Metastatic Heterogeneity of Breast Cancer Cells Is Associated with Expression of a Heterogeneous TGFβ-Activating miR424–503 Gene Cluster

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Abstract

TGFβ signaling is known to drive metastasis in human cancer. Under physiologic conditions, the level of TGFβ activity is tightly controlled by a regulatory network involving multiple negative regulators. At metastasis, however, these inhibitory mechanisms are usually overridden so that oncogenic TGFβ signaling can be over-activated and sustained. To better understand how the TGFβ inhibitors are suppressed in metastatic breast cancer cells, we compared miRNA expression profiles between breast cancers with or without metastasis and found that the miR424–503 cluster was markedly overexpressed in metastatic breast cancer. Mechanistic studies revealed that miR424 and miR503 simultaneously suppressed Smad7 and Smurfl2, two key inhibitory factors of TGFβ signaling, leading to enhanced TGFβ signaling and metastatic capability of breast cancer cells. Moreover, antagonizing miR424–503 in breast cancer cells suppressed metastasis in vivo and increased overall host survival. Interestingly, our study also found that heterogeneous expression of the miR424–503 cluster contributed to the heterogeneity of TGFβ activity levels in, and metastatic potential of, breast cancer cell subsets. Overall, our findings demonstrate a novel mechanism, mediated by elevated expression of the miR424–503 cluster, underlying TGFβ activation and metastasis of human breast cancer. Cancer Res; 74(21):1–12. ©2014 AACR.

Introduction

Breast cancer is second only to lung cancer as the cause of cancer-related deaths in women, and the vast majority of breast cancer–related deaths are due to metastatic diseases (1). It is well established that cancer metastasis, a complex, multistep process, is driven, promoted, and modulated by aberrantly deregulated cellular signals (2). Numerous signaling pathways, such as the Myc, β-catenin, and TGFβ pathways, have been identified to play key roles in breast cancer metastasis (3–5). Among these metastasis-associated cellular signal transduction pathways, the TGFβ cascade is well documented in mediating many of the prometastatic steps (5). High levels of TGFβ or overactivated TGFβ receptors have been linked to the invasiveness and metastasis of breast cancer cells (6, 7). In contrast, low expression of TGFβ receptors in breast tumors correlates with a favorable disease outcome (8). Notably, several studies using inhibitors of TGFβ have shown repression of metastasis of mammary carcinomas (9), further highlighting a critical role of TGFβ signaling in the development and progression of breast cancer metastasis.

TGFβ signaling is triggered by binding of TGFβ receptor ligands to a complex of transmembrane receptor serine/threonine kinases (types I and II), followed by phosphorylation of receptor-activated Smads (R-Smads). Once activated, R-Smads form a complex with a common Smad4 and translocate into the nucleus, where they regulate the transcription of target genes along with various cofactors (10). Every step of the TGFβ signaling cascade is tightly controlled by specialized factors. For example, Smad7 is key to regulating the activity of TGFβ signaling via a negative feedback mechanism (11). Smad7 was found to block the phosphorylation of Smad2/3 upon TGFβ stimulation through binding to the TGFβ receptor complex (12) and inhibit the heterocomplex formation between R-Smads and Co-Smad (13). Importantly, Smad7 binds to the Smad ubiquitination regulatory factor 2 (Smurfl2), a member of the HECT E3 ligases family, resulting in ubiquitination of TGFβ type I receptor and consequent inhibition of R-Smad activation (14). Furthermore, Smurfl2 can target R-Smads for ubiquitin-mediated degradation and hence terminate R-Smads–mediated signaling (15).

Deregulation of TGFβ activation associated with decreases of its key suppressors has been found in various human cancer types. Indeed, recent evidence has shown that low-level expression of Smad7 correlates with lymph node metastasis in pancreatic cancer (16), and knockdown of Smurfl2 in human...
breast cancer cells results in enhanced cell migration in vitro and bone metastasis in vivo (17), suggesting that the quantities of Smad7 and Smurfl2 play an important role in modulating the level of TGFβ signaling activity during the progression of tumor metastasis. How these negative regulators of TGFβ signaling are decreased in cancers, however, remains poorly understood, but it remains an important area of research for future development of anti-TGFβ strategies.

miRNAs are small regulatory RNA molecules that posttranscriptionally downregulate target mRNAs by interacting with their 3’untranslated region (UTR; ref. 18). Differential expression of miRNA in normal and tumor tissues, or between biologic scenarios with or without metastasis, has been analyzed in a variety of cancer types (19, 20). Our present work reports that the miR24–503 miRNA cluster might contribute to TGFβ hyperactivation and thus might represent a new mechanism underlying breast cancer metastasis.

Materials and Methods

Cell culture
Breast cancer cell lines MCF7, T47D, BT474, MDA-MB-231, SKBR3, and MDA-MB-435 were purchased from ATCC in 2008, and according to the provider, the cell lines were authenticated using short tandem repeat (STR) DNA fingerprinting (21). These cell lines were grown in DMEM supplemented with 10% FBS (HyClone) in our laboratory and re-authenticated by STR fingerprinting profiles at the Forensic Medicine Department of Sun Yat-Sen University (SYSU; Guangzhou, China) in May 2014, which revealed fingerprinting profiles identical to those given in the ATCC STR database.

Clinical specimens and patient information
Paraffin-embedded human breast cancer specimens were histopathologically diagnosed at the First Affiliated Hospital of SYSU, and relevant clinical information is presented in Supplementary Table S1. Prior donors’ consents and approvals from the Institutional Research Ethics Committee were obtained. The Cancer Genome Atlas (TCGA) dataset was accessed at https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm.

Plasmids, infection, and transfection
Plasmids were constructed using standard methods (22). Recombinant retrovirus production and infection were performed as previously described (23). Transfection of plasmids or oligonucleotides was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instruction. For detailed description of each plasmid and sequences of synthetic oligonucleotides, see Supplementary Materials and Methods.

miRNA extraction and qPCR
Total miRNA isolation and cDNA synthesis were performed using commercial kits according to the manufacturer’s instructions. miRNA expression was quantified with qPCR using miRNA-specific primers in a real-time PCR system. For details, see Supplementary Materials and Methods.

Wound-healing assay
Cells were seeded in 6-well plates and grown to 90% confluence, followed by serum starvation for 24 hours. A linear wound was created in the confluent monolayer using a pipette tip, and wounds were observed and photographed at various time points. Wound size was measured randomly at five sites perpendicular to the wound.

Transwell matrix penetration assay
Cells (2 × 10⁴) were plated into the top side of polycarbonate Transwell coated with Matrigel (10%; BD) and incubated at 37°C for 22 hours, followed by removal of cells inside the upper chamber with cotton swabs. Migratory and invasive cells on the lower membrane surface were fixed in 4% paraformaldehyde, stained with hematoxylin, and counted (10 random 200× fields per well). Cell counts are expressed as the mean number of cells per field of view.

Three-dimensional spheroid invasion assay
Cells (1 × 10⁴) were mixed with 20% Matrigel and seeded in 24-well plates coated with 100% Matrigel (BD), and medium was changed every other day. Pictures were taken under microscope at various time points.

Western blotting and immunofluorescent assays
Western blotting and immunofluorescent assays were performed according to corresponding standard methods (24, 25). Sources of antibodies used are given in Supplementary Materials and Methods. Fluorescence images were captured using the LSM710 confocal microscopy system (Carl Zeiss).

Luciferase assay
Cells (3.5 × 10⁴) were seeded and settled in 48-well plates for 24 hours. Luciferase plasmid pGL3-control-Smad7-3’UTR, pGL3-control-Smurfl2-3’UTR, or pTAL-basicle1 (100 ng each) plus 1 ng of pRL-TK plasmid (Promega) were transfected into cells. Luciferase and Renilla signals were measured 48 hours after transfection using the Dual-Luciferase Reporter Assay Kit (Promega) according to a protocol provided by the manufacturer.

RNA immunoprecipitation
Coimmunoprecipitation (co-IP) of miRNP with anti-Ago1 (Abcam) was performed as previously described (26). Cell were lysed in buffer containing 100 mmol/L KCl, 5 mmol/L MgCl₂, 10 mmol/L HEPES (pH 7.4), and 0.5% NP-40; and the immune complex captured by protein A agarose was washed in buffer containing 150 mmol/L KCl, 5 mmol/L MgCl₂, 10 mmol/L HEPES (pH 7.4), and 0.1% NP-40 for 6 times. RNA extraction was performed using the RNAeasy Kit (Qiagen).

Immunohistochemical analysis
After sections were stained using anti-Smad7 (Sigma-Aldrich), anti-Smurfl2 (Epitomics), and anti-p-Smad2 antibodies (Abcam), images were captured using the AxioVision Rel.4.6 computerized image analysis system (Carl Zeiss). The degree of immunostaining of indicated proteins was evaluated and scored as previously described (4).
Immuo-laser capture microdissection

All immuno-laser capture microdissection (LCM) tissues were prepared as previously described (27). Samples were inspected and microdissected using Arcturus XT LCM microscope (Life Technologies Corporation). Approximately 600 cancer cells were microdissected from each slide onto a CapSure LCM cap. Total RNA and microRNA from LCM CapSure caps were extracted by QIAamp miRNeasy Mini Kit (QIAGEN GmbH) according to the manufacturer’s instructions.

Tumor metastasis model

Breast cancer cell lines MCF7 (1.5 x 10^6), MDA-MB-231 (1.0 x 10^6), and MDA-MB-435 cells (0.8 x 10^6) stably expressing firefly luciferase, alone or together with miR424-503, or corresponding control vectors, were injected intravenously via the caudal vein of BALB/c nude mice (n = 5). In anagomir systemic treatment experiment, 100 μl miR424 antagomir, miR503 antagomir, or antagomir control (diluted in PBS at 2 mg/mL:Ribo Biotech) was administrated intravenously 3 times per week for 2 weeks, starting day 10 after tumor cells inoculation. For spontaneous metastasis assays, MDA-MB-435-luciferase cells (3.0 x 10^5) were injected into the mammary fat pads (n = 5), and antagomir was administrated intratumorally as previously described when the average volume of grown tumors reached approximately 50 mm^3. Bioluminescence imaging was performed using the IVIS Spectrum Imaging System (Caliper Life Sciences). Image calibration and visualization were performed using the Living Image 4.2 software (Caliper Life Sciences).

Flow cytometry and single-cell cloning

Cells were dissociated into single-cell suspension, and dead cells were excluded by propidium iodide staining. Generation of single-cell–derived cultures was performed by BD InFlux (BD Biosciences) single-cell plating in 96-well plates (Corning). Single, red fluorescent protein (RFP)-positive/GFP-positive cells were gated stringently. When visible cell aggregates appeared, they were transferred to flasks (Corning) and expanded.

Statistical analysis

All statistical analyses were carried out using the SPSS 11.0 statistical software package. All error bars represent mean ± SD derived from 3 independent experiments. In all cases, P < 0.05 was considered statistically significant.

Results

miR424 and miR503 are upregulated in metastatic breast cancer

To identify miRNAs differentially expressed in breast cancer metastases, we retrieved and comparatively analyzed miRNA expression profiles of metastatic versus nonmetastatic subsets from TCGA datasets. Two miRNAs, miR424 and miR503, emerged as highly upregulated miRNAs in metastatic breast cancer specimens as compared with nonmetastatic ones (Fig. 1A). To validate these data, quantitative reverse transcription PCR (qRT-PCR) was conducted with 24 grade 4 breast cancer specimens and 93 low-grade breast cancer specimens (WHO tumor grades 1–3). Consistently, expression of miR424 or miR503 was markedly higher in grade 4 but significantly lower in low-grade, breast cancer samples (Fig. 1B). Furthermore, breast cancer cell lines known to be highly metastatic (MDA-MB-231, SKBR3, and MDA-MB-435) displayed increases of miR424 and miR503 expression relative to non- or low-metastatic cell lines (MCF7, T47D, and BT474; Fig. 1C), indicating an association between miR424/miR503 high expression and breast cancer metastasis.

Notably, miR424 and miR503 levels linearly correlated with each other in breast cancer cell lines and tissue specimens (Supplementary Fig. S1A and S1B). Furthermore, miR424 and miR503 share substantial sequence identity in their seed sequences, and their coding genes are separated by only 216 bp on the X chromosome (28), suggesting that the miR424–503 cluster might play cooperative roles in breast cancer.

The miR424–503 cluster enhances metastasis-associated properties of breast cancer in vitro

To understand whether the miR424–503 cluster is involved in metastasis of breast cancer cells, in vitro study was performed to observe the effect of stable overexpression of the miRNAs on cellular migration and invasions (Supplementary Fig. S2). As shown in Fig. 2A, ectopic miR424, miR503, or miR424–503 enhanced the migratory ability of MCF7 and MDA-MB-231 cells in wound-healing assay, and Transwell matrix penetration assay showed that miR424, miR503, or miR424–503 overexpression drastically increased the invasiveness of both cell lines (Fig. 2B). Consistently, compared with the vector control cells, miR424–503 cluster–transduced cells displayed a highly aggressive penetrating growth in 3-dimensional (3D) culture (Fig. 2C), suggesting that miR424 and miR503 are prometastatic in breast cancer cells.

Next we examined the effect of suppressing endogenous miR424 or miR503 on the phenotype of MDA-MB-231 (moderately metastatic) and MDA-MB-435 (highly metastatic) cells and found that inhibiting miR424 or miR503 markedly weakened the metastasis capability of the tested cells (Fig. 2A–C), further confirming the prometastatic effects of miR424 and miR503.

The miR424–503 cluster promotes metastasis of breast cancer cells in vivo

To further examine whether miR424 and miR503 promotes metastasis in vivo, luciferase-expressing MCF7 and MDA-MB-231 cells transduced with the miR424–503 cluster or control vector were injected into the caudal vein of nude mice. Strikingly, bioluminescence imaging showed that mice injected with MCF7/miR424–503 cells displayed prominent lung metastasis, whereas no visible metastasis was found in mice injected with control MCF7 cells (Fig. 3A). Consistently, MDA-MB-231/miR424–503 mice also generated a significantly larger number of lung metastases than those in vector control cells (Fig. 3A). To further validate whether the endogenous miR424–503 cluster was required for the observed enhanced metastasis in vivo, antagonir–424 and antagonir–503 were
applied to inhibit the endogenous expression of miR424 or miR503 in the experimental metastasis assay. When the endogenous miR424 or miR503 in MDA-MB-231 and MDA-MB-435 cells was reduced by the antagomirs (Supplementary Fig. S3), lung metastasis of both cell lines was significantly abrogated (Fig. 3B). Moreover, 4 of 5 mice bearing miR424–503-transduced MCF7 cells died before 50 days after inoculation, whereas only 2 mice in the vector control group died by day 56 after implantation (Fig. 3C). Similarly, mice injected with miR424–503-transduced MDA-MB-231 cells survived shorter than those injected with the vector control cells (Fig. 3C). When using antagomir, inhibition of miR424 or miR503 extended mice survival (Fig. 3C).

To investigate whether miR424–503 promotes dissemination of breast cancer cells from primary tumors, MDA-MB-435 cells were injected into the mammary gland pads of nude mice and examined for lung metastasis. As Supplementary Fig. S4A shows, while the control animals exhibited marked pulmonary metastases, antagomir treatment inhibited metastasis and prolonged the life of mice without affecting the weights of the
primary tumors (Supplementary Fig. S4B and S4C). Together, these data indicated that the miR424–503 cluster was a strong promoter for breast cancer metastasis in vivo.

**The miR424–503 cluster directly targets and suppresses multiple negative regulators of TGFβ signaling**

In the light that the TGFβ signaling is important for breast cancer metastasis, we then examined the effect of miR424–503 on TGFβ activation. In cells containing the TGFβ reporter gene 3TP-Lux (29), overexpression of miR424, miR503, or miR424–503 significantly elevated, whereas inhibition of miR424 or miR503 dramatically reduced the transactivating activity of TGFβ (Supplementary Fig. S5A). Concordantly, immunofluorescent staining showed that upon TGFβ treatment, overexpression of miR424, miR503, or miR424–503 elevated the nuclear enrichment of Smad3 (Supplementary Fig. S5B). Conversely, inhibition of miR424 or miR503 decreased the quantity of nuclear Smad3 (Supplementary Fig. S5B). These data suggest that the miR424–503 cluster overexpression is able to enhance activity of TGFβ signaling.

Figure 2. The miR424–503 cluster enhances breast cancer metastasis in vitro. A, wound-healing assay was performed with indicated cells. B, representative images (top) and quantification (bottom) of penetrated cells were analyzed using the Transwell matrix penetration assay. C, representative micrographs of indicated cultured cells at day 7 of culture in 3D spheroid invasion assay. **, P < 0.01.
Next, we screened for targets of miR424 and miR503 using the TargetScan Program and found that Smad7 and Smurf2, known as negative regulators of TGFβ signaling, were potential target genes of miR424 and miR503 (Fig. 4A). To validate this prediction, we found that both Smad7 and Smurf2 proteins were reduced in miR424- and/or miR503-overexpressing cells but increased by specific antagonomir(s) (Fig. 4B), whereas no detectable alterations in Smad7 or Smurf2 mRNA were seen (Supplementary Fig. S6A and S6B). When cells were transduced with a luciferase reporter containing Smad7 or Smurf2 3’UTR, ectopic expression of miR424 or miR503 decreased, whereas inhibition of miR424 or miR503 increased the activity of reporter luciferase (Fig. 4C). Furthermore, mutations introduced to miR424 and miR503

Figure 3. The miR424–503 cluster promotes metastasis of breast cancer cells in vivo. A and B, nude mice injected intravenously via the caudal vein with indicated cells were monitored with luciferase live-imaging system at indicated time points (top), and the photon quantity in each mice group was analyzed (bottom). Heatmap scale bar represents photon emission. C, survival curves of mice injected intravenously with indicated cells. **, $P < 0.01$. 

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target sites of the Smad7 or Smurf2 3’UTR abrogated the suppressive effects (Fig. 4D). Moreover, miRNP IP assay revealed high-level enrichment of Smad7 and Smurf2 transcripts in the miRNP of miR424 mimic– or miR503 mimic–transfected cells (Fig. 4E). Collectively, these results establish Smad7 and Smurf2 as targets of the miR424–503 cluster.

Figure 4. The miR424–503 cluster targets Smad7 and Smurf2 expression. A, conserved miR424 and miR503 binding sites in Smad7 and Smurf2 generated by TargetScan analysis. B, Western blot analysis of Smad7 and Smurf2 expression in indicated cells. α-Tubulin was used as a loading control. C, relative activity of reporter luciferase linked to Smad7 or Smurf2 3’UTR measured following miR424 mimic, miR503 mimic, miR424 inhibitor, or miR503 inhibitor transfection in indicated cells. D, relative activity of reporter luciferase linked to mutant Smad7 3’UTR or mutant Smurf2 3’UTR measured following miR424 mimic or miR503 mimic in indicated cells. E, enrichment of Smad7 and Smurf2 3’UTRs in miRNP detected by qPCR following immunoprecipitation against Ago1. **, P < 0.01; *, P < 0.05.
Figure 5. Smad7 and Smurf2 are functionally involved in miR424–503 cluster induced metastasis. A, Western blot analysis of Smad7 and Smurf2 expression in indicated cells. α-Tubulin was used as a loading control. B, representative images (top) and quantification (bottom) of penetrated cells were analyzed using the Transwell matrix penetration assay. C, mice injected intravenously with indicated cells were monitored by luciferase live imaging system at indicated time points (top), and the photon quantity in each mice group was analyzed (bottom). (Continued on the following page.)
Smad7 and Smurf2 repression is essential for miR424–503-mediated metastasis

To determine the functional significance of Smad7 and Smurf2 genes in promoting breast cancer metastasis induced by the miR424–503 cluster, we further stably expressed Smad7 open reading frame (ORF) or Smurf2 ORF, free of 3′UTR in miR424–503-transduced cells (Fig. 5A). As shown in Fig. 5B, restoration of Smad7 or Smurf2 protein expression decreased invasiveness of miR424–503-transduced cells in the Transwell matrix penetration assay.

To determine the effects of Smad7 and Smurf2 on tumor metastasis in vivo, we injected mice intravenously with miR424–503/Smad7 ORF– or miR424–503/Smurf2 ORF–transduced cells, as well as their corresponding vector control cells. As shown in Fig. 5C, Smad7 or Smurf2 abrogated the metastasis-promoting effect of miR424–503, suggesting an essential role of Smad7 and Smurf2 in miR424–503-conferred breast cancer metastasis.

We then examine the clinical relevance of the miR424–503/Smad7-Smurf2/TGFβ axis in human breast cancer specimens. As shown in Fig. 5D, 78.1% (57 cases) and 67.1% (49 cases) of specimens expressing low-level miR424–503 (73 cases), respectively, exhibited high Smad7 and Smurf2 expression, whereas 56.8% (25 cases) and 50% (22 cases) of samples with high miR424–503 expression (44 cases) showed low Smad7 and Smurf2, respectively (both \( P < 0.05 \)). Moreover, those cases expressing high miR424–503 displayed higher p-Smad3 levels (38 of 44 samples; 86.4%) than the low miR424–503 cases (29 of 73 samples; 39.7%; \( P < 0.05 \)), suggesting that the miR424–503 cluster overexpression in clinical breast cancer lesions was associated with downregulation of Smad7 and Smurf2 and activation of TGFβ.

Expression of the miR424–503 cluster correlates with TGFβ activity in breast cancer

Previous evidence has suggested that the intensity of TGFβ signaling is often heterogeneous in the cell population of a breast tumor, and such heterogeneity can be associated with different metastatic behaviors of breast cancer cells (30, 31). Our current immunohistochemical study also showed that nuclear p-Smad3 staining in a breast tumor was not homogeneous (Fig. 5D). To assess whether the differential activities of TGFβ signaling were relevant to the expression levels of miR424–503, we used LCM method to obtain p-Smad3high and p-Smad3low loci from 10 clinical breast cancer specimens. As shown in Fig. 5D, 78.1% (57 cases) and 67.1% (49 cases) of specimens expressing low-level miR424–503, respectively (both \( P < 0.05 \)), suggesting that the miR424–503 cluster correlates with the expression levels of both miRNAs were dramatically higher in TβRE-GFPcells than in TβRE-GFPcells (Fig. 6D). In parallel, the TβRE-GFPcells displayed markedly higher invasiveness than the TβRE-GFPcells in our Transwell matrix penetration assay (Fig. 6E), strongly suggesting close correlations among miR424–503 level, TGFβ activity, and cancer metastasis. Furthermore, when we transiently expressed miR424 or miR503 through transfection of their mimic oligonucleotides in TβRE-GFPcells, increased invasiveness was observed (Fig. 6E). In contrast, inhibition of miR424 or miR303 with inhibitory oligonucleotides decreased invasiveness of TβRE-GFPcells (Fig. 6E). Taken together, our data suggested that the heterogeneous TGFβ activities in breast tumors might be attributable to a differential expression of the miR424–503 cluster.

Discussion

The functional significance of TGFβ signaling has been well-documented in tumor metastasis (5). TGFβ activation–associated accumulation of phosphorylated Smad2 in the nucleus drives formation of bone metastasis of human breast cancer cells (31). Clinical correlations between plasma levels of TGFβ and metastatic disease have been reported in breast cancers, and low expression of TGFβ receptors correlates with a favorable disease outcome (9). The regulatory network that controls the activation level of TGFβ signaling, however, remains to be understood. The finding by this current study demonstrates a new molecular mechanism by which TGFβ is overactivated and thereby provides novel insights in better understanding the regulatory network of TGFβ signaling.
of the signaling pathway under physiologic conditions (12–15). The level of Smad7 or Smurf2 has been found to be crucial in determining the activation level of TGFβ signaling (17, 32), and thus understanding what supervise their quantities in the context of cancer may reveal key mechanisms contributing to metastasis. While previous studies identified loss-of-function mutations or deletions in human cancer (33), Smad7 or Smurf2 expression was found to be regulated also at transcriptional or posttranscriptional levels (34, 35). Our current finding that Smad7 and Smurf2 protein levels are simultaneously and directly suppressed by overexpressed miR424–503 cluster in metastatic breast cancer cells without alterations in their mRNA levels, therefore, represents a novel posttranscriptional model for a coordinated regulation of these 2 factors. Although miRNAs have been reported to target Smad7 (36, 37), and miR424 or miR503 overexpression has been separately found to be associated with poor prognosis in breast cancer (38, 39), our results provides the first demonstration for miRNA
miR424–503 promotes miR424 activity in human breast cancer and found biologically as well as clinically relevant correlations among miR424 activity, miR424–503 expression, and metastatic potential in various subsets of tumor cells in a population. As metastasis is a highly inefficient process, and usually only a small fraction of cancer cells derived from the primary tumors can eventually form metastases in distant organ sites (46), identifying cells or tumor tissues with high miR424–503 expression might be useful for predicting metastatic potential and for identifying the cell population with the highest probability to form metastases in distant organ sites. Whether these tumors and cell populations should represent the major targets for antimetastasis therapies remains to be further studied.

Additionally, the mechanisms underlying the heterogeneous expression of miR424–503 in breast cancer lesions. Importantly, it was reported that miR424 could be regulated by PU.1, a member of Ets transcription factor family (47), and hypoxia could regulate PU.1 to drive miR424 expression (48). As breast tumor cells are exposed to heterogeneous oxygen pressure (49, 50), whether such microenvironmental factors contribute to the heterogeneity of PU.1 levels, and consequently to the heterogeneous TGFβ activation, needs to be investigated in the context of breast cancer metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Li, M. Li
Development of methodology: Y. Li, W. Li, Z. Ying
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Li, W. Li, Z. Ying, H. Tian, M. Li
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Li, W. Li, Z. Ying, H. Tian
Writing, review, and/or revision of the manuscript: Y. Li, J. Li, M. Li
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Li, Z. Ying, X. Zhu
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