Chromatin, Gene, and RNA Regulation

The Heterochronic microRNA let-7 Inhibits Cell Motility by Regulating the Genes in the Actin Cytoskeleton Pathway in Breast Cancer

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Abstract
The heterochronic gene let-7 serves as a tumor suppressor microRNA by targeting various oncogenic pathways in cancer cells. Considerable evidence indicates that reduced expression of let-7 might be associated with poor clinical outcome in patients with cancer. Here, we report that the expression levels of three let-7 family members, let-7a, let-7b, and let-7g, were significantly decreased in the patients with breast cancer with lymph node metastasis compared with those without lymph node metastasis. Enforced expression of let-7b significantly inhibits breast cancer cell motility and affects actin dynamics. Using bioinformatic and experimental approaches, four genes in the actin cytoskeleton pathway, including PAK1, DIAPH2, RDX, and ITGB8, were identified as let-7 direct targets. Blocking the expression of PAK1, DIAPH2, and RDX significantly inhibits breast cancer cell migration induced by let-7b repression. Our results indicate that reconstitution of let-7 expression in tumor cells could provide a novel therapeutic strategy for the treatment of metastatic disease. Mol Cancer Res; 11(3); 240–50. ©2013 AACR.

Introduction
The let-7 family is one of the first tumor suppressor miRNAs to be identified. The let-7 family is composed of 13 members in humans, with both overlapping and distinct functions (1–4). They negatively regulate target gene expression by either translational repression or mRNA cleavage, in a sequence-specific manner (1–4). The role of let-7 in cancer was first discovered when the let-7 family was found to negatively regulate let-60/RAS in Caenorhabditis elegans by binding to multiple let-7 complementary sites in its 3′-untranslated region (UTR; ref. 5). Moreover, having been found that let-7 expression is lower in lung tumors than in normal lung tissue, whereas RAS protein is significantly higher in lung tumors, it was proposed that let-7 is a tumor suppressor gene (5), which is consistent with previous clinical observation in lung cancer (6). Reduced expression of let-7 has been associated with shortened postoperative survival in patients with cancer (7), and forced expression of let-7 family members is able to suppress tumor growth both in vitro and in vivo (2–4). The inhibitory function of the let-7 family in cancer has been corroborated by a number of groups and in various types of tumors (2–4). let-7 probably does these functions by targeting various genes. let-7 inhibits many well-characterized oncogenic proteins, such as KRAS (5, 8, 9), HRAS (5, 8, 9), HMGA2 (9), and cyclin genes (13, 14). A systematic review of 43 published studies shows that let-7 is the miRNA most frequently and significantly associated with clinical outcomes in patients with cancer (7). Furthermore, nanoparticle-based let-7 replacement therapy has been successfully tested in preclinical animal models of cancer (8, 15–18). The suppression of let-7 maturation by LIN28 is mediated by TUTase, which is facile target for pharmacologic inhibition by small chemical compounds (19). Therefore, the characterization of the function of the let-7 family in cancer represents a great opportunity to develop robust biomarkers and novel therapeutic strategies for this disease. In the present study, we analyzed the expression of the let-7 family in invasive ductal carcinoma and determined that lower expression levels of let-7a, let-7b, and let-7g were associated with lymph node metastasis in breast cancer. Functional studies showed that let-7b inhibited breast cancer cell motility by repressing multiple genes in the actin cytoskeleton pathway.

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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doi: 10.1158/1541-7786.MCR-12-0432
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Materials and Methods

Patients and specimens
The breast tumor specimens were collected at the University of Turin (Turin, Italy). All tumors were from primary sites and were immediately snap-frozen and stored at −80°C. Specimens were acquired and processed under procedures approved by the local Institutional Review Boards (IRB) and were compliant with the HIPAA Act.

Cell culture
MDA-MB-231 and MCF7 cells were purchased from the American Type Culture Collection (ATCC) and passaged in the user’s laboratory for fewer than 6 months after resuscitation. Cells were cultured in RPMI-1640 (Cellgro) medium supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen).

Migration and invasion assays
MDA-MB-231 cells were transfected with the let-7b mimic (60 nmol/L), let-7b inhibitor (90 nmol/L), or control oligos. At 48 hours, the cells were serum-starved. At 72 hours after transfection, cells were trypsinized, suspended in serum-free medium, and then seeded into Transwell Permeable Support inserts with 8-μm microporous membranes (Corning) in a 24-well plate (2 × 10^5 cells per well). Culture medium (10% FBS) was in the lower compartment of the plate. At 6 hours, cells on the upper surface of the membrane were removed using a cotton swab. The cells that migrated to the lower surface were fixed and stained with the 3-Step Stain Kit (Richard-Allan Scientific). The stained cells were photographed and counted under a microscope.

Wound-healing assays
MDA-MB-231 cells were seeded into 6-well plates and grown overnight. Cells were transfected with the let-7b mimic (60 nmol/L) or control oligos. After 48 hours, the cells were serum-starved for an additional 24 hours. A sterile 200 μL pipette tip was used to scratch the cells to form a wound. Cells were washed with PBS twice and then cultured in 10% FBS medium (10% FBS) in the lower compartment of the plate. Migration of the cells to the wound was observed and photographed with an inverted phase-contrast microscope, and representative fields were photographed. The healing rate was quantified with measurements of the gap size after culture. Eight different areas in each assay were chosen to measure the distance of the migrating cells to the origin of the wound. The healing rate of cells transfected with the let-7b miRNA was normalized to that of control cells. The healing rate was used to represent the migration of the cells.

Immunofluorescent staining
MDA-MB-231 cells were transfected with the let-7b mimic (60 nmol/L). At 72 hours, the cells were seeded onto fibronectin-precoated coverslips (BD BioCoat; BD Biosciences). After an overnight incubation, the cells were washed twice with cold PBS, fixed with 4% formaldehyde for 20 minutes at room temperature, and permeabilized with 1% Triton X-100 for 4 minutes. The cells were incubated with a vinculin antibody (Sigma-Aldrich) at the ratio of 1:50 and phalloidin-TRITC (0.5 μg/mL; Sigma-Aldrich) in 5% bovine serum albumin at 4°C overnight. The primary antibody was discarded, and the cells were washed 3 times with PBS. The cells were incubated with the appropriate second antibody (Alexa Fluor. 488; Invitrogen) for 30 minutes. After washing with PBS, the coverslips were mounted with mounting medium (Vectashield; Vector Laboratories). Cells were then observed and photographed under a confocal microscope (Zeiss LSM510, Carl Zeiss).

Cell transfection
The pre-miR miRNA precursor and control oligos were purchased from Invitrogen, and miRCURY LNA miRNA inhibitors and control oligos were purchased from Exiqon. For transient transfections, cells were plated 24 hours before transfection at 50% confluence. Plasmids and miRNA oligo transfections were conducted using FuGENE6 transfection reagent (Roche Applied Science) or Lipofectamine RNAiMAX (Invitrogen), respectively.

Real-time PCR
Total RNA was extracted using TRIZOL reagent (Invitrogen) and reverse-transcribed using a high-capacity RNA-to-cDNA kit (Applied Biosystems) under conditions recommended by the manufacturer. The cDNA was quantified by real-time PCR (RT-PCR) using an ABI Prism 7900 sequence detection system (Applied Biosystems). PCR was carried out using SYBR Green PCR core reagents (Applied Biosystems) according to the manufacturer’s instructions. PCR amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out for each sample as a control for sample loading and to allow normalization across samples. Primers are listed in Supplementary Table S1.

TaqMan miRNA assays
Expression of mature let-7 was analyzed using TaqMan miRNA assays (Applied Biosystems) under conditions recommended by the manufacturer. Briefly, single-stranded cDNA was synthesized from 5 ng of total RNA in a 15 μL reaction volume using a TaqMan microRNA reverse transcription kit (Applied Biosystems). The reactions were incubated first at 16°C for 30 minutes, at 42°C for 30 minutes, and then inactivated by incubation at 85°C for 5 minutes. Each cDNA generated was amplified by quantitative PCR using sequence-specific primers from the TaqMan microRNA assay on an 7900HT Sequence Detection System (Applied Biosystems). Each 20 μL PCR included 10 μL of 2× Universal PCR Master Mix (without AmpErase UNG), 1 μL of 20× TaqMan microRNA assay mix, and 2 μL of reverse transcription product. The reactions were incubated in a 384-well plate at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Western blot analysis
Cells were lysed using mammalian protein extraction reagent (Pierce Biotechnology). After quantification using a BCA protein assay kit (Pierce Biotechnology), 30 μg of total protein was separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore).
Membranes were blocked in 5% non-fat milk (Bio-Rad) and then incubated with an anti-PAK1, DIAPH2, RDX, or ITGβ8 primary antibody (1:1,000) followed by incubation with a secondary antibody conjugated with horseradish peroxidase (HRP; 1:2,000; Amersham Pharmacia Biotech) together with an HRP-conjugated primary antibody for actin (1:10,000; Sigma-Aldrich). The PAK1, RDX, and ITGβ8 antibodies were from Cell Signaling Technology. The DIAPH2 antibody was from Novus Biologicals. Protein quantification was conducted using ImageJ software (NIH, Bethesda, MD).

3′-UTR reporter assay
Cells were plated on a 24-well plate. A total of 30 nmol/L of the miRNA mimic was transfected using Lipofectamine RNAiMAX. At 24 hours after transfection, 0.125 μg per well of reporter plasmid was transfected using FuGENE6 transfection reagent. At 48 hours after transfection of the reporter plasmid, cells were harvested and reporter assays were conducted using a dual luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Renilla and firefly luciferase activities were measured using a Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific).

Lentiviral and retroviral transduction
miRZips lentiviral–based miRNA inhibition vectors and control vectors were purchased from System Biosciences. Lentiviral plasmids and packaging vectors were transfected into the packaging cell line 293T (ATCC) using FuGENE6 transfection reagent (Roche Applied Science). The medium was changed 8 hours posttransfection, and the medium-containing lentivirus was collected 48 hours later. MDA-MB-231 cells were infected with lentivirus in the presence of 8 μg/mL polybrene (Sigma-Aldrich). The retrovirus-based human miRNA expression vector (pmiRvec) was purchased from GeneService UK. For retroviral transduction, the PT67-pmi vector and PT67-pre-let-7b stable cells were cultured and the culture medium was collected. Then MDA-MB-231-luciferase cells were infected with retrovirus-containing medium with polybrene.

Transfection of siRNA
siRNA and control oligonucleotides were purchased from IDT. (The sequences are listed in Supplementary Table S2.) Transfections were conducted using the Lipofectamine RNAiMAX transfection reagent (Invitrogen) following the manufacturer’s instructions, and then cells were incubated in the media containing the transfection mixture for 72 hours.

In vivo metastasis assays
MDA-MB-231 cells stably expressing luciferase and the let-7b precursor were used for in vivo metastasis assays. The cells were trypsinized, washed with PBS, and then suspended in PBS at a concentration of 2.5 × 10⁶ cells/mL. A total of 0.2 mL cell suspension was injected into female nude mice through the tail vein (n = 6). The IVIS Lumina II Bioluminescence and Fluorescence Imaging System (Caliper Life Sciences) was used for in vivo bioluminescent imaging of MDA-MB-231-luciferase-pre-let-7b cells every week. Mice were injected intraperitoneally (i.p.) with 150 mg/kg body t-luciferin substrate (Gold Biotechnology). Pilot studies revealed that the peak bioluminescent intensity of tumors was reached at 10 to 15 minutes after t-luciferin injection. As such, 10 minutes were chosen for imaging. Images of the mice were taken under the following settings: exposure time = 60 seconds, f/stop = 16, medium binning, and field of view = 12.5 × 12.5 cm². Living Image software was used to quantify the bioluminescent signal, and this was reported as units of tissue radiance (photons/s/cm²/Sr). At 35 days after injection, the mice were sacrificed, and hematoxylin and eosin (H&E) staining of the lungs was conducted.

Statistical analysis
All data represent the mean ± SD. Statistical analysis was conducted using the Student t test at a significance level of P < 0.05.

Results
let-7b was reduced in patients with metastatic breast cancer and inhibits breast cancer cell migration and invasion
To explore the clinical significance of the let-7 family in breast cancer, we analyzed the expression of all let-7 family members using real-time RT-PCR in a total 118 clinical specimens, including 28 benign breast tumors and 90 invasive ductal carcinoma (IDC) specimens. The real-time RT-PCR results are summarized in Fig. 1A. In 7 of them (except let-7e), we found that the expression levels were remarkably reduced in IDCs compared with benign tumors (all P < 0.05; Fig. 1A). Moreover, the expression levels of let-7a, let-7b, and let-7g were significantly lower in the specimens from IDCs with lymph node metastasis (n = 52) than in those without lymph node metastasis (n = 38, all P < 0.01; Fig. 1A). For example, the median expression level of let-7b was reduced about 3.5-fold in IDCs with lymph node metastasis compared with IDCs without lymph node metastasis (P < 0.001; Fig. 1B and C). This result strongly suggested that decreasing of let-7 expression may be one of the important steps in breast cancer metastasis.

To functionally validate the above clinical observation, we examined the effect of let-7b on migration and invasion in breast cancer cells. We transiently transfected the let-7b mimic or a control into MDA-MB-231 cells. After 72 hours of transfection, the migration and invasion abilities of let-7b or control cells were examined by Transwell assays. We found that enforced let-7b expression significantly inhibited both MDA-MB-231 cell migration (43.06% ± 4.39%, P = 0.00049; Fig. 1D) and invasion (30.24% ± 19.7%, P = 0.012; Fig. 1E) in vitro. To further confirm this observation, we also carried out a loss-of-function experiment. Endogenous let-7b was specifically blocked using a let-7b inhibitor. We found that the let-7b inhibitor significantly increased MDA-MB-231 cell migration (226.31% ± 13.88%, P < 0.01; Fig. 1F) and invasion (195.68% ± 4.44%, P < 0.01; Fig. 1G) in vitro. These experiments were also repeated in other breast
cancer cell lines (Supplementary Fig. S1). Finally, we blocked maturation of all let-7 family members by stably introducing a nature let-7 inhibitory protein, LIN28, into MDA-MB-231 cells. Enforced LIN28 expression was confirmed by Western blot analysis (Supplementary Fig. S2A). As expected, LIN28 overexpression significantly reduced endogenous let-7 expression (including let-7b) in MDA-MB-231 cells (Supplementary Fig. S2B). We found that, consistent with the chemical synthesis let-7b inhibitor, overexpression of LIN28 significantly increased the migration ability of MDA-MB-231 cells (184.2% ± 4.94%, P < 0.01; Supplementary Fig. S2C) compared with control cells.

**Figure 1.** let-7b was reduced in patients with metastatic breast cancer and inhibits breast cancer cell migration and invasion. A, the heatmap of the expression levels of let-7 family in the benign breast tumor (n = 28) and invasive ductal carcinoma (n = 90) specimens. Expression of individual miRNAs was analyzed using a stem-loop real-time RT-PCR assay (TaqMan miRNA assay). B, summary of the let-7b expression between the benign breast tumors and the invasive ductal carcinomas (P < 0.001). C, summary of the let-7b expression between the invasive ductal carcinoma with (n = 52) and without (n = 38) lymph node metastasis (P < 0.001). D and E, MDA-MB-231 cells were transfected with the let-7b mimic or control (60 nmol/L). After 72 hours of transfection, the migration (D) or invasion (E) assays were conducted. F and G, MDA-MB-231 cells were transfected with the let-7b inhibitor or control (90 nmol/L). After 72 hours of transfection, the migration (F) or invasion (G) assays were conducted. The invasion data were normalized by cell proliferation rate. H, pre-let-7b or control was stably expressed in MDA-MB-231 cells by lentiviral infection. Luciferase image of MDA-MB-231 cell metastasis in vivo. MDA-MB-231 cells were i.v. injected into nude mice (6 animals per group). Images were photographed at different time points (2, 28, and 35 days). All data are presented as the mean ± SD. *P < 0.05; **P < 0.01. I, H&E staining of mouse lungs after 35 days following i.v. injection. Arrows indicate metastasis nodes. J, relative number of lung nodes in control and pre-let-7b groups. K, relative size of lung nodes in control and pre-let-7b groups. All data are presented as the mean ± SD. *P < 0.05.
Finally, we stably introduced a pre-let-7b expression vector (containing the genomic DNA of pre-let-7b and its flanking sequences) or a control vector into an MDA-MB-231 cell line that expressed a luciferase reporter. The mature let-7b expression and in vitro migration ability in the stable pre-let-7b–expressing cells was confirmed by real-time RT-PCR and Transwell assays (Supplementary Fig. S3A and S3B). Cells stably expressing the control or pre-let-7b vectors were injected into nude mice through the tail vein (i.v.). The in vivo luciferase activity was detected using an IVIS live imaging system at 2, 28, and 35 days postinjection. We found that enforced let-7b expression remarkably inhibited lung metastasis of MDA-MB-231 cells (Fig. 1H). At 35 days after tumor transplantation, the mice were sacrificed and lung tissues were collected. We found that the metastatic tumor nodes in the pre-let-7b group were remarkably reduced compared with the control group (Fig. 1I and J). Taken together, our results show that let-7b inhibited metastasis of breast cancer cells in vitro and in vivo.

let-7b inhibits breast cancer cell motility and regulates cytoskeleton dynamics

Cell motility is one of the most important factors that can affect both cell migration and invasion in cancer. Interestingly, we observed that the motility of MDA-MB-231 cells was remarkably increased by introducing the let-7b inhibitor (Supplementary Fig. S4A and Supplementary Videos SV1 and SV2). To quantify this observation, we conducted a wound-healing assay under a time-lapse microscope. The let-7b inhibitor and its control were transduced into MDA-MB-231 cells. We found that the motility of MDA-MB-231 cells was significantly increased by the let-7b inhibitor (Supplementary Fig. S4B and S4C and Supplementary Videos SV3 and SV4). Opposite results were observed in the let-7b gain-of-function study using a let-7b mimic (62.7% ± 29%, P < 0.01; Supplementary Fig. S5).

Importantly, we observed that the let-7b mimic dramatically changed the MDA-MB-231 cell shape. MDA-MB-231 cells transfected with the let-7b mimic spread significantly flatter and larger compared with the control cells, indicating that let-7 may regulate cytoskeleton dynamics. It has been shown that cytoskeleton dynamics can have remarkable effects on cell motility (20). The process of cell motility involves assembly and disassembly of focal adhesion and reorganization of actin stress fibers that end in focal adhesions (20). Therefore, our observation that let-7 affected cell shape suggested that let-7b might impact cytoskeleton dynamics and thereby influence cell motility and migration. To test this hypothesis, MDA-MB-231 cells were seeded onto glass coverslips precoated with fibronectin. Vinculin, a focal adhesion marker, and phalloidin-TRITC (F-actin) were used to monitor cytoskeleton dynamics in MDA-MB-231 cells. As shown in Fig. 2, transfection with the...
let-7b mimic inhibited the formation of lamellipodia or filopodia compared with control cells. Persistently stabilized stress fibers and flat cell shape were observed in let-7b mimic–transfected cells. Given previous reports by other groups that inhibition of dynamic changes in focal adhesions and stress fibers can prevent cell motility and migration (21, 22), our results indicated that let-7b might regulate cancer cell motility and migration by controlling cytoskeleton dynamics in cancer cells.

let-7b directly represses multiple genes involved in the actin cytoskeleton pathway

To characterize the mechanism of the let-7 family in the regulation of cytoskeleton dynamics, we analyzed downstream target pathways of let-7 using DIANA-mirPath, a bioinformatic program for the prediction of molecular pathways regulated by certain miRNAs (23). Consistent with our experimental observations, we found that the actin cytoskeleton pathway was indeed one of the pathways that may be significantly repressed by the let-7 family based on in silico prediction \( [-\ln(P)] = 1.1 \). In this pathway, 5 genes that could strongly regulate cytoskeleton dynamics in cancer cells, including PAK1, DIAPH2, RDX, ITGB8, and NWASP, were predicted to be repressed by let-7 (Fig. 3A). These in silico data strongly indicated that the let-7 family may regulate cell motility via direct repression of multiple proteins controlling cytoskeleton dynamics.

To experimentally validate this in silico finding, we first examined the expression levels of the above 5 proteins in let-7b mimic–transfected cells. MDA-MB-231 and MCF7 cells were transfected with the let-7b mimic or control oligo (both 60 nmol/L). Protein was isolated at 72 hours post let-7b mimic transfection. Using Western blot analysis, we found that 4 of 5 the predicted target proteins, including...
PAK1, DIAPH2, RDX, and ITGB8, were remarkably decreased by the let-7b mimic compared with the control (Fig. 3B and D). Moreover, we also transfected let-7b inhibitor or control oligo (90 nmol/L) to the same cell lines. Using Western blot analysis, we found that transfection of the let-7b inhibitor increased the expression levels of the above 4 proteins (Fig. 3C and E).

Next, we examined the mRNA expression of these genes in both let-7b mimic and inhibitor treatment experiments using real-time RT-PCR. The same transfection conditions as the protein experiments were used in the mRNA experiments. We found that the let-7 mimic significantly reduced mRNA levels of the above 4 genes compared with the control (Fig. 3F). A consistently opposite result in mRNA levels was observed in the cells transfected with the let-7b inhibitor (Fig. 3G).

Finally, we separately cloned the 3'-UTRs of PAK1, DIAPH2, RDX, and ITGB8 into the psiCHECK reporter construct. The reporter constructs containing mutant let-7b-binding sites (seed region) in the 3'-UTR of the above genes were also generated. Using reporter assays, we showed that transfection of the let-7b mimic significantly reduced luciferase activity in the wild-type but not the let-7b-binding site mutant 3'-UTR reporters of these 4 genes (Fig. 4A and B). Taken together, our results showed that let-7b regulates multiple genes in the actin cytoskeleton pathway.

Blocking the let-7 target genes in the actin cytoskeleton pathway inhibits migration induced by let-7b repression

It has been well shown that the above 4 novel let-7 target genes identified in our study control cytoskeleton dynamics and regulate cell migration (24–31). However, it is still unclear whether they are functionally involved in let-7 repression–induced migration in cancer cells. To address this question, we used siRNA to block the expression of these genes in MDA-MB-231 cells. The knockdown efficiency of each individual siRNA was first examined by real-time RT-PCR (Fig. 5A). To test whether these let-7 target genes can rescue let-7 repression–induced migration, we stably blocked endogenous let-7b expression.
by miRZips let-7b lentiviral infection, then cells were transfected with each individual siRNA (30 nmol/L). After 72 hours of siRNA transfection, the migration abilities of siRNA or control cells were examined by Transwell assays. We found that knocking down the expression of PAK1, DIAPH2, and RDX significantly inhibited migration induced by let-7b inhibitor (PAK1: 50.87% \pm 8.68%; DIAPH2: 61.36% \pm 2.22%; RDX: 18.6% \pm 0.64%; Fig. 5B and C). However, knocking down expression of ITGB8 did not remarkably affect let-7b inhibitor–induced migration (Fig. 5B and C). Next, we tested transfected siRNA cocktails (10 nmol/L for each siRNA), targeting PAK1, DIAPH2, and RDX, to control and let-7b inhibitor cells. We found that the siRNA cocktail indeed was able to block migration induced by let-7b inhibitor (Fig. 5D). Finally, we tested whether the effect of co-transfection of these 3 siRNAs was able to phenocopy the effect of transfection of let-7b mimic on cytoskeleton dynamics in MDA-MB-231 cells. MDA-MB-231 cells were seeded onto glass coverslips precoated with fibronectin. Vinculin, a focal adhesion marker, and phalloidin-TRITC (F-actin) were used to monitor cytoskeleton dynamics in MDA-MB-231 cells. As shown in Supplementary Fig. S6, transfection with the cocktail of three siRNAs reduced the formation of lamellipodia or filopodia and stabilized stress fibers and flattened cell shape compared with control cells. It was shown similar phenotype as the let-7b mimic transfection (Fig. 2). Taken together, our above results indicate the effect of down-regulation of let-7 on tumor cell motility may be mediated by multiple genes in the actin cytoskeleton pathway such as PAK1, DIAPH2, and RDX.
Discussions

miRNAs are small, endogenous, noncoding RNAs that regulate gene expression in a sequence-specific manner (32, 33). It has been well shown that miRNAs play a critical function in tumor metastasis. Both metastasis-promoting and -inhibiting miRNAs have been identified (34). let-7 is among the founding and best-understood miRNAs in the C. elegans genome, which times seam cell terminal differentiation (1, 35, 36). In human cancer, the let-7 family functions as an important tumor suppressor gene (2–4). It has been reported that expression levels of the let-7 family were globally reduced in tumors compared with normal corresponding tissue (2–4). In human, the let-7 family is composed of multiple members with overlapping or distinct functions (1), and 13 members of the let-7 family have been identified in the human genome (1, 3). We found that most let-7 family members, except let-7e, were remarkably down-regulated in IDC specimens compared with benign breast tumors. Importantly, lower expression levels of let-7 family members (let-7a, let-7b, and let-7g) were significantly associated with lymph node metastasis. Consistent with our findings, it has been reported that the expression levels of the let-7 family members were negatively correlated with tumor metastasis in melanoma (37), liver (38), colon (39), and breast (40, 41). Taken together, this strongly suggests that the let-7 family maybe serve as a critical metastasis-inhibitory miRNA in cancer, although the clinical significance for each individual let-7 family member as a biomarker for predicting tumor metastasis still needs to be further confirmed in large-scale validation studies.

The motility of tumor cells is an important parameter in the migration and metastasis cascade (42). In the present study, we found that inhibiting let-7b dramatically accelerated the motility of breast cancer cells, indicating that reduced let-7b expression in breast tumors may promote metastasis via increased migration. Importantly, we identified 5 genes in the actin cytoskeleton pathway that were let-7b predicted targets, and 4 of them (PAK1, DIAPH2, RDX, and ITGB8) were experimentally confirmed. Importantly, knocking down the expression of 3 of these genes including PAK1, DIAPH2 and RDX significantly blocked migration induced by let-7b inhibitor. PAK1 is a well-known regulator of cytoskeletal remodeling and cell motility. Pak1-depleted cells shows inhibited lamellipodial protrusion and fail to form mature focal adhesions (24). Overexpression of kinase-defective PAK1 in breast cancer cell was accompanied by persistent cell spreading, multiple focal adhesion points, and reduced invasiveness (25). DIAPH2 belongs to diaphanos subfamily of the formin homology family. These proteins are effectors of Rho GTPase that participates in stress fiber formation, cytokinesis, and transcriptional activation of the serum response factor (26). DIAPH2 and Cdc42 together can regulate microtubule attachment to kinetochores (27). RDX is a member of the ezrin–RDX–moesin (ERM) family. The ERM proteins play an important role in organizing membrane domains through their ability to interact with transmembrane proteins and the cytoskeleton (28, 29). RDX can regulate cell migration and invasion in many cancer cells (30, 31). The functions of these proteins in the actin cytoskeleton pathway were summarized in Fig. 4A. Our results suggest that let-7b inhibited breast cancer cell motility by regulating the above genes in the actin cytoskeleton pathway, which may be one of critical mechanisms involved in the inhibition of tumor metastasis by let-7.

Given each miRNA can target hundreds of transcripts (43, 44), the potential regulatory circuitry in cancer afforded by the let-7 family might be enormous. We believe that let-7 may directly or indirectly regulate multiple steps in the metastasis cascaded in cancer cells. In agreement with this hypothesis, several independent mechanisms involving let-7 in tumor metastasis have been recently reported. First, let-7 may reduce tumor cell migration by targeting ITGB3 (37) and MYH9 (45). Second, let-7 may inhibit local invasion by repressing genes that control the extracellular matrix surrounding tumor cells (38–41, 46). Third, let-7 may block the chemotactic response during tumor metastasis by directly targeting CCRT (47) and indirectly reducing CXCR4 (41). Fourth, let-7 may be involved in regulation of epithelial–mesenchymal transition in cancer mediated by targeting NEDD9 and DOCK3 (48). Finally, as a master tumor suppressor miRNA, let-7 may suppress metastasis by regulating critical oncogenic pathways that promote tumor metastasis, such as the RAS/MYC/HMG/2 axis (9, 41, 49, 50) and PAX3 (39). Taken together, the function of let-7 in the inhibition of tumor metastasis may be highly complex. Several key questions still need to be addressed: (i) How do these mechanisms cooperate together during tumor metastasis? (ii) Do these mechanisms function equally or do some of them play a predominant function in a certain cellular- or cancer-type context? (iii) How do these pathways interact with the other tumor suppression functions of let-7?

Given that let-7 simultaneously inhibits multiple molecular pathways involved in the most critical steps of the tumor metastasis cascade, replacement of let-7 expression in tumor cells provides a novel therapeutic strategy to treat metastatic disease, which results in more than 90% of the cases of mortality in patients with cancer. Moreover, let-7 also targets predominant oncogenes such as RAS, MYC, and HMG2 in differentiated tumor cells and induces cancer stem cell differentiation (2–4). Therefore, reconstituting let-7 expression has a strong potential for clinical application as a novel therapeutic strategy in breast cancer. Both lentivirus- and adenovirus-based let-7 gene therapy have been successfully tested in a genetically engineered mouse model for lung cancer (8, 15). Excitingly, a lipid-encapsulated let-7 mimic replacement therapy has been shown to significantly reduce lung cancer growth in vivo in preclinical trials following local or systematic nanoparticle delivery (16, 17). These studies strongly show that reconstituting let-7 expression can be used as an innovative therapeutic for the treatment of human cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
Authors' Contributions
Conception and design: X. Hu, L. Zhang
Development of methodology: X. Hu, J. Guo, L. Zheng, C. Li
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Hu, J. Guo, L. Zheng, J.L. Tanyi, S. Liang, C. Benedetto, M. Mistmedi, D. Katsaras
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Grant Support
This work was supported, in whole or in part, by NIH (R01-CA142776 and P50-CA83638-7951 Project 3 to L. Zhang, SK12HD00849 to J.L. Tanyi); Department of Defense (W81XWH-10-1-0082 to L. Zhang); Ovarian Cancer Research Fund (to L. Zhang). L. Zheng was supported by the China Scholarship Council.

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Received July 11, 2012; revised November 29, 2012; accepted December 20, 2012; published OnlineFirst January 21, 2013.

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