miR-7 Suppresses Brain Metastasis of Breast Cancer Stem-Like Cells By Modulating KLF4

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Abstract

Despite significant improvement in survival rates of patients with breast cancer, prognosis of metastatic disease is still dismal. Cancer stem-like cells (CSC) are considered to play a role in metastatic progression of breast cancer; however, the exact pathologic role of CSCs is yet to be elucidated. In this report, we found that CSCs ($CD24^-/CD44^+/ESA^+$) isolated from metastatic breast cell lines are significantly more metastatic than non-CSC populations in an organ-specific manner. The results of our microRNA (miRNA) profile analysis for these cells revealed that CSCs that are highly metastatic to bone and brain expressed significantly lower level of miR-7 and that this miRNA was capable of modulating one of the essential genes for induced pluripotent stem cell, KLF4. Interestingly, high expression of KLF4 was significantly and inversely correlated to brain but not bone metastasis-free survival of patients with breast cancer, and we indeed found that the expression of miR-7 significantly suppressed the ability of CSCs to metastasize to brain but not to bone in our animal model. We also examined the expression of miR-7 and KLF4 in brain-metastatic lesions and found that these genes were significantly down- or upregulated, respectively, in the tumor cells in brain. Furthermore, the results of our *in vitro* experiments indicate that miR-7 attenuates the abilities of invasion and self-renewal of CSCs by modulating KLF4 expression. These results suggest that miR-7 and KLF4 may serve as biomarkers or therapeutic targets for brain metastasis of breast cancer. *Cancer Res; 73(4); 1434–44. ©2012 AACR.*

Introduction

Breast cancer is becoming a curable disease when diagnosed at an early stage; however, the majority of cancer-related death is still attributed to metastatic disease. Despite this clinical importance, the exact pathologic process of tumor metastasis is as yet poorly understood, and deciphering the exact molecular mechanism of this process is of paramount importance to identify specific therapeutic targets for this devastating disease. It is generally believed that a series of mutations in multiple genes are needed for cancer cells to become metastatic; however, recent cancer stem cell theory, which still remains as a hypothesis, predicts that metastatic tumor cells are, by definition, cancer stem cells and that these cells may exist in tumor mass even at an early stage. This hypothesis well explains the occurrence of metastatic disease that is occasionally observed in early-stage patients. Cancer stem-like cells (CSC) are a minor population of tumor mass and considered to have abilities of tumor initiation, differentiation, and chemoresistance (1). In breast cancer, CSCs or tumor-initiating cells were first identified by using a combination of cell surface markers, $CD24^-/CD44^+/ESA\ (EpCAM)^+$ (2). More recently, aldehyde dehydrogenase 1 (ALDH1) has been identified as a new CSC marker, and the cells isolated by this marker partially overlap with previous identified CSC population (3). $CD44^+/CD24^-$ cells are readily detectable in metastatic pleural effusions, and signature generated from $CD44^+/CD24^-$ cells suggests a metastatic ability of this population (4, 5). Interestingly, $CD44^+/CD24^-$ population can be generated by induction of epithelial–mesenchymal transition (EMT) by treating breast epithelial cells with EMT-inducing agents such as TGF-β1 (6). It should be noted that EMT is considered to be a crucial event in the metastatic process that involves the acquisition of a migratory mesenchymal phenotype (7).

Recent active research in miRNA identified a series of this type of molecules that are involved in tumor progression in various tumors as oncogenes and tumor suppressors (8, 9). In breast cancer, miR-21, 155, 27, 96, 182, and 128 were identified as oncogenes, whereas miR-125, 205, 27, 17, 206, and 145 were found to be tumor suppressor genes, and their corresponding target genes were also defined (10–12). Therefore, miRNA network is considered to play critical roles in tumor initiation

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and progression. However, much less information is available for miRNA in cancer stem cells, particularly in relation to the metastatic ability of CSCs in breast cancer, and therefore, it is of significant interest to identify such miRNAs that may serve as novel therapeutic targets for metastatic breast cancer. Toward this goal, we isolated CSCs population ($CD24^-$/$CD44^+$/ESA^+) from metastatic breast cells and conducted miRNA profile analysis. We found that miR-7 was significantly downregulated in metastatic CSCs and specifically blocked brain metastasis in our animal model by modulating the KLF4 expression.

Materials and Methods

Cells and cell culture

We purchased MDA-MB-231 (MB231) and MCF7 breast cancer cell lines from American Type Culture Collection. The 231BoM-1833, 231BrM-2a, CN34, CN34-BoM2d, CN34-BrM2c, and MCF7-BoM2d cells were a kind gift from Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York). Firefly luciferase-labeled cells were generated by lentivirus infection. 293TN cells were obtained from System Bioscience. MB231, MB231-variant cells, MCF7, MCF7-BoM2d, and 293TN cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics. CN34 and its variant cells were cultured in Medium199 supplemented with 2.5% FBS, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 20 ng/mL EGF, 100 ng/mL cholera toxin, and antibiotics. The immortalized mouse brain microvascular endothelial cells (mBrEC) were supplied by Dr. Isaiah J. Fidler (M.D. Anderson Cancer Center, Houston, TX; ref. 13). mBrEC was maintained at 8% CO_2 at 33°C in DMEM with 10% FBS, 2 mmol/L of L-glutamine, 1 mmol/L of sodium pyruvate, 1% of nonessential amino acids, and 1% of vitamin mixture. Breast tumor cells that were directly transplanted to animal only one generation without *in vitro* culture were obtained from Conversant Biologics. Stem cell populations were isolated as described previously (14). Isolated CSCs were cultured in DMEM/F12 medium with 2% B27, 20 ng/mL EGF, 4 μg/mL insulin, and 0.4% of bovine serum albumin (BSA).

MicroRNA microarray profiling

Total RNAs were isolated from CSCs from MB231, 231BoM, and 231BrM cells using miRNeasy RNA isolation kit (Qiagen). MicroRNA expression profiling was determined by miRNA microarray analysis by using the human miRNA chip (LC Science, miRBase version 14.0). Clustering and its visualization were conducted using Cluster3.0 and TreeView softwares.

Real-time PCR and Western blotting

Total RNA was isolated from cells using TRIzoL reagent (Invitrogen) and from paraffin-embedded human tissue samples using miRNeasy FFEP RNA purification kit (Qiagen), respectively. We used TaqMan MicroRNA Reverse Transcription kit and High Capacity RNA-to-cDNA kit for miRNA and mRNA reverse transcription, respectively (Applied Biosystems). Quantitative PCR (qPCR) was conducted by using TaqMan Universal Master Mix II and TaqMan microRNA assays or TaqMan gene expression assays (Applied Biosystems). A human RNU48 and actin TaqMan probes were used as endogenous controls for miRNA and regular gene expressions, respectively. Western blotting was carried out using a general method with KLF4 antibody (Santacruz). Quantitative reverse transcriptase PCR (qRT-PCR) was conducted using the SYBR Green qPCR Kit (Fermentas) by using following primers: Actin F: TGAGACCTTCAACACCCCAGCCATG, Actin R: CGTAGATGGGCACAGTGTGGGTG, KLF4 F: GTCTTGAGGAAGTGCTGAGC, KLF4 R: ATCGTCTTCCCCTCTTTGGC.

Plasmids

The plasmids expressing hsa-mir-7-1 and hsa-mir-7-2 precursors in lentiviral pCDH-CMV-MCS-EF1-copGFP vector (System Bioscience) were supplied by Yin-Yuan Mo (University of Mississippi Medical Center). pSIN-KLF4-puro lentiviral plasmid was constructed by subcloning from pLM-mCherry-2A-KLF4 purchased from Addgene. KLF4 reporter plasmid, pMir-Report-KLF4-3′-UTR was a kind gift from Dr. Kenneth S. Kosik (University of California, Los Angeles, CA; ref. 15). Psin-miR7-2 plasmid was constructed by subcloning PCR product from pCDH-CMV-MCS-EF1-copGFP-miR7-2. Deletion of miR-7 binding core sequence on 3′-untranslated region (UTR) region of *KLF4* gene was achieved using overlap extension PCR methods (16) by using following primers: pMIR-REPORT-F: GTCCAAATTGCTCGAGTGAT, pMIR-REPORT-R: AGGCGATTAAGTTGGGTA, KLF4 mut1 F : ACTTTTCACACTCGATGAGGGAAG, KLF4 mut1 R : CTTCCCTCATCGAGTGTGAAAAGT, KLF4 mut2 F : AAATCTATATTTGATCAACATTTA, KLF4 mut2 R : TAAATGTTGATCAAA-TATAGATTT.

3′UTR Reporter assay

Ten nanograms of reporter plasmid and 200 ng of miR7 expression plasmid were cotransfected with 1 ng of phRG-TK *Renilla* luciferase internal control plasmid (Promega) into 293TN cells using Lipofectamine 2000 reagent (Invitrogen). After 24 hours, luciferase activities were measured by using dual-luciferase reporter assay system (Promega).

Transfection

For reporter assay, cells were transfected with Lipofectamine 2000. For knockdown of miR7, cells were transfected with Locked nucleic acid (LNA) targeting miR-7 (Exiqon) using RNAifectin reagent (Applied Biological Materials).

Matrigel invasion and transmigration assays

For Matrigel invasion assay, CSCs were labeled with Cell tracker green (Invitrogen) and 50,000 cells were seeded into Matrigel-coated Transwell insert (Corning) supplemented with DMEM with 10% serum. The bottom side of Transwells were filled with DMEM with 20% serum. For transmigration assay, 100,000 of mBrEC were seeded into Transwell insert (Corning, pore size 3 μm) and allowed to grow to confluence for 1 day. CSCs were labeled with Cell tracker green and 50,000 cells were seeded into Transwell inserts supplemented with DMEM with 10% serum. The bottom side of Transwells were filled with DMEM with 20% serum. After 24 hours, labeled cells were counted under the fluorescent microscope.

Animal experiments

For experimental metastasis assay, nude mice (7–8 weeks) were injected with 50,000 luciferase-labeled CSCs in PBS into left cardiac ventricle in a total volume of 100 μL. To confirm a successful injection, the photon flux from whole body of the mice was immediately measured using IVIS Xenogen bioimager (Caliper). The brain metastasis progression was monitored and the luminescence was quantified. At the endpoint of this study, whole brain was removed, incubated in RPMI-1640 medium with 0.6 mg/mL luciferin for 15 minutes, and photon flux was monitored.

Sphere formation assay

Metastatic variant of MCF7 (MCF7-BoM2d cells) was suspended in DMEM-F12 medium supplemented with 2% of B27 supplement, 0.4% BSA, 4 μg/mL insulin, 20 ng/mL basic fibroblast growth factor (bFGF), and 20 ng/mL EGF (Invitrogen). Cells were then seeded in 96-well Ultra-low attachment plates (Corning) as a density of 500 per well. Eight days later, mammospheres in the plate were counted under the microscope. For passage culture, MCF7-BoM2d cells were seeded in low-attachment 10 cm dish. After 8 days, mammospheres were collected by using 40 μm mesh cell strainer, trypsinized, and seeded in another 10 cm dish. This passage culture was repeated 4 times.

MTS assay

Two-thousand CSCs were seeded in a 96-well plate with DMEM medium with 1% FBS for 72 hours. After the incubation, cell proliferation was measured by the MTS dye method (Promega).

Statistical analysis

For *in vitro* experiments, *t* test or one-way ANOVA was used to calculate the *P* values. Wilcoxon rank-sum test was used to calculate the *P* value for *ex vivo* bioluminescence from brain and expression level of miR-7 and KLF4 in human specimens. The Kaplan–Meier method was used to calculate the survival rates and was evaluated by the log-rank test.

Results

miR-7 Is downregulated in metastatic CSCs

To identify miRNAs which are specific to metastatic CSCs, we first isolated CSCs population using well established markers, $CD24^-$, $CD44^+$, and ESA^+, from human breast cancer cell line MDA-MB231, and also from its variants, 231BoM and 231BrM. The latter 2 cell lines were established by Massague and colleagues as highly metastatic variant to bone and brain, respectively (35). These cells were examined for their tumor initiating ability by injecting them into mammary fat pad of nude mice. The results of our limiting dilution analysis confirmed that the isolated CSCs ($CD24^-/CD44^+/ESA^+$) population has significantly stronger ability of generating tumors compared with non-stem cell population (Table 1). We then examined the metastatic ability of these cells by implanting various doses of CSCs into mice via intracardiac injection. As shown in Table 1, we found that CSCs from 231BoM and 231BrM were more metastatic to bone and brain, respectively,

Table 1. Limiting dilution analysis for tumor incidence and metastasis of CSCs in nude mice

Strain	Population	10^4	10^3	10^2	10	CSC frequency (95% confidence interval)
Incidence of tumors/number of injected cells per injection						
MB231	Stem cells	6/6	5/6	2/6		(1/183–1/1,097)[a]
	Non-stem cells	1/2	0/2	0/2		(1/2,356–1/118,284)
231BrM	Stem cells		4/4	5/6	2/6	(1/19–1/110)[a]
	Non-stem cells		5/6	1/6	0/6	(1/236–1/1,374)
231BoM	Stem cells		7/7	7/7	0/1	(1/7–1/89)[a]
	Non-stem cells		2/4	0/4	0/4	(1/419–1/6,668)
Incidence of brain metastasis/number of injected cells per injection						
231BrM	10^5	10^4	10^3			Frequency of brain metastatic cell (95% confidence interval)
Stem cells	4/4	6/6	3/6			1/1425 (1/4,282–1/474)[a]
Non-stem cells	3/4	3/6	0/5			1/40,529 (1/112,528–1/14,598)
Incidence of bone metastasis/number of injected cells per injection						
231BoM	10^5	10^4	10^3			Frequency of bone metastatic cell (95% confidence interval)
Stem cells	6/6	4/5	1/5			1/5,802 (1/15,427–1/2,182)[a]
Non-stem cells	2/6	0/5	0/5			1/274,471 (1/1,094,044–1/68,859)

NOTE: CSCs isolated from MB231, 231BoM and 231BrM were injected subcutaneously into the mammary fat pad of nude mice, and the growth of tumors was monitored by BLI. CSCs isolated from 231BoM and 231BrM were injected intracardially into the nude mice, and the growth of brain and bone metastasis was monitored by BLI.
[a]$P < 0.0001$.

compared with non-stem cells. These results strongly support our notion that metastatic cells derive from CSCs and they metastasize in an organ-specific manner.

Next, we conducted miRNA profile analysis of CSCs prepared from MDA231, 231BoM, and 231BrM by extracting RNAs from these cells followed by hybridizing them to a microRNA chip (LC Science), which contains 894 human microRNAs. The results of our array analysis revealed that there are 8 miRNAs whose expressions were significantly altered among these CSCs, and that 2 miRNAs, miR-7 and miR-21, were significantly reduced in both 231BoM and 231BrM compared with MDA-MB231 (Fig. 1A). We then confirmed the expression of miR-7 and miR-21 in CSCs of these cells by TaqMan qPCR and found that only miR-7 was significantly downregulated in CSCs in both 231BoM and 231BrM, suggesting that miR-7 may contribute to the metastatic ability of CSCs (Fig. 1B). To further clarify this notion, we examined the specificity of miR-7 expression in CSCs and non-stem cell population from MDA-MB231, 231BoM, and 231BrM. As shown in Fig. 1C, the expression of miR-7 was significantly reduced in CSCs compared with non-stem cells in both 231BoM and 231BrM, but not in CSCs of MDA-MB231, strongly suggesting that miR-7 is specific to metastatic CSCs. The generality of this observation was further confirmed by examining the expression of miR-7 in 2 different cell lines, CN34 and MCF7, and their metastatic variants, CN34BoM, CN34BrM, and MCF7BoM. Again, we found that the expression of miR-7 was significantly suppressed in the bone and brain metastatic variants of these cells in a CSC-specific manner (Fig. 1C). We also found that the expression of miR-7 was significantly lower in mammospheres of MCF7BoM compared with the parental cells, suggesting that miR-7 plays a role of self-renewal of CSCs (Fig. 1D). Indeed, we

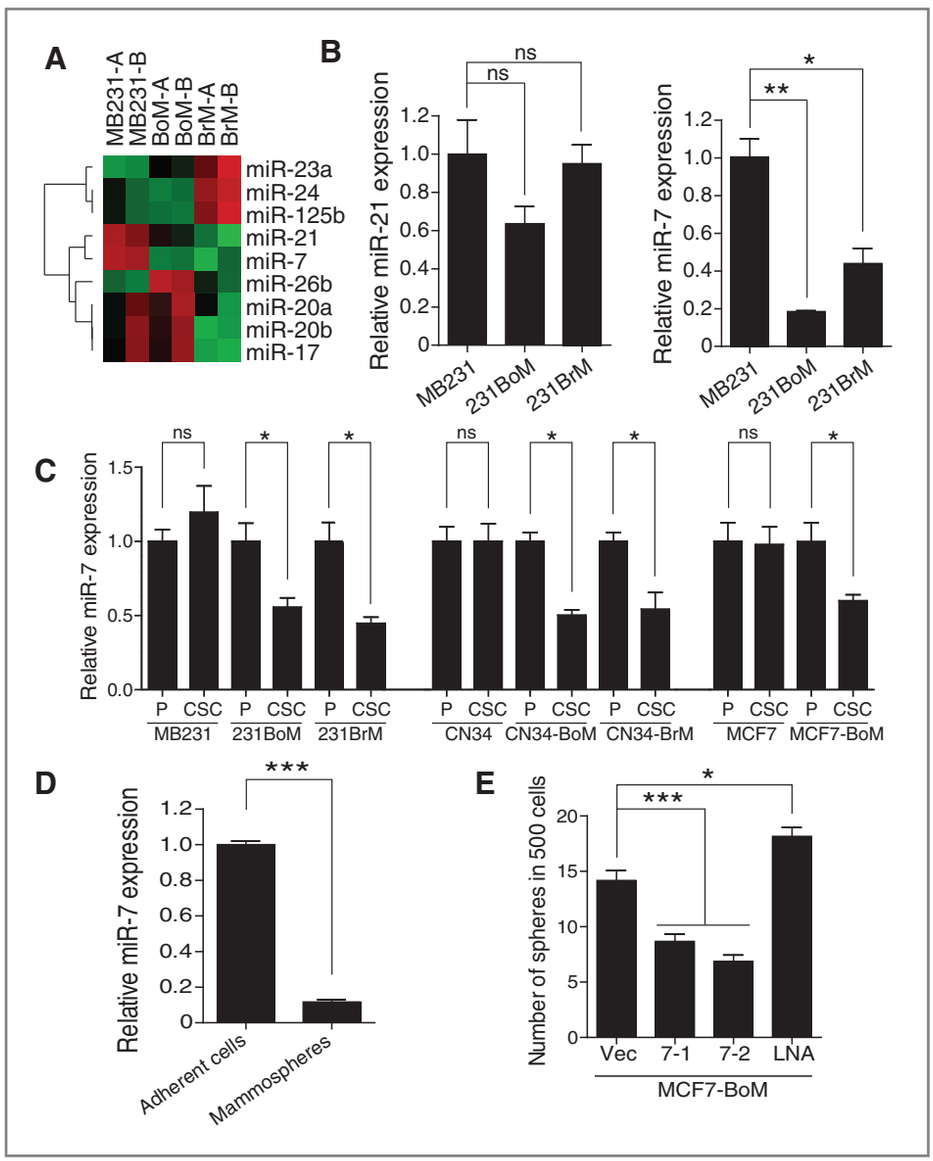

Figure 1. miR-7 is downregulatedin cancer stem-like cells (CSCs). A, CSCs from MB231, 231BoM, and 231BrM were isolated, and their RNAs were subjected to miRNA array analysis. A heatmap was generated for the MicroRNAs that were significantly up- or downregulated in CSCs among 231BrM and 231BoM compared with MB231. B, the expression of miR-21 and miR-7 were examined by TaqMan PCR in CSCs prepared from MB231, 231BoM, and 231BrM. C, miR-7 expressions in both parental cells and CSCs from different cell lines were measured by TaqMan PCR. P, unsorted parental cells; CSC, cancer stem cell. D, the expression of miR-7 was examined in adherent cells and mammospheres derived from MCF7-BoM cells by TaqMan PCR. E, Sphere-forming ability was measured in MCF7BoM cells that are ectopically expressing miR-7 or miR7-LNA. Data are represented as mean ± SEM ($n = 3$). ***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.05$; ns, not significant.

found that ectopic expression of miR-7 significantly suppressed the ability of sphere formation of MCF7BoM cells, whereas miR7-specific LNA enhanced the sphere-forming ability (Fig. 1E).

miR-7 Suppresses the expression of *KLF4* gene

To understand the functional role of miR-7 in CSCs, we first searched its potential target genes that may be involved in stem cell physiology using multiple database including TargetScan, PicTar, miRanda, and SLOAN-Kettering. We found that KLF4, which is one of the IPS genes, is among the highest scored genes on the target list and that there are 2 potential miR7 target sites in 3′ UTR region of this gene. The target site 1 is conserved in mammals, whereas the target site 2 is well conserved among vertebrates (Fig. 2A). To examine the effect of miR-7 on the expression of KLF4, we ectopically expressed miR-7-1 or miR-7-2 in 231BoM and 231BrM cells by infecting corresponding lentivirus expression vector followed by qRT-PCR analysis. As shown in Fig. 2B, we found that both miR-7-1 and miR-7-2 significantly suppressed the expression of KLF4 in these cells, and the similar suppressive effect of miR-7 was observed when we used CN34BoM, CN34BrM, and MCF7BoM cells. These results were also confirmed by Western blot analysis (Fig. 2C). To further validate our results, we cotransfected lentiviruses carrying miR-7 expression vector and the luciferase reporter plasmid with 3′-UTR of KLF4 into 293TN cells. As shown in Fig. 2D, the reporter activity of 3′UTR of KLF4 was significantly suppressed by miR-7, whereas cotransfection of LNA targeting miR-7 stimulated the expression of this gene. We then introduced mutations in the 2 target sequences of 3′UTR, either alone or in combination, and examined their abilities to respond to miR-7. We found that the deletion of target site 1 (MT1) partially inhibited the suppressive effect of miR-7, whereas the deletion of the target site 2 (MT2) or a combination of both sites almost completely attenuated the effect of miR-7 (Fig. 2E). These results strongly suggest that miR-7 is capable of suppressing the expression of KLF4 by binding to these target sequences of 3′-UTR.

miR-7 Suppresses brain metastasis of CSCs *in vivo*

The results of our *in vitro* experiments strongly suggest that miR-7 plays a role in metastatic CSCs by modulating the expression of KLF4. Interestingly, when we analyzed the relationship between KLF4 and metastatic status of breast cancer using the existing GEO database for combined 710 patients (GSE12276, GSE2034, GSE2603, GSE5327, and GSE14020), we found that the expression of KLF4 was significantly and inversely correlated with brain but not bone metastasis-free survival (Fig. 3A). To validate this observation, we next examined the effect of miR-7 in metastatic ability of CSCs *in vivo*.

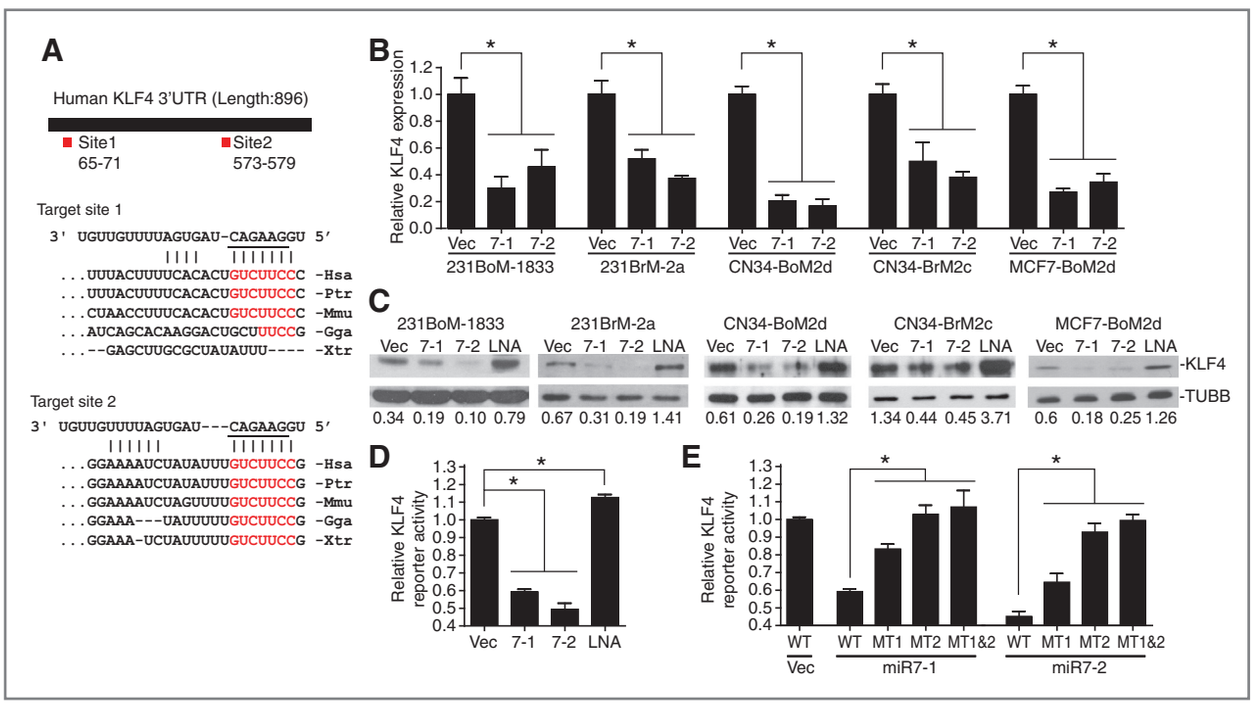

Figure 2. miR-7 directly targets KLF4. A, schematic representation of the KLF4 3′-UTR with 2 miR-7 binding sites and sequence alignment of predicted miR-7 binding sites on KLF4 3′-UTR. Complementary sequences of miR7 to mammalian KLF4 3′-UTR are shaded red. Hsa, human; Ptr, chimp; Mmu, mouse; Gga, chicken; Xtr, frog. B, 231BoM-1833, 231BrM-2a, CN34-BoM2d, CN34-BrM2c, and MCF7-BoM2d were infected with lentivirus expressing pri-miR-7-1, pri-miR-7-2, or vector only and the KLF4 mRNA levels were measured by RT-PCR. C, Western blotting analysis of KLF4 in the same set of CSCs as described in (B). D, KLF4 3′-UTR luciferase reporter plasmid was transfected in 293TN cells with indicated miRNAs or LNA. The luciferase activities were then measured after 24 hours of transfection. E, constructs carrying KLF4 3′ UTR luciferase reporter or deletion mutant of miR7 binding sites (site 1; MT1, site 2; MT2) were transfected in 293TN cells with indicated miRNAs. Cells were harvested and luciferase activities were measured after 24 hours transfection. *, $P < 0.05$.

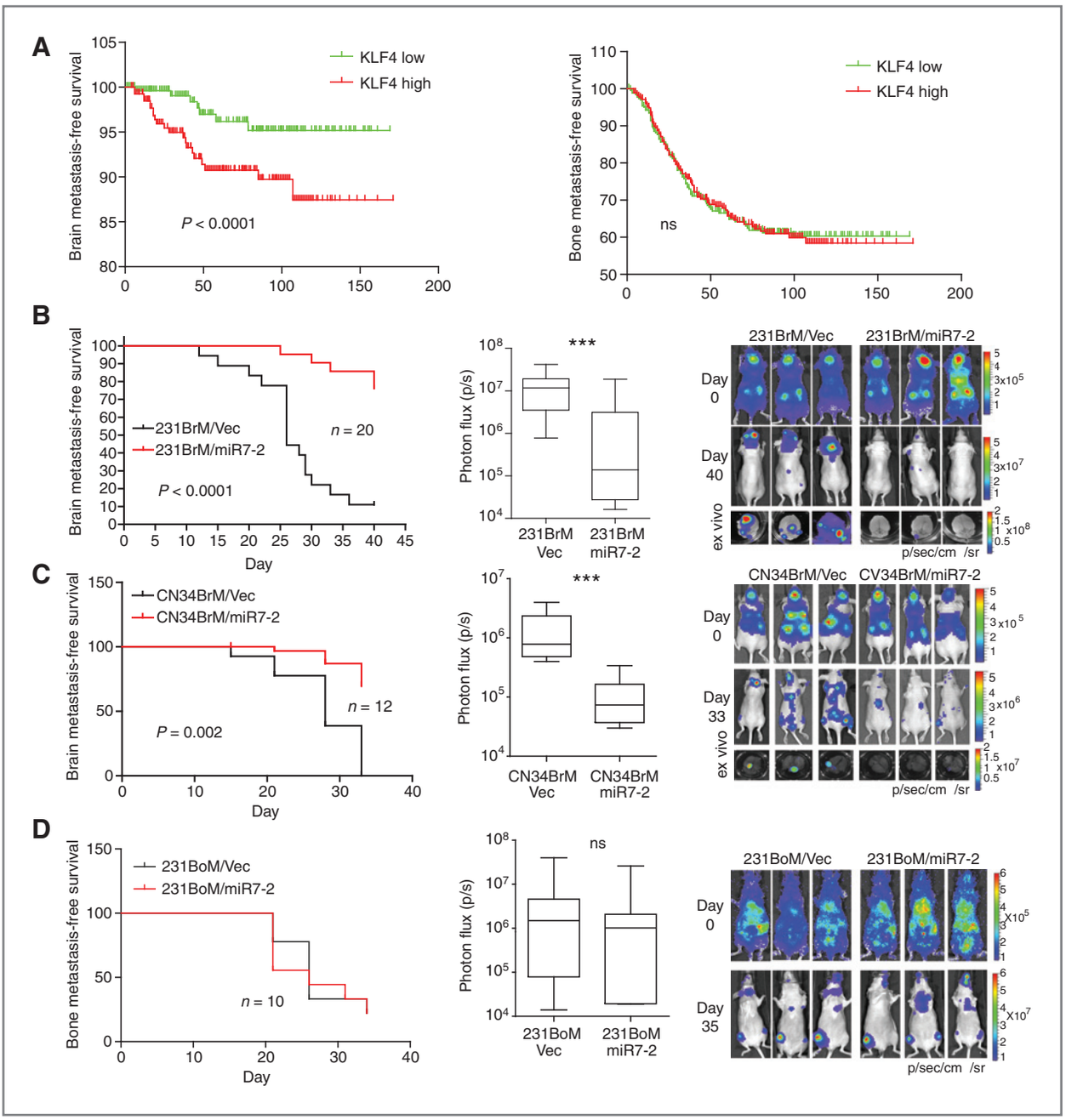

Figure 3. miR-7 suppresses brain metastasis *in vivo*. A, Kaplan–Meier analysis for brain and bone metastasis-free survival of 710 patients with breast cancer in GEO databases (GSE12276, GSE2034, GSE2603, GSE5327, and GSE14020). Patients were divided into 2 groups based on the expression status of KLF4 in their primary tumors. B, left, Kaplan–Meier analysis for brain metastasis-free survival of mice inoculated with CSCs that were isolated from 231BrM/Vec or 231BrM/miR7-2. Middle, total photon flux of brain metastatic lesions was measured by BLI at the end point. Right, BLI images of brain metastatic lesions of 3 representative mice from each experimental group. C, left, Kaplan–Meier analysis for brain metastasis-free survival of mice inoculated with CSCs that were isolated from CN34BrM/Vec or CN34BrM/miR7-2. Middle, total photon flux of brain metastatic lesions was measured by BLI at the end point. Right, BLI images of brain metastatic lesions of 3 representative mice from each experimental group. D, left, Kaplan–Meier analysis for brain metastasis-free survival of mice inoculated with CSCs that were isolated from 231BoM/Vec or 231BoM/miR7-2. Middle, total photon flux of bone metastatic lesions was measured by BLI at the end point. Right, BLI images of bone metastatic lesions of 3 representative mice from each experimental group. ***, indicates $P < 0.0001$.

CSCs were prepared from 231BrM and CN34BrM cells that were infected with lentivirus carrying with or without miR-7-2, and they were transplanted into nude mice through intracardiac injection followed by monitoring metastatic tumor growth in the brain. As shown in Fig. 3B and C, bioluminescent image right after the tumor injection showed distribution of

tumor cells in the whole body indicating a successful intracardiac injection. After 5 weeks, metastatic tumor growth in the brain was prominent in all mice that received CSCs carrying vector only. On the other hand, animals that received CSCs carrying miR-7-2 showed significantly less signals in the brain, suggesting that miR-7-2 was indeed capable of suppressing brain metastasis of CSCs (Fig. 3B and C). We then conducted a similar experiment using the CSCs prepared from the bone metastatic cell line, 231BoM and MCF7BoM, which carried lentivirus with or without miR-7-2. Interestingly, however, we found that miR-7-2 did not affect the incidence or growth of bone metastasis (Fig. 3D and Supplementary Fig. S1A). We also found that overexpression of miR7 in 231BrM cells decreased the tumor size by around 30%, and this effect was blocked by ectopic expression of KLF4, indicating that miR7 does affect primary tumor but not to the level of metastasis (Supplementary Fig. S1B). Ectopic expression of KLF4 alone in 231BrM did not affect the primary tumor growth *in vivo* and this is due to the fact that the endogenous level of KLF4 in 231BrM cells is already quite high. These observations are consistent with the results of clinical cohort analysis in Fig. 3A and strongly support the notion that miR-7-2 specifically suppresses brain metastasis by downregulating KLF4.

miR-7 Blocks invasion and proliferation of CSCs

To further understand the effect of miR-7 and KLF4 on tumor metastasis, we examined the transmigrating ability of CSCs of 231BrM through brain endothelial cells that were originated from mouse brain blood vessel. As shown in Fig. 4A, we found that miR-7 significantly suppressed transmigration of CSCs of 231BrM, whereas LNA that was targeted to miR-7 significantly stimulated the transmigrating ability of CSCs from MB231. We also examined the invasive ability of these cells using Matrigel invasion chamber and found that miR-7 was also able to block the invasion of CSCs through Matrigel (Fig. 4A, right). We then tested the effect of KLF4 on transmigrating and invasive ability of CSCs using the same assay systems. As shown in Fig. 4B, suppressive ability of miR-7 on

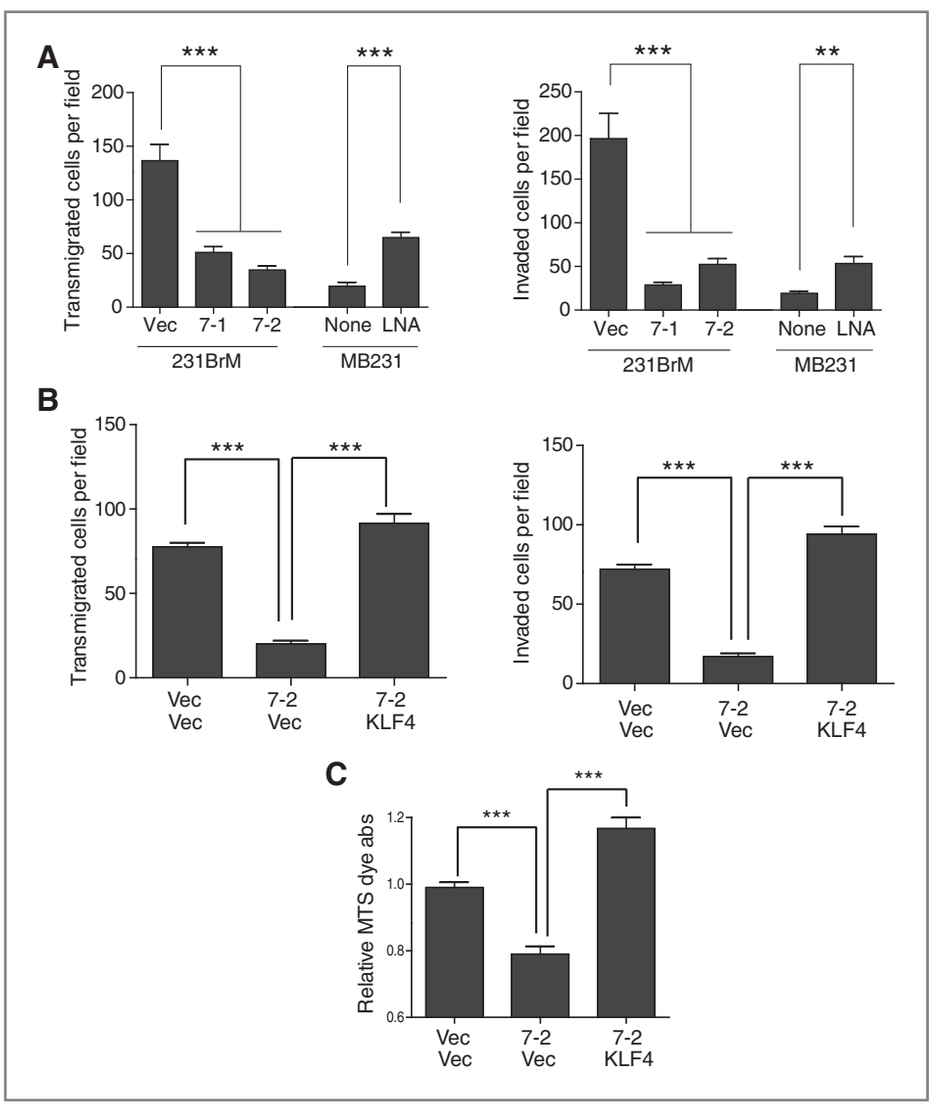

Figure 4. miR7 Suppresses the metastatic ability of 231BrM through inhibition of KLF4. A, CSCs isolated from 231BrM and MB231 with the ectopic expression of miR7 and miR7-LNA were seeded on top of mouse brain endothelial cells or Matrigel. The number of invaded cells was counted after 24 hours. B, CSCs isolated from 231BrM infected with lentivirus of miR7 or KLF4 expression plasmids were seeded on top of mouse brain endothelial cells or Matrigel. The number of invaded cells was counted after 24 hours. C, cell growth was measured by MTS assay in the CSCs isolated from 231BrM infected with lentivirus of miR7 or KLF4 expression plasmids. ***, $P < 0.0001$; **, $P < 0.001$.

both transmigration through endothelial cells and Matrigel was significantly attenuated by ectopic expression of KLF4, suggesting that miR-7 suppresses invasive ability of CSCs by blocking KLF4 expression. Next, because KLF4 is involved in self-renewal of stem cell, we also examined the effect of both miR-7 and KLF4 on proliferation of CSCs by MTS assay and found that miR-7 was capable of blocking the growth of CSCs, whereas ectopic expression of KLF4 significantly mitigated this effect of miR-7 (Fig. 4C).

KLF4 Promotes self-renewal of CSCs

One of the most important features of CSCs is their ability to maintain undifferentiated status and self-renewal. To evaluate whether KLF4 regulates the self-renewal of CSCs, we conducted fluorescence-activated cell sorting (FACS) analysis of 231BrM cells with or without ectopic expression of KLF4 followed by sorting the cells using the CSCs markers (CD24, CD44, and ESA). As shown in Fig. 5A, ectopic expression of KLF4 indeed significantly increased CSCs population in 231BrM cells. We also found that KLF4 significantly enhanced the mammosphere-forming ability of 231BrM cells, which is another hallmark of CSCs (Fig. 5B). To further validate our result in clinical samples, we obtained primary tumor from patients with advanced breast cancer and the tissue was passaged once in nonobese diabetic/severe combined immunodeficient mouse without *in vitro* culture. The tumor cells were dissociated and the cells were infected with KLF4 lentivirus and cultured in low-attachment plates. We then measured the population of CSCs by FACS after 72 hours as well as their mammosphere-forming ability by counting the number of spheres after 10 days. As shown in Fig. 5C and D, we again found that KLF4 significantly enriched the CSCs populations and mammosphere-forming ability. On the other hand, overexpression of miR7 significantly decreased the mammosphere-forming ability of primary breast tumor cells (Fig. 5D).

The expressions of miR-7 and KLF4 are inversely correlated in brain metastatic lesions of breast cancer patients

To further validate the clinical significance of miR-7 and KLF4 in brain metastasis of breast cancer, we microdissected tumor tissues from both primary and brain metastatic lesions followed by conducting TaqMan qRT-PCR. We found that the expression of miR-7 was significantly lower in brain metastatic tumors than that in primary tumor tissues (Fig. 6A, left), whereas the expression of KLF4 was significantly higher in brain metastatic tissue compared with primary tumors (Fig. 6A, right). The results of correlation analysis between miR-7 and KLF4 indicate that there was a significant inverse correlation between these 2 genes in brain metastatic tissues (Fig. 6B). Furthermore, we examined the expression of KLF4 in brain metastatic tissues by immunohistochemistry and found that KLF4 was expressed more in the nucleus and significantly less in cytosol in brain metastatic tissues compared with primary tumors (Fig. 6C and D). These clinical data are consistent with

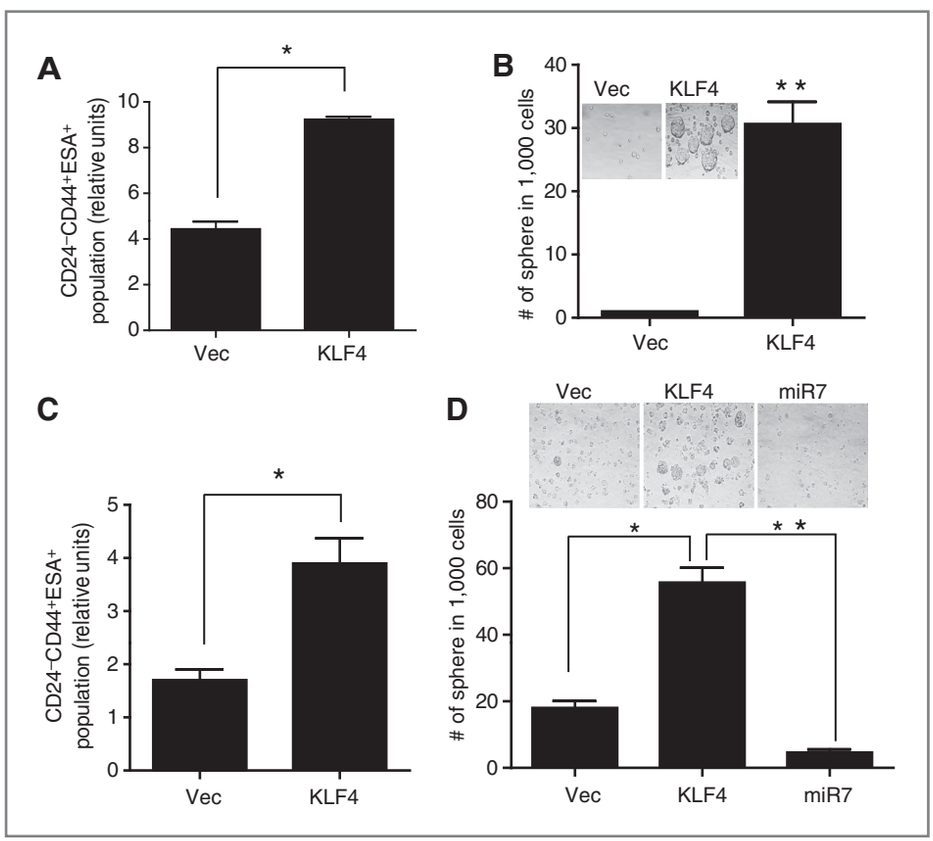

Figure 5. KLF4 promotes self-renewal of CSCs. A, 231BrM was infected with KLF4 or control lentivirus and the CSC population (CD24- CD44+ ESA+) was measured by FACS after 72 hours. B, CSCs were isolated from 231BrM infected with KLF4 or control lentivirus and cultured in low-attachment plates. The number of mammospheres was counted after 10 days. C, primary breast cancer cells isolated from patients with advanced cancer were directly infected with KLF4 or control lentivirus, and the CSC population (CD24− CD44+ ESA+) was measured by FACS after culturing the cells for 72 hours in low-attachment plates. D, the same primary breast cancer cells infected with KLF4 or control lentivirus were cultured in low-attachment plates and the number of mammospheres was counted after 10 days. *, $P < 0.05$; **, $P < 0.001$.

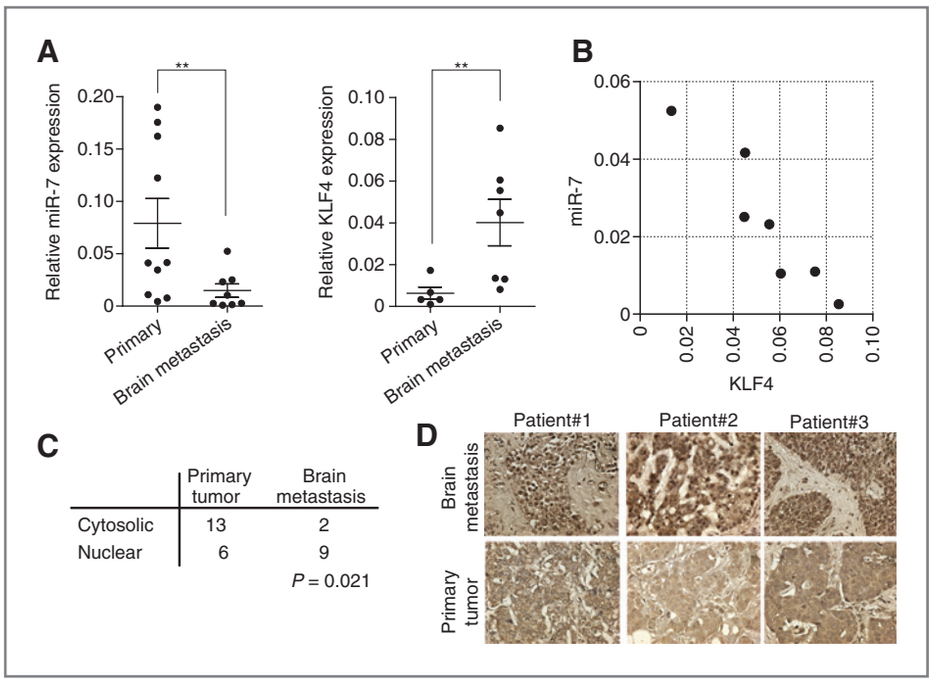

Figure 6. miR-7 and KLF4 are down- and upregulated, respectively, in brain metastatic lesion of patients with breast cancer. A, expression of miR-7 and KLF4 was measured by TaqMan PCR in primary and brain metastasis samples after microdissection. The relative expression level of miR-7 to RNU48 ($n = 8$–10/group) and the expression level of KLF4 to ACTB were plotted ($n = 7$–10/group). B, correlation of the miR-7 and KLF4 expression in each sample. C, immunohistochemical analysis for KLF4 expression was conducted for primary breast tumors and brain metastatic tumors, and the numbers of nuclear or cytosolic staining were assessed under microscope. D, representative photos of KLF4 staining in primary tumor and brain metastasis samples. **, $P < 0.001$.

our notion that miR-7 suppresses brain metastasis through inhibition of KLF4 expression.

Discussion

According to the recent cancer stem cell theory, metastatic cells are considered to have the typical characteristics of CSCs including self-renewal capability in addition to their invasiveness, and they also need to adapt themselves to the distant organs that have totally different microenvironment from the primary tumors (17, 18). Therefore, it is plausible that there are distinct populations of metastatic CSCs that can proliferate in the niche of specific distant organs. In this report, we indeed showed that CSCs isolated from both 231BoM and 231BrM have strong tumor-initiating abilities; however, they preferentially metastasize to bone and brain, respectively, despite the fact that these cells were isolated using the same markers ($CD24^-/CD44^+/ESA^+$). In addition, these metastatic CSCs showed significantly lower level of miR-7 and higher expression of KLF4 compared with CSCs from less metastatic parental cells. These results suggest an existence of wide range of heterogeneous population of CSCs although they still share the self-renewal ability and same surface markers. This is also exemplified by the fact that CSCs from different organs have been isolated by using different markers (1, 19). While these observations add further complexity to cancer stem cell biology, it opens a window of opportunity to identify specific targets for metastatic and recurrent diseases.

Mature miR-7 is individually transcribed and processed from 3 different gene locus in human genome, and it is highly expressed in parts of the brain, eye, and pancreas, suggesting its role in the development of these organs (20–22). On the other hand, aberrant expression of miR-7 was observed in glioblastomas, and it has been characterized as a putative tumor suppressor by targeting EGFR and AKT (23, 24). In breast cancer, miR-7 was shown to block invasiveness and tumorigenic potential by targeting PAK, which is consistent with the idea of tumor suppressor function of miR-7 (25). Interestingly, Bos and colleagues recently reported that EGFR and its ligand were highly expressed in brain metastatic cells and knockdown of EGFR ligands significantly attenuated cell motility and invasion *in vitro* and in *vivo*. Notably, inhibition of EGFR with cetuximab also inhibited brain metastasis in mice (35). However, another report found that miR-7 expression correlated with poorer prognosis of breast cancer, and these apparently conflicting results suggest that miR-7 may be differentially expressed in a specific cell population of breast cancer (26). We found that miR-7 is indeed downregulated specifically in highly metastatic CSCs to brain and bone but not in CSCs isolated from the parental cell (MDA-MB231), suggesting that miR-7 has a specific function in metastatic CSCs in addition to its tumor suppressor activity. In fact, we found that miR-7 was downregulated in mammosphere, a hallmark of self-renewal of CSCs, and that ectopic expression of miR-7 significantly reduced the ability in mammosphere formation. How miR-7 attenuates CSCs is a clinically relevant question. A range of potential targets of miR-7 were reported including EGFR, IRS-1, PAK-1, RAF-1, and SATB1 that are involved in key signaling of tumor progression (23, 25). In this report, we identified KLF4 as a critical downstream target of miR-7 in CSCs. KLF4 is one of IPS genes that are required for maintenance of stemness of progenitor cells (27, 28). Interestingly, Yu and colleagues recently showed that knockdown of KLF4 decreased the proportion of cancer stem cells, whereas overexpression of this gene led to an increase in the cancer stem cell population, suggesting that KLF4 plays a strong oncogenic role in

mammary tumorigenesis likely by maintaining stem cell-like futures of breast cancer cells (29). In support of this notion, KLF4 has been found to be frequently overexpressed in 70% of breast cancers, and its localization in the nucleus of breast cancer cells has been identified as a marker of aggressive phenotype in early-stage infiltrating ductal carcinoma (30). On the contrary, the expression level of KLF4 was downregulated in the gastrointestinal cancer due to the promoter methylation, and overexpression of this gene was shown to attenuate the tumorigenicity of colonic and gastric cancer cells *in vivo* (31, 32). Therefore, the clinical picture of KLF4 is complex and context dependent. It should be noted that KLF4 is a transcription factor that can both activate and suppress genes that are related to the proliferation and differentiation. In fact, KLF4 blocks senescence and apoptosis by repressing transcription of P53, whereas it can activate P21-dependent cell-cycle arrest, and therefore, KLF4 can function both as a tumor suppressor and an oncogene (33, 34).

How the miR7-7/KLF4 pathway specifically links to brain metastasis is an intriguing question. The result of our cohort data analysis including 710 patients with breast cancer clearly indicates significant correlation of KLF4 expression with brain but not bone metastasis-free survival. Our TaqMan PCR analysis also revealed that the expression of miR-7 and KLF4 are significantly down and upregulated, respectively, in brain metastatic lesions of patients with breast cancer and that their expressions are inversely correlated. Importantly, ectopic expression of miR-7 significantly blocked the brain metastasis but not bone metastasis in our animal model. The downregulation of MiR-7 expression and hence activation of *KLF4* gene is specific to metastatic CSCs but not to organ tropism of metastasis. Therefore, the brain specificity of miR-7/KLF4 is likely due to the downstream targets of this pathway or related to microenvironmental factors of brain. In this context, it should be noted that Bos and colleagues recently identified a brain-metastatic signature which includes 17 specific genes based on comprehensive expression array analysis of patients with breast cancer (35). Interestingly, the results of our promoter analysis for all these genes revealed that 9 of 17 genes (COL13A1, CSF3, FSCN1, HG18, LTBP1, PELI1, PLOD2, SCNN1A, and TNFSF10) have perfectly matched consensus sequences of KLF4 binding site on the promoter regions, suggesting a potential possibility of controlling these genes by KLF4 although it is not clear whether these genes are cause or consequence of brain metastasis at this point. Several other targets of KLF4 have been identified including TGF-β and Notch (36, 37) and these genes are known to be involved in stem cell self-renewal and tumor progression. Our results also indicate that miR-7 significantly inhibits mammosphere formation, whereas KLF4 promotes self-renewal of CSCs, suggesting that miR-7/KLF4 pathway is critical in CSC physiology in conjunction with TGF-β and Notch. Furthermore, it is becoming clear that CSCs require niche for their growth and they need to either adapt themselves to the existing niche or to generate their own niche in the distant organs that have totally different microenvironment from the primary tumor (38). The brain consists of highly specialized cells such as neuron, glia, and astrocytes. Therefore, miR-7/KLF4 pathway may facilitate a reciprocal interaction of CSCs and these brain cells that generates a suitable niche for CSCs, although this hypothesis needs to be experimentally tested. Nevertheless, our results indicate that miR-7/KLF4 axis may serve as a potential therapeutic target for brain metastasis of breast cancer, which is almost always incurable at the present time.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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