Original Article
The GTP binding activity of transglutaminase 2 promotes bone metastasis of breast cancer cells by downregulating microRNA-205

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Abstract: Previous studies have shown that transglutaminase 2 (TG2) induces epithelial to mesenchymal transition (EMT) in various tumors. Several studies have also demonstrated the critical role of microRNAs (miRNAs) in regulating EMT of various types of tumors. However, the relationship between TG2 and miRNAs is not well understood. In the present study, we investigated if miR-205, which is known to inhibit EMT and is commonly regulated by TG2, contributes to TG2-induced EMT of human breast cancer cells. We have analyzed the expression of miR-205 in TG2-expressing and TG2-non-expressing breast cancer cells by quantitative real-time PCR (qRT-PCR) and the expression of TG2 and EMT related markers, such as ZEB1 and Vimentin, by western blotting. We also have studied the regulation of tumor metastasis by miR-205 and TG2 using matrigel invasion assays, intracardiac injection of breast cancer cells into mice and in vivo bioluminescent imaging. MiR-205 was significantly downregulated in high TG2-expressing or TG2-transfected breast cancer cells than in low TG2-expressing or mock-transfected breast cancer cells. Overexpression of miR-205 reduced the bone metastasis of MCF7/TG2-C277S cells that express transamidase-activity deficient TG2 and inhibits the invasiveness of MDA-MB-231 breast cancer cells that express TG2. Bioluminescent imaging showed that intracardiac injection of MCF7/TG2-C277S cells in mice promoted bone tumors, especially in the knee and jaw, but MCF7/TG2-C277S cells ectopically expressing miR-205 did not metastasize. The GTP binding activity, but not transamidase activity, of TG2, induces EMT in breast cancer cells by inhibiting the expression of miR-205 that suppresses EMT by downregulating the expression of ZEB1, an EMT marker. Moreover, in vivo experiments demonstrate that miR-205 down-regulation by TG2 induces bone metastasis of breast cancer cells.

Keywords: MicroRNA, transglutaminase 2, metastasis, breast cancer

Introduction

Breast cancer is the second most commonly diagnosed cancer and the second most common cause of cancer-related deaths in the USA. In 2017, an estimated 252,710 women and 2,470 men were expected to be diagnosed with invasive breast cancer, and an estimated 63,410 women were to be diagnosed with in situ breast carcinoma. Furthermore, 40,610

women and 460 men were expected to die from breast cancer in 2017 [1]. Breast cancer is generally classified by the status of the estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2 or c-erbB2). Patients with triple-negative breast tumors are deficient in ER, PR and HER2 expression and their effective treatment remain a challenge in the clinic because of the lack of ER and HER2 expression which is a target for

hormonal therapy or targeted therapy. These patients show worse prognosis because of the lack of effective targeted therapeutics to prevent metastasis [2]. Metastasis is associated with drug resistance and is a significant reason for treatment failure in cancer patients. Therefore, understanding the unknown mechanisms underlying metastasis is critical to overcoming resistance to each anti-cancer drug. Although several mechanisms related to metastasis have been discovered and several biomarkers of Triple-Negative Breast Cancer (TNBC) such as CDCP1, miR-770 and TG2 are known, further in-depth studies are necessary to understand the complex metastatic mechanisms underlying TNBC for development of more effective drugs [3, 4].

Transglutaminase 2 (TG2) is an EMT marker protein with diverse functions such as protein cross-linking, post-translational modification of proteins, scaffolding, cell adhesion and cell signaling [5-8]. The most well-known role of TG2 in cancer is depletion of tumor suppressors via protein cross-linking [6]. The crosslinking activity of TG2 activates FAK, AKT and NFκB signaling, which induces EMT in various tumors and promotes resistance to several anti-cancer drugs [9-14]. Moreover, TG2 plays a critical role in EMT induction via cross-talk with several signaling pathways such as Transforming Growth Factor β1 (TGFβ1), Wnt, β-catenin and Nuclear factor-kappaB (NF-κB) [15, 16]. Previous clinical studies have shown a correlation between TG2 expression, metastatic cancer and poor survival outcomes in ovarian, breast and colon cancer patients [17, 18]. TG2 is also known to mediate motility, invasion, and growth of breast cancer cells [19, 20].

MicroRNAs (miRNAs) are small, single-stranded non-coding RNAs (about 23 nucleotides long) that play critical roles in the post-transcriptional regulation of gene expression by targeting the complementary mRNA sequence in the 3'-untranslated regions of various target genes [21]. The miRNAs play tumor suppressor or oncogenic roles, and their expression is altered in breast cancer; the miRNAs regulate all the different stages of cancer progression, such as cell proliferation, apoptosis, cell migration, angiogenesis and stem cell maintenance [21, 22]. Previous studies showed that the miR-200 family, which includes miR-21 and miR-205, regulates tumor cell proliferation and invasion in various tumors including breast tumors [23, 24]. MiR-205 is highly expressed in multiple tissues such as the esophagus, trachea, prostate, thymus and breast tissues [25, 26]. The aberrant miR-205 expression is found in many solid tumors. While miR-205 is over-expressed in non-small cell lung cancer [27-29] and endometrial cancer [30], its expression is down-regulated in prostate cancer [31], melanoma [32-34] and breast cancer [24, 35]. Especially, miR-205 is highly expressed in the normal ducts of the breast but dramatically reduced in the breast tumor tissues [24, 36]. MiR-205 is negatively regulated by HER2, which is one of the biomarkers for worse prognosis in breast cancer [37]. Moreover, the miR-200 family members and miR-205 inhibit epithelial to mesenchymal transition (EMT) via E-cadherin regulation and ZEB1 inhibition [23]. The known tumor suppressive functions of miR-205 in breast cancer include direct repression of several oncogenes such as VEGFA and HER3, as well as suppression of EMT by inhibiting ZEB1 and ZEB2 expression [36, 38, 39].

It was recently established that GTP binding activity, but not the transamidase activity of TG2 was required for expression of EMT markers in melanoma [40] and breast cancer [41] cells. However, to date, the miRNAs that are regulated by TG2 have not been identified. Therefore, this study aimed to verify if miR-200s or miR-205 are down-regulated by TG2 in breast cancer cells. Moreover, we investigated the EMT induction mechanism by TG2 using the MDA-MB-231 cell line that represents the aggressive TNBC phenotype and shows high TG2 expression.

Material and methods

Cancer cell lines and antibodies

The human breast cancer cells, MCF7 and MDA-MD-231, were purchased from ATCC. The MCF7 and MDA-MB-231 cells were grown in RPMI1640, and L-15 media supplemented with 10% FBS and 1 × antibiotics (Gibco, Waltham, MA, USA), respectively. The anti-TG2 antibody was purchased from Abcam (CUB 7402). The others antibodies for western blot analysis were obtained from Cell Signaling.

Generation of stable transgenic breast cancer cell lines

The control (pcDNA3.1) and TG2-expressing (pcDNA3.1-TG2) plasmid vectors were kindly provided by Dr. Soo-Youl Kim (National Cancer Center, South Korea). The MCF7/mock and MCF7/TG2 cells were established using G418 selection (Gibco, 500 mg/ml). The mutant TG2 constructs (C277S and R580A) were stably expressed in the MCF7 cell line by retroviral transfection and selection against puromycin as previously described [7]. The control shRNA (sc-108080) and TG2 shRNA (sc-37514-V) lentiviral particles were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). We generated MDA-MB-231/mock and MDA-MB-231/shTG2 cells by infecting MDA-MB-231 cells with control-shRNA and TG2-shRNA lentiviruses for 48 h followed by puromycin (Gibco, Waltham, MA, USA) selection. We cloned the scrambled control miRNA clone (CmiR0001-MR04, GeneCopoeia) and the partial length of the miR-205 primary precursor sequence into the pcDNA3.1 vector. We transfected the MCF7/TG2-C277S cells with these constructs and selected the stable clones using G418 (Geneticin). The miRNA inhibitor control clone (CmiR-AN0001-AM04) and the anti-miR-205 vector (HmiR-AN0307-AM04) were also purchased from GeneCopoeia (MD, USA) and selected by hygromycin (Gibco, Waltham, MA, USA).

Western blotting

For western blot analysis, cells were lysed on ice in RIPA buffer (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Fifty micrograms of total protein lysates from all samples were electrophoresed on a NuPAGE (4-20%) Tris-Glycine gel in SDS running buffer (Invitrogen, Carlsbad, CA, USA). The separated proteins were transferred to nitrocellulose membranes using the iBlot transfer apparatus (Invitrogen, Carlsbad, CA, USA). Then, the membranes were blocked in Tris-buffered saline containing 0.5% Tween 20 (TBS-T) and 5% BSA for 1 hr at room temperature followed by incubation with the primary antibodies overnight at 4°C. After the membranes were washed thrice with TBS-T for 10 min each, the blots were incubated with the HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA) in TBS-T containing 1% BSA for 1 hour at room temperature. Then the protein bands were visualized using the G-box Chemi Systems (SynGene, India).

MicroRNA extraction and quantitative RT-PCR

Quantitative RT-PCR was performed on RNA isolated from breast cancer cells. Total RNA was extracted from various breast cancer cell lines using the miRNeasy Mini Kit (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions. Then, cDNA was synthesized from purified mRNA samples using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). Quantitative RT-PCR was performed on a LightCycler using SYBR Green (Roche Diagnostics, Switzerland) in the 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster, CA, USA). GAPDH expression was used as an internal control to normalize input cDNA. TaqMan gene expression assays for miR-205 (Hs04231469_s1) and GAPDH (Hs02758991_g1) were purchased from Invitrogen (Carlsbad, CA, USA).

Invasion assay

CytoSelect™ 24-Well Cell Invasion and Migration Assay kits were purchased from Cell Biolabs (CA, USA) and invasion assays were performed according to the manufacturer's instructions. First, the cells were serum-starved overnight, followed by adding 5×10^5 cells in 300 µl of medium without FBS in the upper chamber of transwell plates, whereas the lower chamber was filled with 500 µl of medium with 10% FBS. We stained the cells that migrated and invaded into the lower chamber with crystal violet.

In vivo metastasis assay using intracardiac injection and bioluminescent imaging

Animal experiments were performed in compliance with RARC guidelines of NIH. Five to six-week-old female athymic NCr-nu/nu mice (NCI, Frederick, MD, USA) were anesthetized, laid on their back, and the cell suspension was injected into the left ventricle of the heart through a transdiaphragmatic access with a 27.5-gauge needle. The baseline luciferase activity of MCF7/Mock/LUC, MCF7/TG2-C277S/LUC and MCF7/TG2-C277S/miR-205/LUC cells was assessed by *in vitro* bioluminescent imaging using the IVIS Imaging System (Xenogen, Alameda, CA). Then, we added 150 µg/ml D-luciferin (Caliper Life Science, Hopkinton, MA) to the cell culture medium. Then, equal
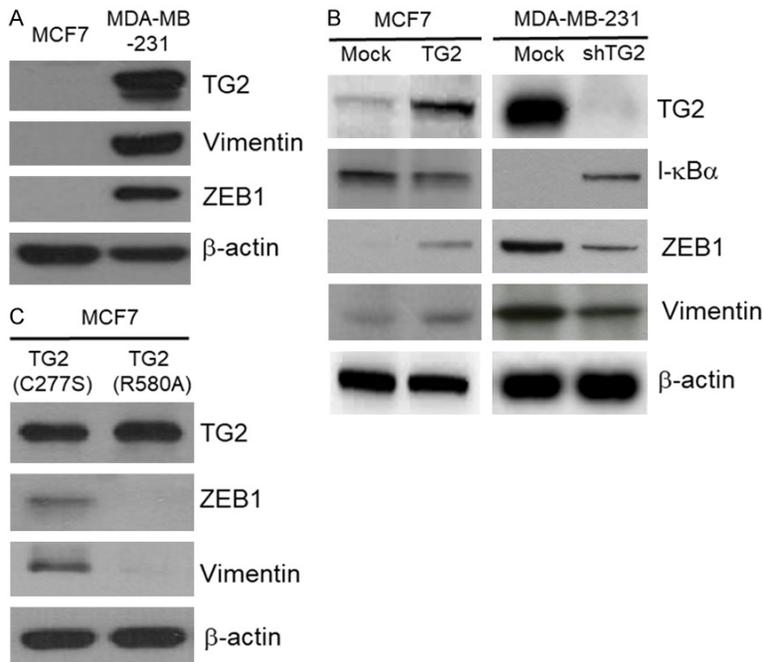
Figure 1. GTP binding activity, but not transamidase activity of TG2 induces EMT. Western blot analysis of TG2, Vimentin and ZEB1 in (A) MCF7 and MDA-MB-231 cells; and (B) mock-transfected MCF7, TG2-transfected MCF7, control shRNA-transfected MDA-MB-231 and TG2 shRNA-transfected MDA-MB-231 cells. (C) Representative immunoblot shows TG2 expression in MCF7 stably transfected with vectors containing transamidase-inactive TG2 (TG2-C277S) and GTP-binding-inactive TG2 (TG2-R580A) constructs.

ing between proteins. To determine if the GTP-binding or the transamidase activities of TG2 play a role in the EMT induction in breast cancer cells, we selected the MCF7 and MDA-MB-231 breast cancer cell lines for this study. The TG2-expressing breast cancer cell line, MDA-MB-231, showed higher expression of EMT-related proteins, Vimentin and ZEB1, than the TG2-deficient MCF7 cell line (**Figure 1A**).
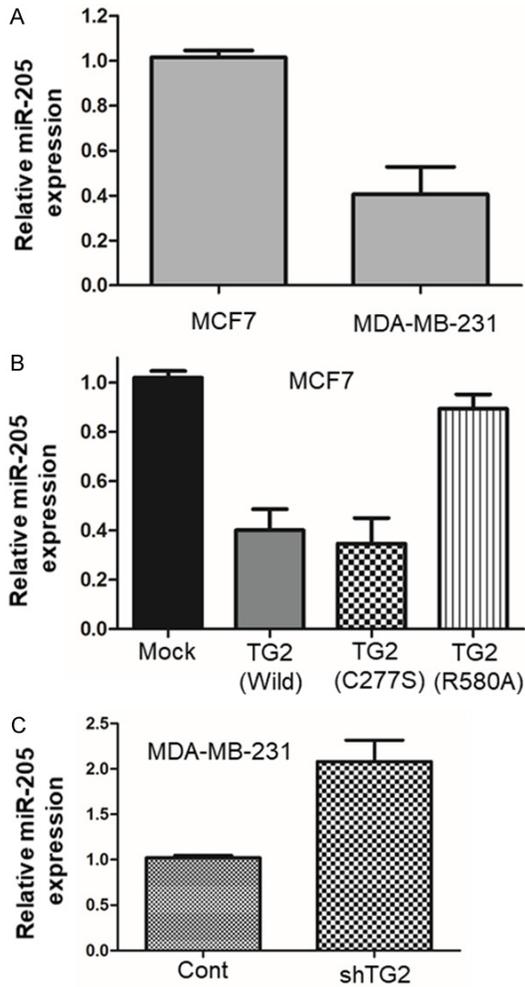
Next, we analyzed ZEB1 and Vimentin levels in TG2-over-expressing and TG2-knock-down breast cancer cells to determine the role of TG2 in activating EMT. TG2-overexpressing MCF7 cells showed higher ZEB1 and Vimentin levels than the mock-transfected comparator MCF7 cells. In contrast, the TG2 knockdown MDA-MB-231 cells showed decreased EMT signaling (low ZEB1 and Vimentin levels) than the control shRNA-transfected MDA-MB-231 cells (**Figure 1B**). Furthermore, the catalytically inactive TG2 (TG2-C277S) effectively induced EMT in the MCF7 breast cancer cells, whereas the GTP-binding-deficient TG2 (TG2-R580A) showed decreased ability to induce EMT (**Figure 1C**). These results suggested that the GTP binding activity of TG2 played a crucial role in induction of EMT in breast cancer cells.

TG2 downregulates miR-205 in breast cancer cells

We performed real-time quantitative RT-PCR in MCF7 and MDA-MB-231 breast cancer cells to detect the expression of miR-205 using U6 small nuclear RNA as an internal control. The MDA-MB-231 cells showed 50% reduced miR-205 expression relative to the MCF7 cells (**Figure 2A**). TG2 overexpressing MCF7 cells showed a 50% decrease in miR-205 levels than the mock-transfected control MCF7 cells. Moreover, MCF7 cells expressing TG2 deficient in GTP binding activity (R580A) showed higher miR-205 expression than the MCF7 cells

numbers (3×10^5) of MCF7/Mock/LUC, MCF7/TG2-C277S/LUC and MCF7/TG2-C277S/miR-205/LUC cells (n=3 each) in 100 µl were injected into the left ventricle of the individual mice.

In vivo bioluminescent imaging was performed using the IVIS Imaging System as previously described [42]. In brief, 150 mg/kg body weight D-luciferin in D-PBS (Dulbecco's phosphate-buffered saline) was injected intraperitoneally into mice, 5 min before imaging. Then, the anesthetized mice were imaged dorsally for 3 min and then ventrally for another 3 min in an imaging box. We acquired the images and analyzed the bioluminescent signals using the Living Image software (Xenogen). Serial bioluminescent imaging was performed every week for 10 weeks.

Results

GTP binding activity, but not transamidase activity of TG2 contributes to EMT induction in breast cancer cells

Previous studies showed that the transamidase activity of TG2 contributed to cross-link-

Figure 2. TG2 downregulates miR-205 in breast cancer cells. A. qRT-PCR analysis of miR-205 expression relative to the housekeeping U6 snRNA in the TG2-deficient MCF7 and TG2-positive MDA-MB-231 breast cancer cell lines. B. QRT-PCR analysis of relative expression of miR-205 in MCF7 cells stably transfected with vectors containing the wild-type TG2, transamidase-inactive TG2 (TG2-C277S) and GTP-binding inactive TG2 (TG2-R580A) constructs. C. QRT-PCR analysis of relative expression of miR-205 in MDA-MB-231 cells stably transfected with vectors containing control shRNA and TG2 shRNA (shTG2) constructs.

expressing TG2 deficient in transamidase activity (C277S), which suggested that GTP binding activity was required for reducing miR-205 expression in MCF7 breast cancer cells (**Figure 2B**). Furthermore, TG2 knockdown by shRNA in MDA-MB-231 cells restored miR-205 expression (**Figure 2C**). These data demonstrate that TG2 regulates miR-205 expression in breast cancer cells.

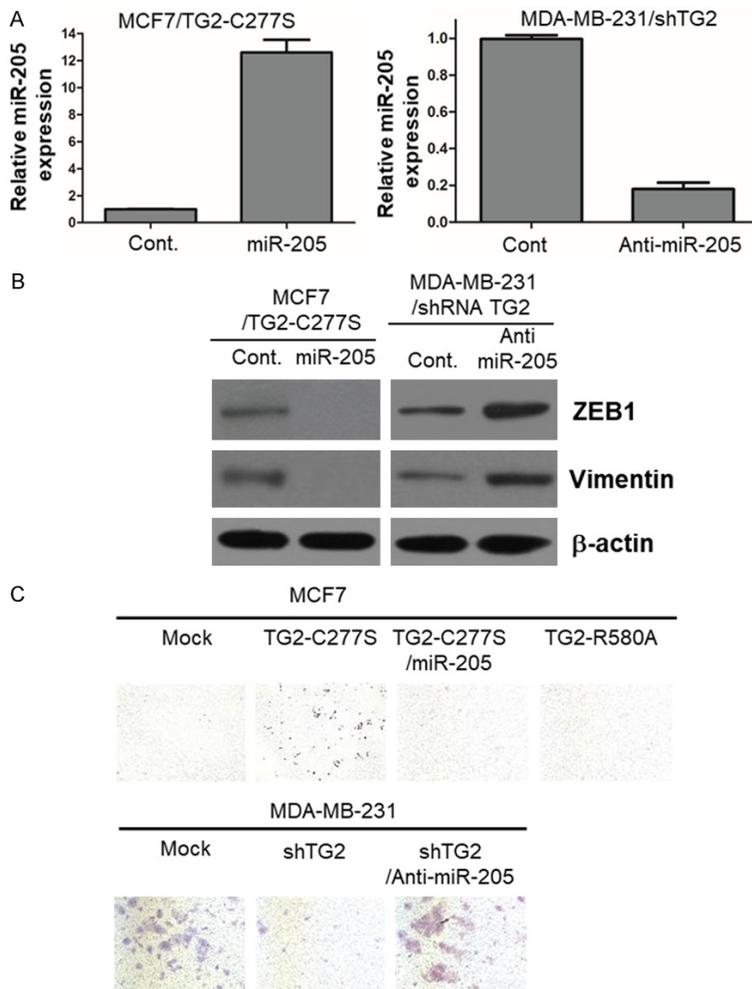*MiR-205 overexpression inhibits EMT induction in breast cancer cells by TG2 harboring GTP binding activity*

Next, we tested if ectopic expression of miR-205 expression in TG2-transfected cells promotes EMT in breast cancer cells. Therefore, we analyzed miR-205 expression by qRT-PCR in MCF7/TG2-C277S cells that ectopically express miR-205 (**Figure 3A**, Left). As shown in **Figure 3B**, ectopic miR-205 expression in MCF7/TG2-C277S cells significantly downregulated ZEB1 and Vimentin expression and concurrently reduced cell invasiveness (**Figure 3C**, Top). Conversely, knockdown of miR-205 by the miR-205 inhibitor (**Figure 3A**, Right) upregulated ZEB1 and Vimentin expression accompanied by increased cell invasiveness (**Figure 3C**, Bottom). These results suggested that TG2 activates ZEB1 and Vimentin expression by downregulating miR-205.

GTP binding activity of TG2 in breast cancer cell contributes to in vivo bone metastasis by inhibiting miR-205 expression

Next, we investigated if the enforced expression of transamidase-deficient TG2 that harbors GTP binding activity was sufficient to promote bone metastasis by using MCF7/TG2-C277S cells that co-expressing luciferase (**Figure 4**). We monitored the progression of bone metastasis after intracardiac injection of tumor cells by bioluminescence imaging (BLI) on a weekly basis. As expected, we demonstrated bone metastasis in the mice injected with TG2-overexpressing MCF7 cells, MCF7/TG2-C277S/Luc cells within 10 weeks, whereas mice injected with mock-transfected MCF7 cells did not show bone metastasis in 10 weeks (**Figure 4A**). These findings indicate that enforced expression of TG2 is sufficient to promote bone metastasis of MCF7 breast cancer cells.

We further tested if miR-205 overexpression could functionally inhibit bone metastasis of TG2 expressing breast cancer cells. We stably transfected miR-205 expression vector into the MCF7/TG2-C277S/Luc cells. Then, we monitored bone metastasis weekly by bioluminescence imaging (BLI) mice injected intracardially with miR-205 expressing MCF7/TG2-C277S/Luc cells. Our data showed that miR-205 induction effectively inhibited bone metastasis of

Figure 3. MiR-205 overexpression inhibits EMT induction by transamidase-inactive TG2 that harbors GTP binding activity in breast cancer cells. qRT-PCR analysis of the relative expression of (A) miR-205 and (B) EMT markers ZEB1 and Vimentin in TG2-overexpressing MCF7 (MCF7/TG2-C277S) cells that are stably transfected with a vector containing control and miR-205 constructs, and TG2-knockdown MDA-MB-231 cells (MDA-MB-231/shTG2) that are stably transfected with vector harboring control and anti-miR-205 constructs. (C) Representative photographs show in vitro invasion assay results of transamidase-inactive TG2 (TG2-C277S) expressing MCF7 cells that are stably transfected with vectors containing control miRNA and miR-205, and TG2-knockdown MDA-MB-231 cells (MDA-MB-231/shTG2) that are stably transfected with anti-microRNA control and anti-miR-205 constructs.

MCF7/TG2-C277S/Luc cells after 10 weeks relative to control-miR expressing MCF7/TG2-C277S/Luc cells (**Figure 4A**). Intracardiac injection of the MCF7/TG2-C277S cells that express the transamidation-inactive form resulted in bone metastases, especially jaw and knee, in the mice in 10 weeks. However, mice that received an intracardiac injection of MCF7/TG2-R580A cells, which express the GTP-binding-deficient form of TG2, did not show any bone metastases in 10 weeks (**Figure 4B**). These findings indicate that over-expression of miR-205 inhibits bone metastasis of breast cancer cells expressing the transamidation-inactive form of TG2 (TG2-C277S).

Discussion

During the past decades, several studies have reported increased expression of TG2 in various types of cancer cells [9, 43]. Moreover, several reports have demonstrated that high expression of TG2 in cancer cells is associated with metastasis and drug-resistance [10, 12, 44-48]. Our research focused on the role of TG2 in the EMT induction of breast cancer cells. Previous studies have reported that TG2 induces EMT in various tumors. The GTP-binding domain of TG2 is essential for epithelial-to-mesenchymal transition (EMT) in mammary epithelial cells [41] and ovarian cancer [16, 49]. Besides, inhibition of TG2 by siRNA and small-molecule TG2 inhibitors renders drug-resistant cancer cells sensitive to chemotherapeutic drugs and inhibits EMT in both in vitro and in vivo models [17, 50-52].

First of all, we confirmed that the TG2-expressing MDA-MB-231 cells showed higher expression of EMT markers than the TG2-deficient MCF7 cells that didn't express EMT markers (**Figure 1A**, **1B**). This data was consistent with previous data showing that TG2 induces EMT via ZEB1 and Vimentin [14] and NF-κB activation via I-κBα depletion, which are well known in literature [48].

The GTP-binding and transamidation activities of TG2 are known to be mechanistically exclusive to each other and are regulated by GTP and
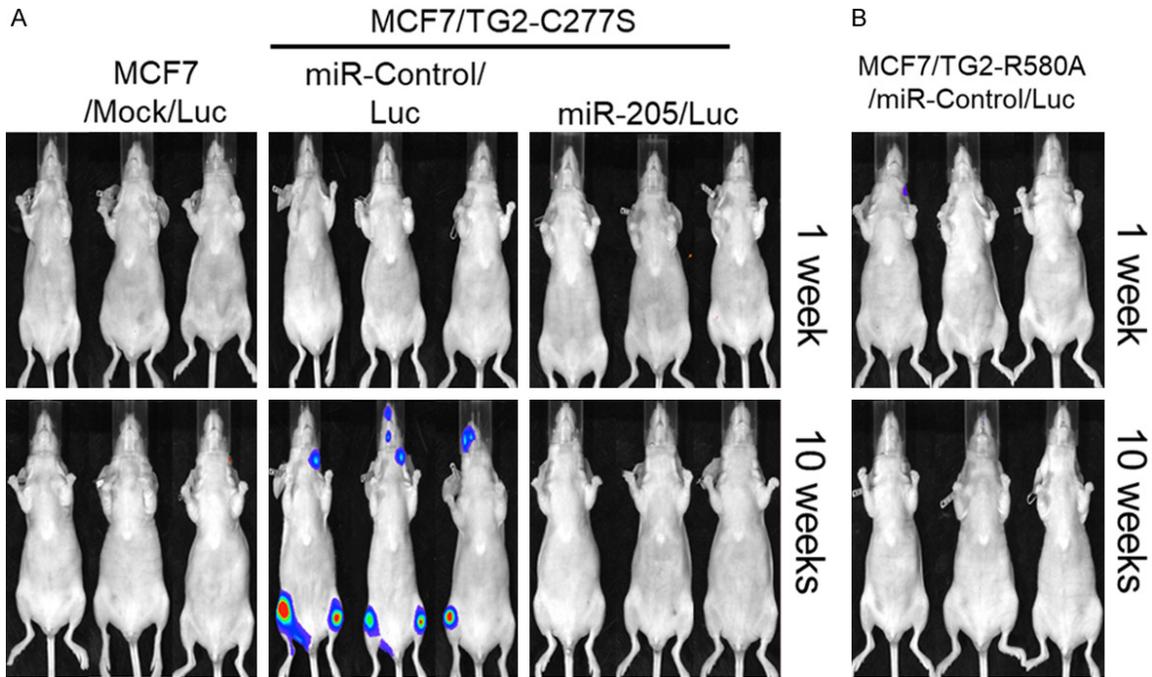
Figure 4. MCF7 breast cancer cells expressing TG2 with GTP binding activity promotes in vivo bone metastasis via miR-205 inhibition. Representative images show bioluminescence monitoring of mice at 1 week and 10 weeks after intracardiac injection of equal number (3×10^5 cells) of (A) luciferase-transfected MCF7/mock cells, transamidase-inactive TG2 (TG2-C277S) expressing MCF7 cells (MCF7/TG2-C277S) and miR-205 overexpressing MCF7/TG2-C277S cells and (B) GTP-binding inactive TG2 (TG2-R580A) expressing MCF7 cells (MCF7/TG2-R580A).

calcium, respectively. The transamidase activity of TG2 is promoted by calcium and blocked by GTP, whereas, the GTPase activity of TG2 is inhibited by calcium [33]. It is still not clear whether intracellular TG2 acts as a transamidase or as a GTPase under physiological conditions.

We used breast cancer cells expressing either the wild-type TG2 (TG2-WT), transamidation-inactive TG2 (TG2-C277S) or the GTP-binding-deficient TG2 (TG2-R580A mutant) to determine the contribution of the transamidation and GTP binding activities of TG2 to EMT induction in breast cancer cells (**Figure 1C**).

Our results confirmed previous reports that transamidation activity of TG2 did not correlate with EMT in various tumor cells [16, 41, 49]. We showed that transamidation-inactive TG2-C277S was as competent as TG2-WT in inducing EMT (**Figures 1B**, **1C** and **3C**). On the contrary, the GTP-binding-inactive R580A mutant inhibited EMT induction, despite high expression and possessing transamidation activity (**Figure 3C**). These results are consistent with previously reported findings [40, 41,

53-55]. However, previous reports had not investigated the mechanistic details of EMT induction by TG2.

Previous studies demonstrate that microRNAs such as miR-495, miR-622 and miR-1 regulate the metastasis of various tumors including gastric cancer [56], thyroid carcinoma [57] and squamous cell carcinoma [58]. Recently, preclinical and clinical studies are underway to determine the therapeutic relevance of several noncoding RNAs such as miRNAs [59]. We measured the expression of miR-205 and miR-200 in MCF7 (TG2-negative) and MDA-MB-231 (TG2-positive) breast cancer cells by qPCR because these two microRNAs are associated with regulation of ZEB1 expression [23, 24, 38]. Moreover, miR-205 modulates breast cancer metastasis [26]. Furthermore, miR-205, but not miR-200, is down-regulated in TG2-expressing MDA-MB-231 cells compared to TG2-negative MCF7 cells (**Figure 2A**). TG2 inhibition induces miR-205 expression in MDA-MB-231 cells, whereas, TG2-transfected MCF7 cells show decreased miR-205 expression than the mock-transfected MCF7 cells. We also demonstrate that the GTP-binding activity of

TG2 plays a critical role in regulating miR-205 in the breast tumor cells (**Figure 2B**).

Next, we tested if miR-205 regulates EMT signaling induction by TG2. As expected, miR-205 blocked TG2-induced EMT signaling by reducing ZEB1 and Vimentin levels (**Figure 3B**) and cell invasiveness (**Figure 3C**). These results suggest that miR-205 acts as a potential tumor suppressor by inhibiting EMT in TG2 expressing breast tumor cells.

Finally, we confirmed that mice intracardially injected with MCF7 cells transfected with the transamidation-inactive TG2 (TG2-C277S) form tumors in the bones, especially jaw and knees. On the contrary, mice intracardially injected with MCF7 cells transfected with the GTP-binding-deficient TG2 (TG2-R580A) did not generate any bone tumors. Moreover, ectopic miR-205 expression inhibited bone metastasis of TG2-C277S transfected MCF7 cells (**Figure 4**). These results suggest that miR-205 potentially mediates inhibition of ZEB1 by binding to its putative target sequences in the 3'-UTR of ZEB1 [38]. Our study indicates that TG2 induces EMT via downregulation of miR-205. Further studies are necessary to investigate the mechanisms by which TG2 regulates miR-205 expression and also address if miR-205 restores drug sensitivity in TG2-induced drug-resistant tumor cells.

In summary, we demonstrate that TG2 (especially the GTP binding activity of TG2) induces EMT by increasing ZEB1 expression via miR-205 inhibition. Therefore, our study suggests that TG2 inhibition or ectopic miR-205 expression is a potential rational approach for the treatment of TG2-induced metastatic breast cancer patients.

Disclosure of conflict of interest

None.

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