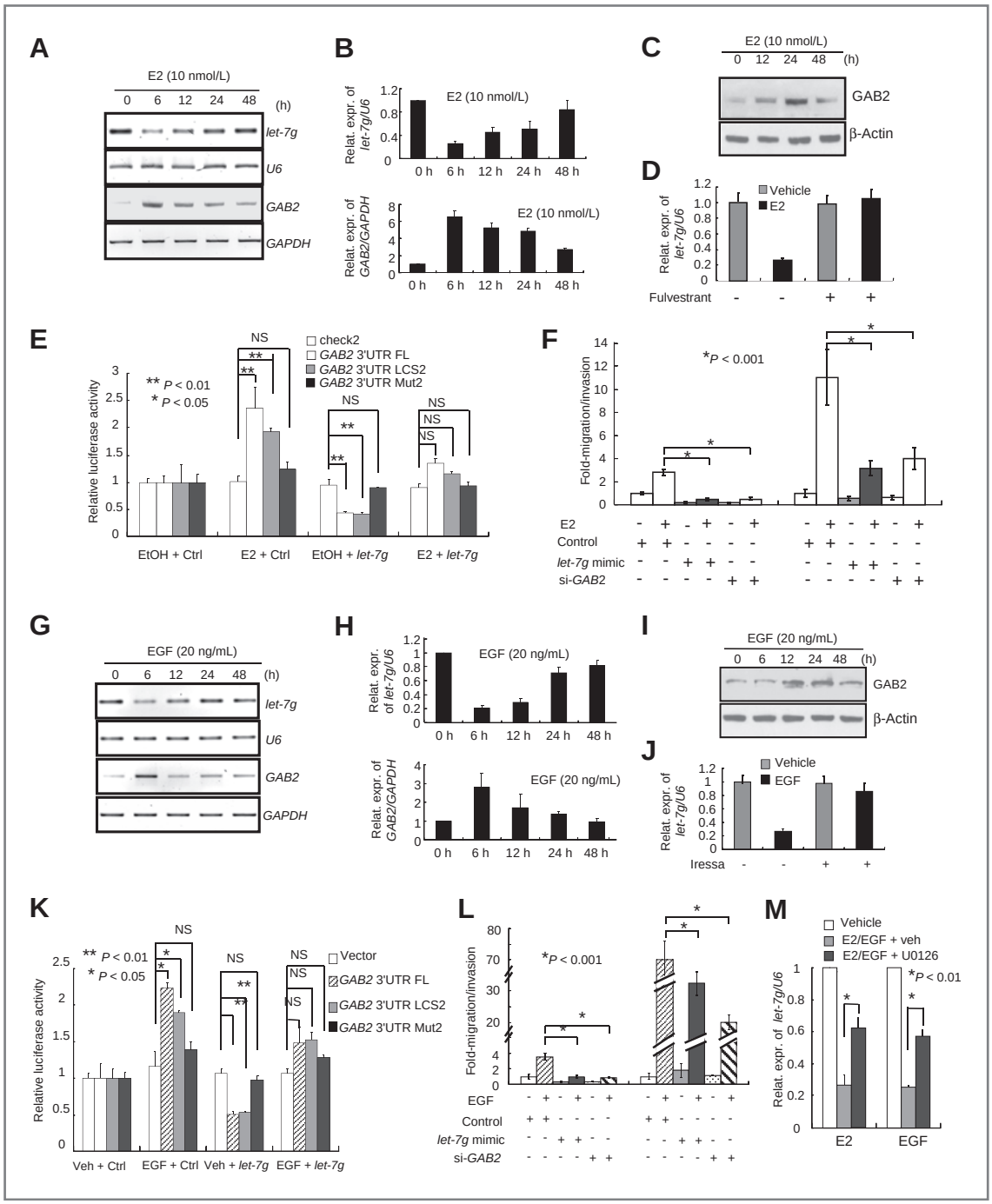


Figure 6. Estrogen and EGF promoted mammary carcinoma cell migration and invasion through reduction of *let-7g* expression. A, estrogen transiently reduced *let-7g*. B, estrogen stimulation repressed *let-7g* expression but enhanced *GAB2* expression. C, estrogen transiently increased GAB2 protein expression. D, estrogen depletion of *let-7g* expression was mediated through ERα. Estrogen-deprived MCF-7 cells were treated with estrogen in the presence or absence of pure ERα antagonist fulvestrant. E, estrogen regulated GAB2 expression via its 3′-UTR. F, estrogen promoted cell migration and invasion through regulating *let-7g* and GAB2. MCF-7 cells were transfected with *let-7g* mimic or GAB2 siRNA as indicated. Transwell migration assay and invasion assay were conducted. G, EGF transiently reduced *let-7g* expression. H, EGF stimulation repressed *let-7g* expression but enhanced *GAB2* expression. I, EGF transiently increased GAB2 protein expression. J, EGF depletion of *let-7g* expression was mediated through the EGF receptor. MCF-7 cells were treated with EGF in the presence or absence of EGFR antagonist Iressa. K, EGF regulated GAB2 expression via its 3′-UTR. L, EGF promoted cell migration and invasion through regulating *let-7g* and GAB2. MCF-7 cells were transfected with *let-7g* mimic or GAB2 siRNA as indicated. Transwell migration assay and invasion assay were conducted. M, estrogen/EGF-induced depletion of *let-7g* was mediated by p44/42 MAPK. Estrogen-deprived and serum-deprived MCF-7 cells were pretreated with MEK-specific inhibitor U0126 for half an hour and followed by 6-hour treatment with E2 or EGF. The expression levels of mature *let-7g* were measured by quantitative real-time PCR. Ctrl, control; Veh, vehicle; NS, nonsignificant.

due to complications of metastasis (42), a process that *let-7a* is not correlated with clinically nor mechanistically. We have therefore garnered both clinical and mechanistic evidence to propose a paradigm, used by specific growth factors upstream and mediated by specific signaling molecules downstream, that *let-7g* is pivotal to promote breast cancer invasion and metastasis.

It is of interest to note that *let-7g* has also been reported to play a unique role among the *let-7* family members in the irradiation response of lung cancer (43). Contrary to other *let-7* miRNAs, only *let-7g* exhibited an increased expression in response to γ-irradiation in 3 lung cancer cell lines. However, the mechanism whereby *let-7g* played the unique role in lung cancer was not determined.

In this study, we showed that E2 and EGF, major prometastatic growth factors in breast cancer (44–46), specifically repress *let-7g* expression and subsequently increase GAB2 and FN1 expression, which, in turn, cooperatively increased the activity of p44/42 MAPK and MMP-2/MMP-9. It has been reported recently that the p44/42 MAPK cascade may repress *let-7* expression either via activating LIN28 transcription to affect primary *let-7g* processing (47) or via phosphorylating TAR RNA–binding protein, a critical component of miRNA-generating complex, to suppress the expression of *let-7* miRNAs (36). Our study thus adds another dimension to the signaling network, that reduced expression of *let-7g* specifically increased p44/42 MAPK activity, which, in turn, further repressed *let-7* expression, generating a positive feedback loop to potentially enhance the biological processes such as metastasis and proliferation (Figs. 4–6, Supplementary Tables S2 and S4). Our results have thus identified a feedback loop (E2/EGF-p44/42 MAPK-*let-7g*-GAB2/FN1-p44/42 MAPK) used by *let-7g* to integrate multiple signaling molecules for the facilitation of mammary carcinoma metastasis (Supplementary Fig. S6). We propose that the critical growth factors for breast cancer (estrogen and EGF) might specifically deplete *let-7g* expression and further trigger synergistic interaction between *let-7g* and p44/42 MAPK to promote mammary carcinoma cell invasion and metastasis via positive feedback loops.

It should be noted that *let-7g* should be expected to possess a wide range of functionalities due to its pleiotrophic regulation of genes. Further studies will be required to fully understand its functional roles and interactions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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