Downregulation of miR-106b induced breast cancer cell invasion and motility in association with overexpression of matrix metalloproteinase 2

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Breast cancer (BC) is one of the most common cancers in women, and it can often metastasize to the bone. The mechanism of BC bone metastasis remains unclear and requires in-depth investigation. In a previous study, we found the expression of matrix metalloproteinase 2 (MMP2) to be significantly more pronounced at metastatic bone sites than at orthotopic sites. MicroRNA expression profiling showed miR-106b to be markedly downregulated during BC bone metastasis. However, the specific manner in which MMP2 and miR-106b are involved in the BC bone metastasis is still unclear. In the present study, we found MMP2 expression in orthotopic tumor tissue to be related to the risk of bone metastasis in BC patients. MiR-106b levels in orthotopic tumor tissue showed a negative correlation with MMP2 expression and breast cancer bone metastasis. MMP2 was shown to be a direct target of miR-106b. Both gain- and loss-of-function studies showed that MMP2 could promote the migration and invasion of BC cells and that miR-106b could suppress both. The blockage of MMP2 by RNA interference mimicked the anti-migration and anti-invasion effects of miR-106b, and introduction of MMP2 antagonized the function of miR-106b. MMP2 was also found to regulate the ERK signaling cascade and so adjust the bone microenvironment to favor osteoclastogenesis and bone metastasis. These results suggest that MMP2 upregulation plays an important role in BC bone metastasis through ERK pathways, and miR-106b directly regulates MMP2 expression. The miR-106b/MMP2/ERK pathway may be a promising therapeutic target for inhibiting BC bone metastasis.

Breast cancer (BC) is one of the most common malignancies in women, worldwide. Bone metastasis is the most frequent complication of BC. It can lead to bone fractures, severe pain, nerve compression, and hypercalcemia.1–4 Effective therapies for bone metastasis require an in-depth understanding of the molecular mechanism of the processes underlying the condition.

In a previous study, we established a novel mouse model using human-derived orthotopic breast tissue, metastatic bone, and BC cell lines. This model reflects the complete, natural, species-specific process of BC metastasizing to bone.5 Using this model, we isolated two BC cell lines: SUM1315-br (from SUM1315 orthotopic tumor in implanted breast) and SUM1315-bo (from SUM1315 metastatic tumor in implanted bone). Gene and miRNA profiling analyses revealed that matrix metalloproteinase 2 (MMP2) was overexpressed and miR-106b downregulated in the bone metastasis tissues relative to the orthotopic BC tissues. This illustrates possible targets related to BC bone metastasis.5

Matrix metalloproteinase 2 has already been proven useful as a marker in the prediction of tumor progression and metastasis.6,7 Recent studies in BC patients have shown that increases in the activity level of MMP2 in the sera are a strong, independent predictor of aggressive disease.8–10 Although the function of MMP2 in tumor progression is well known, its role in BC bone metastasis is still under intense investigation.

MicroRNA 106b (miR-106b) is located in a cluster with miRs 93–25 on intron 13 of the Mcm7 on chromosome 7q22. It can be actively co-transcribed with the Mcm7 primary RNA transcript.11 Ectopic expression of miR106b in chronic lymphocytic leukemia (CLL) cells has demonstrated that miR106b can lead to an accumulation of p73.12 Until now, no comprehensive study on the function of miR106b in BC has been conducted.

In recent years, the idea that osteolytic bone metastasis is driven by intricate cellular and molecular interactions among tumor cells and the host stromal cells commonly found in the bone microenvironment has drawn much attention.1–3 The function of tumor-derived bone metastasis factors hinges upon their ability to increase RANKL production and decrease osteoprotegerin (OPG) secretion by osteoblasts, ultimately promoting osteoclast differentiation and activation.1–4 The present study revealed that miR106b/MMP2/ERK pathways affect the balance of RANKL and OPG production through osteoblasts that promoted BC bone metastasis.
Materials and Methods

Human tumor cell lines and cell culture. Breast cancer cell lines MDA-MB-231, BT474, MCF-7, MDA-MB-468, HCC1937, T47D, and ZR-75-30, were purchased from ATCC (Manassas, VA, USA) and cultured in complete medium. Cell line SUM1315 was provided by Stephen Ethier (University of Michigan). Two BC cell lines were established and named SUM1315-br and SUM1315-bo, as in our previous study. All cells were incubated at 37°C in a humidified chamber supplemented with 5% CO2.

Patient selection. Faure marin fixed and paraffin embedded (FFPE) tumor samples (n = 50) were collected in Department of Breast Surgery of Jiangsu Province Hospital between February 2002 and March 2007, from patients treated surgically for clinical stage I–III breast cancer (aged 34–65 years). Study patients were followed up for the judgment of bone metastasis-positive or bone metastasis-negative until June 2012, with median follow-up times of 46 months (range, 32–60 months). Patients’ characteristics are listed in Table 1. All of the patients gave their informed consent, and the study has been approved by the Ethical and Scientific Committee of our Institution.

Preparation of conditioned medium. SUM1315-bo cells were grown until sub-confluence and then starved in serum-free DMEM, for 24 h. Conditioned media (CM) were then collected, centrifuged, concentrated, aliquoted, and stored at −20°C until use.

Osteoblast differentiation and osteoclastogenesis assay in vitro. Human mesenchymal stem cells (HMSCs) were purchased from ScienCell (Corte Del Cedro, Carlsbad, CA, USA), plated in cell culture flasks and expanded in mesenchymal stem cell growth medium (MSCM; ScienCell) at 37°C in a 5% CO2 atmosphere for 7–10 days. After cells were allowed to grow to an adequate density, adherent cells were trypsinized and then seeded at a density of 5000 cells/cm² on a 24-well plastic plate. After 24 h, MSCM was removed and osteogenic induction medium (ObM; ScienCell) was added to induce osteoblast differentiation (Day 0). On the seventh day, the osteoblast cells were plated at 1 x 10⁶ cells per well in 24-well plates at a 1:1 ratio of basal culture medium/filtered CM (harvested from 24 h incubation of confluent tumor cells). The medium was changed every 2 days.

Molecular techniques and reagents. Details of the molecular techniques (viral production, luciferase assay, cell transfection, real time PCR, Western blotting, transwell invasion and migration assay) and reagents (antibodies, plasmids, and DNA constructs) are summarized and listed in the Supplementary Data S1.

Immunohistochemistry. Paraffin-embedded tissue sections were deparaffinized, rehydrated, rinsed, immersed in 10 mM sodium citrate, microwaved for 20 min, and cooled for 20 min. For cytoimmunohistochemistry, slides were fixed in PBS (pH 7.4) solution containing 4% paraformaldehyde for 10 min and then rinsed. After incubation in methanol containing 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, it was then microwaved in 0.01 mM sodium citrate (pH 6.0) for antigen retrieval, incubated with rabbit polyclonal (Rp) antibody against MMP2 (1:1000; Cell Signaling Technology, Beverly, MA, USA) for 2 h at 37°C, then incubated with HRP labeled rabbit anti-goat secondary antibody for 1 h at 37°C, then incubated with 3, 3'-diaminobenzidine solution for 10 min and counterstained with hematoxylin.

Evaluation of immunohistochemistry results. Immunohistochemical staining results were interpreted by two experienced pathologists and the mean staining density was determined using ImagePro Plus 6.0 (ImagePro, Bethesda, MD, USA). MMP-2 expression was evaluated under a light microscope at a magnification of 400×. For each specimen, five images of representative areas were acquired and a total of 1000–2000 tumor cells were counted. For human samples, IHC scoring was performed using a modified Histo-score (H-score), which included a semiquantitative assessment of both fraction of positive cells and intensity of staining. The extent of the staining, defined as the relative area of positive staining within the tumor cells relative to the whole tissue area, was scored on a scale of 0–4 as follows: 0%, 0, −10%; 1, 11–25%; 2, 26–50%; 3, 51–75%; and 4, >75%. The sum of the staining-intensity and staining-extent scores was used as the final staining score for MMP2 (0–7). For statistical analysis, a final staining score of 0–5 was considered indicative of low expres-

| Table 1. Associations between the expression of matrix metalloproteinase 2 (MMP2) in the primary tumor and the clinicopathological characteristics of breast cancer patients |
|------------------|------------------|------------------|------------------|
|                  | No. patients     | No. (%) expressing high levels of MMP2 | P-value |
| Age              |                  |                  |                  |
| ≤ 50             | 28               | 9 (32.1)         | 0.085            |
| > 50             | 22               | 2 (9.0)          |                  |
| Bone metastasis  |                  |                  |                  |
| Yes              | 25               | 13 (52)          | 0.001            |
| No               | 25               | 2 (8)            |                  |
| Tumor size†      |                  |                  |                  |
| T1               | 20               | 4 (20)           | 0.434            |
| T2               | 22               | 4 (18.1)         |                  |
| T3               | 8                | 3 (37.5)         |                  |
| Node stage†      |                  |                  |                  |
| N0               | 9                | 1 (11.1)         | 0.097            |
| N1               | 23               | 3 (13.0)         |                  |
| N2               | 14               | 6 (42.8)         |                  |
| N3               | 4                | 1 (25)           |                  |
| Histological grade |          |                  |                  |
| Grade 1          | 2                | 0 (0)            | 0.728            |
| Grade 2          | 39               | 9 (23.0)         |                  |
| Grade 3          | 9                | 2 (22.2)         |                  |
| ER               |                  |                  |                  |
| Positive         | 17               | 3 (17.6)         | 0.728            |
| Negative         | 33               | 8 (24.2)         |                  |
| PR               |                  |                  |                  |
| Positive         | 14               | 3 (21.4)         | 1.000            |
| Negative         | 36               | 6 (22.2)         |                  |
| HER2‡            |                  |                  |                  |
| Positive         | 10               | 3 (30)           | 0.671            |
| Negative         | 40               | 8 (20)           |                  |

ER, estrogen receptor; IDC, infiltrating ductal carcinoma; PR, progesterone receptor. TNM 6 classification according to the Union Internationale Contre le Cancer criteria. HER2 positivity = positive fluorescent in situ hybridization test. Significance was assessed using a 2 x 2 table and the χ² test.
tion and 6–7 was considered indicative of high expression.\(^{(13)}\)

The immunostained slides were evaluated by two board-certified pathologists at two separate institutions. They independently examined the entire tissue section while blinded to the clinical data. Reading agreement was found to be 96% concordant between the two pathologists. Non-concordant cases were resolved by a third pathologist blindly scoring these cases and using the two out of three rules for the determination of final scores.

**Statistical analyses.** Data are presented as mean ± SD. The Student’s \( t \)-test (two-tailed) was used to determine the statistical significance of the differences between groups. \( P < 0.05 \) was considered statistically significant. The intensity of MMP2 expression in human breast cancer samples was analyzed using the \( \chi^2 \) test. The association between MMP2 and miR-106b expression in human breast cancer samples was analyzed using the Spearman rank correlation test. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

**Results**

**Association between MMP2 overexpression and BC bone metastasis.** Matrix metalloproteinase 2 breast tumor tissue expression was evaluated in 50 BC patients using immunohistochemistry. Representative images of MMP2 staining are shown in Supplementary Figure S1. Matrix metalloproteinase 2 (MMP2) tumor tissue expression is associated with increased risk of bone metastasis in BC patients. Significantly more cells were found to be positive for MMP2 cytoplasmic staining in BC cells, in 52.0% (13/25) and 8% (2/25) of the patients, respectively (Table 1). The relationship between expression of MMP2 and BC bone metastasis was analyzed. Higher levels of MMP2 expression were closely associated with BC bone metastasis (\( P = 0.001 \)).

**Association between miR-106b and MMP2 expression.** MiR-106b breast tumor tissue expression was also evaluated in the previous 50 BC patients one-by-one corresponding using RT-PCR. A negative correlation was observed between the expression of miRNA 106b and MMP2 tumor expression (Spearman correlation \( R_s = -0.786, P = 0.001 \)).

**MMP2 and miR-106b expression in a panel of BC cells.** To validate the results of our earlier gene expression and microRNA expression profiling analyses,\(^{(5)}\) we confirmed MMP2 expression in two SUM1315-derived sub lines isolated and purified from the orthotopic and metastatic xenograft using Western blotting, as shown in Supplementary Figure S2a. SUM1315-bo showed higher protein levels of MMP2 and lower levels of miR106b than SUM1315-br or SUM1315 (Suppl. Fig. S2a,b). Differential mRNA expression of MMP2 was confirmed on a panel of eight human BC cell lines. Breast cancer cell lines such as MDA-MB-231 and SUM1315 showed higher MMP2 expression, as shown in Fig. 1. (a) The mRNA of MMP2 was transfected into SUM1315-bo. This was confirmed at both the gene and protein levels. (b) The invasiveness of MMP2 knockdown SUM1315-bo and the negative control (si-NC) cells were assessed using transwell assays. The invasiveness through 8 μm pore transwells was significantly lower in MMP2 knockdown SUM1315-bo than in the negative control (\( P < 0.05 \)). Original magnification \( \times 100 \). (c) The constructed expression vector pEGFP-C2-MMP2 was stably transfected into MCF-7, which was MMP2-negative. Transfection was confirmed at the protein level. (d) The invasiveness of MMP2 overexpressing MCF-7 (MCF7-MO) and the control cells were assessed by transwell assays. The invasiveness through 8 μm pore transwells was found to be significantly higher in MMP2 over-expressing MCF-7 than in controls (\( P < 0.05 \)). Original magnification \( \times 100 \).
mRNA and protein levels but lower miR-106b levels than MCF-7 (Suppl. Fig. S2c,d,e). Besides this, BC cell line BT474 showed higher protein level than T47D, ZR-75-30, MDA-MB-468 and HCC1937. Because SUM1315 and SUM1315-bo cells have greater migratory potential than MCF-7 cells, we speculate that MMP2 might be involved in BC migration and invasion.

Inhibition of endogenous MMP2 and migration and invasion of SUM1315-bo cells. The role of MMP2 on cell migration and invasion of SUM1315-bo cells was demonstrated by knocking down MMP2 using siRNA. We used SUM1315-bo cells because they have a high migratory potential and express endogenous MMP2 in large quantities. Three small interfering RNA (siRNA)-coding oligos against human MMP2 were
designed and compared. The two most effective MMP2 siRNA constructs had target sequences of AGU AGA UCC AGU AUU CAU UCC CUG C (si-M1) and AGA AGU UGU AGU UGG CCA CAU CUG G (si-M2). These sequences were verified at the mRNA and protein levels (Fig. 1a). Notably, silencing of MMP2 was found to be associated with significantly decreased invasive and migratory ability in SUM1315-bo cells (Fig. 1b).

### Overexpression of MMP2 and invasion and migration of MCF-7 cells.

We transfected MCF-7 cells with the constructed expression vector pGFP-LV5-MMP2 to determine whether overexpression of MMP2 could increase cell migration and invasion. Using Western blot analysis, we found that the level of MMP2 increased 9.12-fold after transfection (Fig. 1c). We analyzed the effects of MMP2 on the migratory and invasive behavior of MCF-7 cells. Results showed a 3.93-fold increase in cell motility and a 2.70-fold increase in cell invasiveness after transfection of the constructed expression vector pGFP-LV5-MMP2 (Fig. 1d). These results indicate that overexpression of MMP2 promoted both migration and invasion of MCF-7 cells in vitro.

### Interaction between MiR-106b and MMP2 in BC cells.

Bioinformatic analysis indicated the presence of a conserved binding site for miR-106b in MMP2 3′UTR (Suppl. Fig. S3). We found the expression level of miR-106b at the cancer site of BC patients to be lower than at the normal site (Fig. 2a). The expression level of miR-106b was found to be inversely correlated with MMP2 in MCF-7 and SUM1315-bo cells. MCF-7 cells expressed more miR-106b but less MMP2 than SUM1315-bo cells (Fig. 2a, Suppl. Fig. S2c,d). These results suggest a possible role for miR-106b in the regulation of MMP2. We therefore evaluated MMP2 as a target of miR-106b. To validate this hypothesis, we first investigated the effects of miR-106b overexpression on MMP2 mRNA and protein levels in SUM1315-bo cells using real-time PCR and Western blot analysis. As expected, transfection of SUM1315-bo cells with miR-106b significantly decreased MMP2 mRNA and protein levels (Fig. 2b). We next investigated the effects of miR-106b-specific inhibitor (miR-106bI) on MMP2 mRNA and protein levels in MCF-7 cells. Again, miR-106bI significantly increased MMP2 mRNA and protein levels in MCF-7 cells (Fig. 2b). We then focused on the potential of MMP2 as a target of the events through which miR-106b mediates the invasive and migratory activity of BC cells.

We performed a luciferase reporter assay to confirm that miR-106b interacted with MMP2 3′UTR directly. A fragment of MMP2 3′UTR including the binding site of miR-106b (Suppl. Fig. S3) was inserted downstream of the luciferase open reading frame in the reporter plasmid. The reporter vector was cotransfected in SUM1315-bo cells with miR-106b mimic or negative control. Results showed that luciferase activity decreased by approximately 71.78% in the presence of miR-
106b mimic (Fig. 2c). Mutations in the miR-106b-binding site in MMP2 3′UTR were found to abrogate this repression, supporting the hypothesis that the effect of miR-106b is exerted through direct interaction with the mRNA target. We also found that transfection of MCF7 cells with miR-106bI caused a marked increase in the luciferase activity (Fig. 2d). However, transfection of SUM1315-bo cells with miR-106bI failed to increase luciferase activity (data not shown). This may be because the endogenous expression level of miR-106b in SUM1315-bo cells is much lower than in MCF7 cells. Conversely, miR-106bI did not increase the luciferase activity of PGL3-M-MUT, demonstrating that mutations of the miR-106b-binding site in the MMP2 3′UTR abolish the ability of miR-106b to regulate its expression. These results indicate that miR-106b regulates the expression of the MMP2 by binding directly to its 3′UTR in BC cells.

Overexpression of miR-106b and invasion and migration of SUM1315-bo cells. Because SUM1315-bo cells have higher migratory potential than MCF-7 cells, we speculated that miR-106b might be involved in BC migration. To confirm this hypothesis, we transfected SUM1315-bo cells with 200 nM miR-106b mimic to determine whether overexpression of miR-106b would decrease migration and invasion. Using miRNA-specific quantitative real-time PCR, we found that the level of endogenous miR-106b had increased by 10.55-fold after transfection (Fig. 3a). We further analyzed the effects of miR-106b on the migratory and invasive behavior of SUM1315-bo cells. Results showed a 1.83-fold decrease in cell motility and a 5.01-fold decrease in cell invasiveness, respectively, after transfection of the miR-106b mimic (Fig. 3b). These results indicate that overexpression of miR-106b can inhibit migration and invasion of SUM1315-bo cells in vitro to a considerable extent.

Inhibition of endogenous miR-106b and migration and invasion of MCF-7 cells. To determine whether endogenous miR-106b can regulate tumor invasion and migration, MCF-7 cells were transfected with miR-106bI or control miR-NC inhibitor (miR-NCI). We used MCF-7 cells because they express more endogenous mature miR-106b than SUM1315-bo cells. After treatment with miR-106bI, the intracellular level of miR-106b was significantly lower than in cells treated with miR-NCI (Fig. 3c). Transwell assay indicated that the migration and the invasiveness of MCF7 cells increased 2.35- and 2.37-fold, respectively, after transfection with miR-106bI (Fig. 3d).

miR-106b and proliferation and apoptosis of breast cancer cells. To determine whether endogenous miR-106b can regulate tumor cell proliferation and apoptosis, we transfected SUM1315-bo cells with 200 nM miR-106b mimic or MCF-7 cells with 200 nM miR-106b inhibitor. Results showed a 0.76-fold decrease (Fig. 4a) in proliferation and a 3.8-fold increase (Fig. 4b) in cell apoptosis, respectively, after transfection of the miR-106b mimic. The proliferation of MCF7 cells increased 1.23-fold (Fig. 4c) and the apoptosis of MCF7 cells decreased 1.56-fold (Fig. 4d), respectively, after transfection with miR-106bI.

miRNA-106b and the MMP2/ERK pathway. Because the MAPK pathway has been linked to the metastatic cascade,(14) we hypothesized that MMP2 may regulate BC bone metastasis and may be associated with the components of the MAPK pathway. To test our hypothesis, we performed Western blot analysis for p-ERK and ERK protein in untransfected cells (N), miR-106b (106b), miR-106bI (106bI), or si-MMP2 and in cells overexpressing MMP2 (M-O). Cells treated with miR-106b showed higher levels of the ratio p-ERK/ERK protein than controls (P < 0.01), while miR-106b and si-MMP2 cells expressed lower levels of the ratio p-ERK/ERK (P < 0.01) (Fig. 5a). These data indicate that miR-106b’s ability to decrease invasion and migration is attributable, in part, to its capacity to inhibit MMP2 and decrease the level of activated ERK expression.

Factors secreted from SUM1315-bo cancer cells and the balance of RANKL and OPG production by osteoblasts. To determine whether MMP2 can influence osteoclast differentiation by altering the expression or release of RANKL and OPG in osteoblasts, we used SUM1315-bo CM to culture HMSC that had fully differentiated into osteoblasts with osteogenic induction medium. We then determined the level of RANKL and OPG released from osteoblasts using immunoblotting (Fig. 5b). Human mesenchymal stem cell-derived osteoblasts secreted low levels of RANKL and relatively high levels of OPG (RANKL/OPG ratio = 0.28). This pattern was reversed by CM from tumor cells (RANKL/OPG ratio = 3.17). **P < 0.01.
Mitogen-activated protein kinase (MAPK)/ERK signaling pathways are highly conserved in all eukaryotes. They are involved in many different cellular responses. Deregulation of MAPK/ERK signaling is often associated with human diseases, including cancer. We found that by suppressing MMP2, miR-106b decreased the expression of p-ERK, ERK which regulated cell invasion and migration. These results provided a new insight into the complex regulatory pathway composed of miR-106b, MMP2, and the MAPK/ERK pathway.

Furthermore, we investigated the role of MMP2 and miR106b in tumor-stimulated osteoclast activation. HMSC-derived osteoblasts were treated with the CM from tumor cells and showed many TRAP-positive multinucleated mature osteoclasts (data not shown). Human mesenchymal stem cell-derived osteoblasts were cultured with CM from tumor cells and showed many TRAP-positive cells. The CM of the SUM1315-bo cell line induced extensive differentiation of osteoclasts. However, the osteoclast-activating ability of the CM was significantly reduced when the expression of MMP2 was suppressed.

This is the first attempt to illuminate the function of miR-106b in BC bone metastasis. In this way, chemotherapies targeting miR106b/MMP2/ERK pathway could be a suitable strategy for the reversal of the metastatic potential of BC cells.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Materials and methods.

Fig. S1. Representative images of matrix metalloproteinase 2 (MMP2) immunohistochemical staining in breast cancer bone metastasis patients.

Fig. S2. Matrix metalloproteinase 2 (MMP2) expression in a panel of breast cancer cells.

Fig. S3. Analysis of matrix metalloproteinase 2 (MMP2) 3′UTR sequence using TargetScan showing a putative miR-106b-binding site.