MiR-200c suppresses TGF-β signaling and counteracts trastuzumab resistance and metastasis by targeting ZNF217 and ZEB1 in breast cancer

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Resistance to trastuzumab and concomitantly distal metastasis are leading causes of mortality in HER2-positive breast cancers, the molecular basis of which remains largely unknown. Here, we generated trastuzumab-resistant breast cancer cells with increased tumorigenicity and invasiveness compared with parental cells, and observed robust epithelial–mesenchymal transition (EMT) and consistently elevated TGF-β signaling in these cells. MiR-200c, which was the most significantly downregulated miRNA in trastuzumab-resistant cells, restored trastuzumab sensitivity and suppressed invasion of breast cancer cells by concurrently targeting ZNF217, a transcriptional activator of TGF-β, and ZEB1, a known mediator of TGF-β signaling. Given the reported backward inhibition of miR-200c by ZEB1, ZNF217 also exerts a feedback suppression of miR-200c via TGF-β/ZEB1 signaling. Restoration of miR-200c, silencing of ZEB1 or ZNF217 or blockade of TGF-β signaling increased trastuzumab sensitivity and suppressed invasiveness of breast cancer cells. Therefore, our study unraveled nested regulatory circuits of miR-200c/ZEB1 and miR-200c/ZNF217/TGF-β/ZEB1 in synergistically promoting trastuzumab resistance and metastasis of breast cancer cells. These findings provide novel insights into the common role of EMT and related molecular machinery in mediating the malignant phenotypes of breast cancers.

Breast cancers remain the most common malignancies in women with one million newly diagnosed cases and 400,000 deaths worldwide per year.1 The vast majority of these deaths are attributed to distal metastasis and resistance to available therapeutics.1 Overexpression of ErbB2/HER2 occurs in 20–25% of human breast cancers, and is associated with poor prognosis and reduced disease-free survival.2,3 Trastuzumab (Herceptin; Genentech, San Francisco, CA), a humanized monoclonal antibody against the human epidermal growth factor receptor 2 (HER2), has been successfully used for therapy of early-stage and metastatic breast cancers. However, most patients responsive to the initial trastuzumab treatment develop resistance within a year.4 Furthermore, trastuzumab-resistant breast cancers or cell populations are characterized by enhanced metastatic potentials.5 Despite accumulating studies unraveling the mechanisms underlying trastuzumab resistance and metastasis of breast cancers, it remains elusive whether these malignant phenotypes are conjugated and how they are regulated by canonical oncogenic pathways.

Epithelial–mesenchymal transition (EMT) correlates closely with cancer progression by facilitating invasion of malignant cells.6,7 As a key regulator of EMT, TGF-β signaling promotes cancer cell invasion and conferring resistance to various therapies via canonical proliferative and pro-survival pathways involving Ras/MAPK or PI3K/Akt signaling.8 In particular, activated TGF-β signaling is sufficient and necessary for multiple drug resistance in lung cancer and colorectal cancer.9 In breast cancers, the functional crosstalk between HER2 and TGF-β signaling is critically involved in the development of resistance to chemotherapy and HER2-targeted therapy.10 A cohort of transcriptional factors, for
Epithelial-mesenchymal transition (EMT) enables invasion and metastasis by malignant cells. A family of microRNAs (miRNAs) called miR-200 is known to suppress tumor progression by targeting genes involved in EMT, including TGF-β. In this study, the authors found that miR-200c deregulation can cause both resistance to trastuzumab (Herceptin) and metastasis in HER2-positive breast cancers. They also identified several molecular factors that regulate TGF-β activity and form nested, synergistic regulatory circuits involving miR-200c. These findings provide novel insights into the role of EMT and related molecular machinery in mediating the malignant phenotypes of breast cancers.

What's new?

Epithelial-mesenchymal transition (EMT) enables invasion and metastasis by malignant cells. A family of microRNAs (miRNAs) called miR-200 is known to suppress tumor progression by targeting genes involved in EMT, including TGF-β. In this study, the authors found that miR-200c deregulation can cause both resistance to trastuzumab (Herceptin) and metastasis in HER2-positive breast cancers. They also identified several molecular factors that regulate TGF-β activity and form nested, synergistic regulatory circuits involving miR-200c. These findings provide novel insights into the role of EMT and related molecular machinery in mediating the malignant phenotypes of breast cancers.

Example, Snail, Twist, ZEB1, are activated by TGF-β signaling, which causes altered gene expression profiles culminating in uncontrolled proliferation, inhibited apoptosis and elevated invasiveness. Meanwhile, TGF-β signal is regulated by diverse cancer-related genes encoding transcriptional factors or microRNAs (miRNAs). For example, the Krüppel-like zinc finger protein ZNF217 promotes TGF-β signaling by transcriptionally activating TGF-β2 and TGF-β3. Given the documented role of EMT in tumor progression, deciphering the role of TGF-β signaling in trastuzumab resistance and metastasis of HER2-positive breast cancers will provide novel insights into the molecular crosstalk underlying multifaceted malignant phenotypes of breast cancers.

MiRNAs are 20–22 nucleotide noncoding RNAs which post-transcriptionally silence target genes by annealing to the 3′-untranslated region (UTR) of the respective mRNAs. The miR-200 family is well known to suppress tumor progression by targeting multiple genes involved in EMT, metastasis and angiogenesis of neoplasms. In this study, we generated trastuzumab-resistant breast cancer cells with increased tumorigenicity and invasiveness, screened for candidate miRNAs responsible for these malignant phenotypes, and focused on the role of miR-200c, which was the most remarkably downregulated miRNA in these cells. We defined ZNF217, a transcriptional activator of autocrine TGF-β, and ZEB1, a mediator of TGF-β signaling as direct targets of miR-200c, and unraveled nested feedback circuits in the context of TGF-β signaling involved in concomitant regulation of trastuzumab resistance and metastasis of breast cancers.

Material and Methods

Cell culture, drugs and generation of trastuzumab-resistant cells

Human breast cancer SKBr-3 cell line was purchased from ATCC (Manassas). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified incubator with 5% CO2. Recombinant TGF-β2 (BioLegend, San Diego) and Docetaxel (Sigma, St. Louis) were used for incubation with cells at working concentrations of 20 ng/ml and 4 nM, respectively. Trastuzumab (Herceptin) was obtained from Roche (Basel, Switzerland) and dissolved in sterile water. Trastuzumab-resistant (TR) cells were developed by continuous culture of SKBr-3 cells in the presence of 5 μg/ml trastuzumab for 6 months as reported. Thereafter, TR and parental (WT) SKBr-3 cells were cultured in parallel with or without trastuzumab, respectively.

MiRNA microarray and data submission

MiRNA microarray analysis was performed using parental and trastuzumab-resistant SKBr-3 cell lines. Briefly, total RNAs were extracted from the cultured cells using TRIzol (Invitrogen, Carlsbad, CA). The samples were then labeled using the miRCURY™ Hy3™/Hy5™ Power Labeling Kit (Exiqon, Denmark) and hybridized with the miRCURY LNA Array (Exiqon, version 11.0). Scanning was performed using an Axon GenePix 4000B microarray scanner, and the independent microarray analyses were performed for the paired samples. GenePix Pro software (version 6.0) was used to determine the raw intensities of the images. Randomly hierarchical clustering was performed on the miRNA expression profiles.

The above microarray data have been deposited in Gene Expression Omnibus (GEO, assigned accession #: GSE47011).

Plasmid construction and preparation of lentivirus

The coding sequence of ZNF217 was amplified from cDNAs prepared with trastuzumab-resistant cells. The following paired primers were used: 5′-TTGCTAGCCACATGCAATC GAAAGTGACAGGAAAC-3′ and 5′-TACTGGTAA CTGG GATCCGCCTC-3′, and 5′-CTCCGTTGAGCCGGATCCC AAGTTAC-3′ and 5′-TTTCTTAGATCCCCCTTAATTAGTGA ATCAAG. The resulting 1.1 kb and 2.3 kb fragments were cloned to the NheI/BamHI and BamHI/XhoI sites of pcDNA3.1(+) (+). The ZNF217 cDNA was then subcloned into the lentivirus-based expression plasmid pLenti6/V5 (Invitrogen), and then virus packaging and infection were performed according to protocols as recommended by the manufacturer.

MiRNA mimics, miRNA inhibitors (miR-ASO) and siRNA transfection

All synthetic miRNAs and miRNA inhibitors including negative control (scrambled RNAs), miR-200c, miR-200c inhibitor and negative control (scrambled RNAs) were purchased from Shanghai Genechem (Shanghai, China). Silencer Select Negative Control siRNAs (scrambled RNAs), TGFBR2 siRNA, ZEB1 siRNA and ZNF217 siRNA were obtained from...
Genechem. TGFBR2 siRNA sense 5'-GGUCGCUUUGCU GAGGUCUC-3' and antisense 5'-AGACCUAGCUUAAAGCGA CC-3', ZEB1 siRNA sense 5'-GGCGCCGAUAACGCUU CAAA-3' and antisense 5'-UGUAACGUAUUGCCCGC CG-3', and ZNF217 siRNA sense 5'-GGUCUCCUAAUCCCA AUATT-3' and antisense 5'-UAUUGGAAUAGGAAG CCTT-3' were annealed. MiR-200c mimic was transfected at a concentration of 30 nM and miR-200c inhibitor at a concentration of 60 nM.

**MTT assay and soft agar colony formation assay**

Cells (3,000/well) were seeded in 96-well plates and exposed to trastuzumab in complete medium after overnight serum starvation. Cell proliferation was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-H-tetrazolium] assay with minor modifications. Briefly, 48 hr after transfection, the transfection medium was replaced with 100 µl fresh serum-free medium with 0.5 g/l MTT. After incubation at 37°C for 4 hr, the MTT-containing medium was removed by aspiration and 50 µl DMSO was added. After incubation at 37°C for a further 10 min, absorbance was measured at a wavelength of 490 nm in a plate reader. Soft agar colony formation was performed as described previously and measured after 20 days.

**Apoptosis assay**

Cells were plated in six-well plates (4 × 10⁵ cells/well) and transfected with miR-200c or miR-ASO using Lipofectamine 2000 (Invitrogen). SKBr-3 cells were exposed to trastuzumab at a final concentration of 5 (WT cells) and 10 µg/ml (TR cells), respectively. Twenty-four hours after adding trastuzumab, cells were stained with annexinV-FITC and propidium iodide (PI) and flow cytometry was performed to detect apoptosis of the transfected cells.

**Wound healing assay and matrigel invasion analysis**

For wound healing assays, wound closure was observed by taking photographs under a microscope at 0, 24 and 48 hr after scratching. Invasion assay was performed with matrigel (BD Biosciences, Heidelberg, Germany) following the manufacturer's instructions. Photographs of three randomly selected fields of fixed cells were taken and cells were counted in high power fields (hpf) by light microscopy.

**Quantitative RT-PCR for miRNAs and mRNAs**

Total RNA was extracted by Trizol reagent (Invitrogen) according to the manufacturer's protocol. For miRNA, reverse transcription reaction was performed with SuperScript® II reverse transcriptase (Invitrogen). cDNA was detected using SYBR® Premix Ex Taq™ (TaKaRa Bio Group, Shiga, Japan). For quantifying miRNAs, a miScript reverse transcription kit (Qiagen, Hilden, Germany) was used for reverse transcription, followed by amplification using SYBR® Premix Ex Taq™ (Takara). GAPDH and U6 RNA were used as internal loading controls for mRNAs and miRNAs, respectively. The following primers were used for qRT-PCR amplification of miRNAs: universal primer (UP) in the miScript reverse transcription kit (Qiagen) and 5'-TAATACTGGCAGAT GTAGGA-3' or 5'-GTTGGCTGCTTCCGGCAACATAT-3' for miR-200c or U6 RNA, respectively. Primers used for PCR quantification are listed in Supporting Information Table S1.

**Western blot analysis**

Cells were washed in phosphate buffered saline (PBS) twice before proteins were extracted, and proteins were separated on a SDS/PAGE gel, transferred onto a PVDF membrane and subjected to immunoblot analysis. Blotting was performed with antibodies against E-cadherin (BD Biosciences), vimentin (Santa Cruz, CA), ZEB1 (Santa Cruz), ZNF217 (Abcam, Cambridge, UK), smad-3 (Cell Signaling Technology, Boston), p-smad3 (Cell Signaling Technology) and GAPDH (Abcam). Goat anti-rabbit and goat anti-mouse immunoglobulin horseradish peroxidase-linked F(ab)2 fragments (ZB-2305, Zhong Shan JinQiao, Beijing, China) were used as secondary antibodies.

**Construction of ZEB1 and ZNF217 untranslated region luciferase plasmids and reporter assay**

The 3' untranslated region (UTR) fragments of ZEB1 and ZNF217 containing the miRNA target sites were amplified from the cDNA of TR cells. The intact 3'UTRs of ZEB1 and ZNF217 and mutant 3'UTRs with nucleotide substitutions in the putative binding sites corresponding to the seed sequence of miR-200c were cloned downstream of the firefly luciferase gene in the pGL3 vector (Promega, Madison, WI). Primers used for PCR amplification of ZEB1 and ZNF217 3'UTRs are listed in Supporting Information Table S2. These constructs were co-transfected with miR-200c or a control miRNA into HEK293A cells. Cells were rinsed in PBS 48 hr later, and luciferase activity was assessed using the dual luciferase reporter assay system with a luminometer (Promega).

**In vivo tumor development and growth assay**

Athymic nude mice (Institute of Zoology, Chinese Academy of Sciences, Shanghai, China), 4–6 weeks old, were randomly assigned into groups. TR cells (3 × 10⁶) were injected in the right mammary fat pad in situ. When the xenograft tumors formed by the TR cells reached 50 mm³, miR-200c and control miRNA (15 µg/injection, twice a week) complexed with a lipid-based delivery agent were injected into the tumors 72 hr prior to intravenous injection of trastuzumab (10 mg/kg, twice a week). Tumor volume was calculated as abc/6. After the last in vivo optical imaging, all mice were sacrificed for further analysis. All experimental protocols were performed in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guideline of the UK and were approved by the Institutional Animal Care and Use Committee of Fourth Military Medical University.

**Metastasis assay in nude mice**

TR cells (1 × 10⁶), transfected with miR-200c or control miRNA, were injected through the tail vein into athymic nude mice. MiR-200c and control miRNA were injected every 2 days (1 mg of oligos per kg of body weight). Mice became moribund and were sacrificed after pulmonary...
perfusion 4 weeks later. The number of tumor nodules formed on the lung surfaces was counted. Lungs were excised and fixed in 10% formaldehyde, after which preparation of 0.3 μm paraffin-embedded sections were prepared and stained with hematoxylin and eosin (H&E).

Statistical analysis
Statistical analysis was performed using the software SPSS 16.0 for Windows. Student’s t-test was used for analyzing the results expressed as mean ± SD. Differences were considered significant when p < 0.05.

Results
Acquisition of trastuzumab resistance causes concomitantly enhanced invasiveness of breast cancer cells
To develop an in vitro model of acquired trastuzumab resistance, the HER2-overexpressing breast cancer cell line, SKBr-3 (wild-type cells or WT cells), was continuously exposed to 5 μg/ml trastuzumab for 6 months until cells had acquired resistance to trastuzumab (trastuzumab-resistant cells or TR cells).17 Resistance of the cells to trastuzumab was confirmed by MTT assays (Fig. 1a). Consistent with previous studies characterizing trastuzumab-resistant cancer cells,2 we observed significantly decreased HER2 and PTEN expression in TR cells (Supporting Information Fig. S1).

We next investigated the malignant behaviors of trastuzumab-resistant breast cancer cells. Compared with WT cells, TR cells exhibited significantly increased colony formation on soft agar (p < 0.001, Fig. 1b) and enhanced invasiveness in a transwell assay (p < 0.001, Fig. 1c). More interestingly, we found that TR cells appeared to have lost their tight cell–cell contacts and grew as loosely packed spindle-like fibroblastic cells and began spreading as individual cells with pseudopodia, strongly suggesting the acquisition of mesenchymal properties (Fig. 1d). Consistent with the morphological changes of EMT, significant downregulation of the epithelial marker E-cadherin and upregulation of mesenchymal protein Vimentin

Figure 1. High invasiveness and EMT of trastuzumab-resistant breast cancer cells. Human breast cancer SKBr-3 cells were cultured in the presence of trastuzumab (5 μg/ml) for 6 months to obtain trastuzumab-resistant (TR) cells. TR and wild-type (WT) SKBr-3 cells were exposed to trastuzumab (5 μg/ml) for 24 hr and subjected to MTT assays (a). Cells were subjected to colony formation assay in soft agar (b) or transwell assay of invasion (c). Cells were photographed using a phase contrast microscope (d) or subjected to Western blotting (e) or qRT-PCR assay (f) for expression of indicated genes. FN, fibronectin. GAPDH was used to normalize the qRT-PCR data. Data are represented as the mean ± SD of n = 3 replicates or representative of three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 (Student’s t-test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
were observed in the TR cells (Fig. 1e). We also observed significantly increased mRNA levels of EMT markers including Snail1, Twist1, ETS1, ZEB2 and fibronectin (Fig. 1f). These data suggest that the acquisition of trastuzumab resistance in breast cancers correlates with enhanced invasion potential probably driven by EMT.

**Downregulation of miR-200c accounts for high invasiveness and trastuzumab resistance of breast cancer cells**

MiRNAs have emerged as critical regulators of cell signaling including the TGF-β pathway. To identify miRNAs involved in the regulation of TGF-β signaling in trastuzumab-resistant cells, we performed microarray screening of differentially expressed miRNAs between parental and resistant SKBr-3 cells (Fig. 2a and GEO accession No. GSE47011). Given the established suppressive role of miR-200 family in EMT, we proposed miR-200c, which is among the most significantly decreased miRNAs in the TR cells in microarray assay, is involved in the aforementioned malignant phenotypes of trastuzumab-resistant cells. Quantitative RT-PCR confirmed the significant downregulation of miR-200c in the TR cells (p < 0.001, Fig. 2b). We found that introduction of miR-200c mimics could significantly reverse a mesenchymal phenotype of TR cells, which was consistent with a dramatic upregulation of E-cadherin and downregulation of Vimentin (Figs. 2c and 2d). MiR-200c enhanced the sensitivity of the TR cells to trastuzumab, and miR-200c inhibition by ASO could confer resistance in the WT cells when treated with increasing concentrations of trastuzumab in MTT assays (Fig. 2e). Concurrently, miR-200c mimics caused a marked increase of TR cells undergoing apoptosis when treated with trastuzumab for 3 days (p < 0.001, Fig. 2f), and inhibition of miR-200c by ASO in both WT SKBr-3 cells and another HER2-positive breast cancer cell line, BT474, caused significantly suppressed apoptosis of the WT cells exposed to trastuzumab (p < 0.001, Fig. 2f). In addition, inhibition of miR-200c desensitized SKBr-3 to the chemotherapeutic drug docetaxel (Supporting Information Fig. S2). In parallel with its role in trastuzumab resistance, miR-200c could suppress the migration of TR cells in a wound healing assay (Fig. 2g), and dramatically inhibited the invasion capacity of TR cells on a matrigel (p < 0.001, Fig. 2h). Intriguingly, the expression of HER2 was also restored by introduction of miR-200c in TR cells (Supporting Information Fig. S3). Therefore, a defect in miR-200c expression may underlie the acquisition of trastuzumab resistance and invasiveness of breast cancer cells.

**Elevated TGF-β signaling in response to miR-200c suppression is involved in invasiveness and trastuzumab resistance of breast cancer cells**

TGF-β signaling is a key regulator of EMT and is sufficient for multiple drug resistance in various malignancies. Thus, we examined the levels of TGF-β signaling in trastuzumab-resistant breast cancer cells. As expected, elevated Smad3 phosphorylation was observed in TR cells compared with the parental cells, suggestive of the involvement of TGF-β signaling in acquired resistance of breast cancers to trastuzumab (Fig. 3a). Consistently, as the predominant inducers of TGF-β signaling, the autocrine TGF-β2 and TGF-β3 were significantly upregulated in TR cells (Fig. 3b). Blockade of TGF-β signaling by knockdown of the type 2 TGF-β receptor (TGFBR2) increased trastuzumab sensitivity (Fig. 3c) and inhibited the invasiveness (p < 0.01, Fig. 3d) of TR cells, which was in line with decreased expression of the documented EMT markers (Fig. 3e).

We next probed the regulatory role of miR-200c in TGF-β signaling of breast cancer cells. We found that transfection of miR-200c suppressed the expression of TGF-β2 and TGF-β3 and dampened TGF-β signaling in TR cells (Figs. 3f and 3g). Conversely, transfection of allele-specific oligonucleotide (ASO) inhibitor of miR-200c resulted in upregulated TGF-β isoforms and elevated Smad3 phosphorylation in WT breast cancer cells (Figs. 3f and 3g), suggesting that miR-200c suppresses TGF-β signaling in HER2-positive breast cancer cells. These data in synergy with previous reports showed that TGF-β signaling is elevated due to miR-200c downregulation and is critically involved in acquisition of trastuzumab resistance and enhanced invasiveness of breast cancer cells.

**MiR-200c suppresses malignant phenotypes of breast cancer cells by targeting ZNF217 and ZEB1**

MiRNAs modulate gene expression by inhibiting the translation or promoting the degradation of specific mRNAs. We searched for the potential targets of miR-200c using TargetScan, pictar, DIANA-MICROT and miRanda softwares. MiR-200c was predicted to target ZEB1, which was consistent with previous study, and ZNF217, a Krüppel-like zinc finger protein family member (Fig. 4a). Consistently, we found that both the mRNA and protein levels of ZEB1 were significantly upregulated in TR cells compared to WT cells, whereas transfection of TR cells with miR-200c significantly hampered the expression of ZEB1 (Figs. 4b and 4c). In parallel, the protein but not the mRNA level of ZNF217 was much higher in TR cells than in WT cells, suggesting a potent translational inhibition of ZNF217 in TR cells (Figs. 4b and 4c). Consistently, miR-200c mimics did not affect the mRNA level of ZNF217 but downregulated the ZNF217 protein significantly in these cells (Figs. 4b and 4c). To further validate the silencing of ZEB1 and ZNF217 by miR-200c, we performed a dual luciferase reporter gene assay. The fragments of the 3'-UTR of ZEB1 and ZNF217, containing the potential miR-200c binding site and mutations in their seed sequences, were cloned into a vector with the firefly luciferase reporter gene. Then these constructs were co-transfected with miR-200c or a control miRNA into the HEK 293A cells. As expected, miR-200c dramatically decreased the expression of luciferase flanked by the 3'-UTR of ZEB1 or ZNF217, whereas the mutations in the predicted miR-200c binding sites of 3'-UTR abrogated the suppression of luciferase.
Figure 2.
activity (Fig. 4d). These results showed that ZEB1 and ZNF217 are direct targets of miR-200c in breast cancer cells.

We next investigated whether ZEB1 and ZNF217 are responsible for the high invasiveness and trastuzumab resistance of breast cancer cells. We found that knockdown of either ZEB1 or ZNF217 caused suppressed invasion and migration of TR cells in transwell and wound-healing assays (p < 0.01, Figs. 4e–4g). In addition, ZEB1 or ZNF217 silencing significantly expanded the cytotoxicity of trastuzumab to TR cells as revealed by suppressed cell growth (Fig. 4h) and efficient induction of apoptosis (p < 0.001, Fig. 4i). Thus, knockdown of ZEB1 and ZNF217 in trastuzumab-resistant breast cancer cells repressed cell invasion and at least partially restored sensitivity of cells to trastuzumab.

ZNF217 exerts feedback inhibition of miR-200c by TGF-β signaling in trastuzumab-resistant breast cancer cells

ZNF217 was reported to promote EMT by transcriptionally activating TGF-β, whereas ZEB1 was a mediator of TGF-β signaling in EMT.11,12 Consistently, we found that both knockdown of ZEB1 and ZNF217 reversed the mesenchymal transition observed in the TR cells (Figs. 5a and 5b). These observations were in line with suppressed autocrine production of TGF-β2 and TGF-β3 and impaired TGF-β signaling in ZNF217 knockdown TR cells (Figs. 5c and 5d). As a result, silencing of either ZNF217 or TGFBR2 suppressed the expression of ZEB1, which is a target gene in TGF-β pathway (Figs. 5d and 5e). ZEB1 was reported to transcriptionally repress miR-200c, thus forming a feedback inhibitory mechanism in epithelial-derived tumors.23 Indeed, we found that ZEB1 knockdown significantly increased the miR-200c level in TR cells (Fig. 5f). Consistently, the downregulation of ZEB1 by knockdown of ZNF217 or TGFBR2 resulted in restored expression of miR-200c (Fig. 5g). Co-silencing of ZNF217 and ZEB1 showed a suppressive effect on miR-200c comparable to solely ZEB1 knockdown, suggesting the common pathway involved in miR-200c downregulation by ZNF217 and ZEB1 (Fig. 5h). Conversely, ZNF217 overexpression in WT SKBr-3 cells caused pronounced inhibition of miR-200c, which is dependent on TGF-β signaling, whereas treatment of cells with recombinant TGF-β2 suppressed miR-200c regardless of cellular ZNF217 levels (Fig. 5i). Therefore, miR-200c was inhibited in trastuzumab-resistant breast cancer cells by nested feedback signaling circuits of miR-200c/ZEB1 and miR-200c/ZNF217/TGF-β/ZEB1 (Fig. 5j).

MiR-200c re-sensitizes trastuzumab-resistant cells to trastuzumab and suppresses metastasis in vivo

To investigate whether miR-200c could restore the trastuzumab sensitivity in breast cancers in vivo, we inoculated the mammary fat pads of athymic nude mice in situ with TR SKBr-3 cells. When the xenograft tumors of the TR cells reached 50 mm³, miR-200c and control miRNA (15 µg/injection, twice a week) complexed with a lipid-based delivery agent were injected into the tumors 72 hr prior to intravenous injection of trastuzumab (10 mg/kg, twice a week)17 and mice were sacrificed at day 30. As expected, trastuzumab inhibited the growth of TR cells transfected with miR-200c but not that of TR cells transfected with control miRNA (Figs. 6a–6c). These data suggested that miR-200c is effective in restoring trastuzumab sensitivity in a xenograft breast cancer model.

We next explored the effect of miR-200c on metastasis of tumors developed from TR breast cancer cells. TR cells were transfected with miR-200c or control miRNA and were randomly injected into athymic nude mice via tail vein, followed by administration of miR-200c and control miRNA every 2 days at a dose of 1 mg/kg of body weight.21 Mice became moribund and were sacrificed after pulmonary perfusion 4 weeks later. Metastatic nodules were observed on the surface of the lungs in mice injected with control miRNA, but no nodules were found in mice injected with miR-200c (Figs. 6d and 6e). Consistent with these results, an H&E assay confirmed the presence of nodules found in the lungs of the mice injected with control miRNA, but not in those of mice injected with miR-200c (Fig. 6f). These data showed that miR-200c could inhibit the metastasis of TR cells in vivo.

Discussion

Trastuzumab resistance and distal metastasis are leading causes of mortality in patients with HER2-positive breast cancers.2,24 The molecular machinery underlying either of these malignant phenotypes has been extensively deciphered.2,24 For instance, resistance to trastuzumab can be attributed to reduced HER2
expression or antibody affinity, increased pro-survival signaling through alternative receptor tyrosine kinases and altered intracellular signaling such as the loss of PTEN. Nevertheless, the clinical observations that trastuzumab displayed potent inhibition of multi-organ metastases and that loss of HER2 expression facilitated distal metastasis of breast cancers receiving trastuzumab treatment suggest a strong link between trastuzumab resistance and metastasis. Our study demonstrated that the acquisition of trastuzumab resistance is characterized by enhanced invasiveness of breast cancer cells with concomitant EMT and elevated TGF-β signaling. Downregulation of miR-200c underlies the modulation of the aforementioned

Figure 3. TGF-β signaling is activated by miR-200c suppression in trastuzumab resistance and EMT of breast cancer cells. (a, b) Western blotting (a) and qRT-PCR (b) were performed using WT and TR SKBr-3 cells. p-smad3, phosphorylated smad3. (c–e) SiRNAs were used for knockdown of TGF-β receptor type 2 (TGFBR2), followed by MTT assay of cells treated with indicated doses of trastuzumab (c), and transwell (d) and qRT-PCR (e) assays of transfected cells. (f, g) Cells were transfected with synthesized miR-200c mimics (for WT cells), and qRT-PCR (f) and Western blot (g) analysis were performed. GAPDH was used to normalize the qRT-PCR data. Data are represented as the mean ± SD of n = 3 replicates or representative of three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 (Student’s t-test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Figure 4. ZEB1 and ZNF217 are direct targets of miR-200c in suppression of breast cancer cell invasion and trastuzumab resistance. (a) The predicted binding sequences of human miR-200c to the wide-type and mutant 3′ UTR of ZEB1 and ZNF217. (b, c) Untransfected cells or TR cells transfected with miR-200 mimics or control oligonucleotides were subjected to qRT-PCR (b) or Western blot analysis (c). (d) pGL3 constructs containing intact or mutant 3′ UTR of ZEB1 or ZNF217 were co-transfected with scrambled or miR-200c mimics into HEK293A cells. Luciferase activity was measured 48 hr after transfection. (e–j) TR cells were transfected with siRNAs targeting the indicated genes, and were subjected to qRT-PCR (e), transwell (f) or wound healing (g) assays, to MTT assays after incubation with indicated doses of trastuzumab (h), or to annexin V/PI staining for flow cytometry of apoptotic cells after incubation with trastuzumab (10 μg/ml) for 24 hr (i). GAPDH was used to normalize the qRT-PCR data. Data are represented as the mean ± SD of n = 3 replicates or representative of three independent experiments. **p < 0.01 and ***p < 0.001 (Student’s t-test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Figure 5. ZEB1 and ZNF217 promote EMT and exert feedback inhibition of miR-200c in trastuzumab-resistant breast cancer cells. (a–h) TR cells transfected with control or indicated siRNAs were photographed using a phase contrast microscope (a), immunoblotted (b, d), and subjected to qRT-PCR assays (c, e–h). (i) WT SKBr-3 cells were infected with control or ZNF217-expressing recombinant lentiviruses (upper panel), or incubated with recombinant TGF-β2 (lower panel), combined with transfection with indicated siRNAs. Cells were then subjected to qRT-PCR assay for miR-200c. (j) Schematic diagram of the nested feedback loops of miR-200c/ZEB1 and miR-200c/ZNF217/TGF-β2/ZEB1 in breast cancer cells. GAPDH was used to normalize the qRT-PCR data. Data are represented as the mean ± SD of n = 3 replicates or representative of three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with the control or mock group (Student’s t-test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
phenotypes of breast cancer cells via two nested regulatory loops, that is, miR-200c/ZEB1 and miR-200c/ZNF217/TGF-β/ZEB1. Re-expression of miR-200c or knockdown of ZNF217 and ZEB1 could trigger a shift and re-balance of this regulatory cycle and reverse both trastuzumab resistance and metastasis in HER2-positive breast cancer cells.

Mesenchymal transition has been well-characterized as a driver of metastasis and hallmark of cancer-initiating cells.22 Meanwhile, accumulating evidence has suggested a strong link between EMT and drug resistance.27 For instance, hepatocellular and lung cancer cells resistant to EGFR inhibitors undergo EMT.28,29 EMT-like transition promoted by increased Wnt3 or β1-integrin was also observed in trastuzumab-resistant breast cancer cells.30,31 We established an in vitro model of trastuzumab resistance in breast cancer cells and found that these cells displayed greater invasive capacities than the wide-type cancer cells, and exhibited significant morphological and biochemical properties of EMT. As a master regulator of EMT, TGF-β signaling plays essential roles in regulating the malignant phenotypes, for example, drug resistance, of breast cancers.11,22 In accordance with these findings, we detected in trastuzumab-resistant cells substantially elevated TGF-β signaling, and blockade of TGF-β signaling caused increased trastuzumab sensitivity and suppressed invasiveness of breast cancer cells. Although it remains to be resolved how mesenchymal transition confers a trastuzumab-resistant phenotype on carcinoma cells, our observations in synergy with previous reports suggest that EMT may represent a common cellular mechanism of tumor progression as well as a causative link between trastuzumab resistance and metastasis in breast cancers.2,22
MiRNAs are key modulators in carcinogenesis and the maintenance of malignant phenotypes, for example, multiple drug resistance.\textsuperscript{14,32} Particularly, miR-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1\textsuperscript{13}; miR-216a/217-induced EMT was found to promote drug resistance in liver cancer\textsuperscript{34}; upregulation of miR-21 mediates resistance to trastuzumab therapy for breast cancers.\textsuperscript{17} We discovered here that elevated activation of TGF-β signaling is attributed to downregulation of miR-200c in trastuzumab-resistant breast cancer cells; restoring miR-200c was sufficient to inhibit TGF-β signaling, thereby re-sensitizing cells to trastuzumab and reversing the malignant phenotypes of trastuzumab-resistant breast cancer cells. We also identified ZEB1 and ZNF217 as two direct targets of miR-200c involved in trastuzumab resistance and metastasis. ZEB1 promotes EMT in many cell types by initiating the expression of a subset of mesenchymal genes.\textsuperscript{35} Furthermore, ZEB1 suppresses miR-200c, a miRNA which directly targets ZEB1, forming a negative miR-200c/ZEB1 feedback loop.\textsuperscript{23} We defined here miR-200c, a miRNA which directly targets ZEB1, forming a feedback crosstalks, whereby trastuzumab resistance may represent a common phenotype of cells with survival or proliferative advantages as outcomes of diverse signaling events.\textsuperscript{2,36} Therefore, the miR-200c/TGF-β-related pathway we demonstrated here may also play a role in overcoming stresses induced by other tumoricidal drugs. Meanwhile, our findings do not exclude that alternative mechanisms, for example, PTEN deficiency as reported by other groups, also exert a role on the malignant phenotypes of the trastuzumab-refractory cell subsets, which was manifested by altered expression of multiple miRNAs including miR-21, a miRNA reported to target PTEN in promoting trastuzumab resistance of breast cancers.\textsuperscript{17} Similarly, the downregulation of HER2, which is believed as a driver of trastuzumab resistance,\textsuperscript{2} was also observed in trastuzumab-resistant cells. Whereas we found that miR-200c is involved in the regulation of HER2 expression, systemic studies are needed to unravel the molecular links between HER2 and the miR-200c/TGF-β pathway we addressed here. In addition, feedback circuits represent a reversible regulatory pattern of cell signaling during transitions of physiological or pathological states.\textsuperscript{36} Here, we established in breast cancers that HER2 overexpression caused HER2-mediated EMT in HER2+ breast cancer by expanding the cancer stem cell population.\textsuperscript{37} These regulatory circuits also have implications in targeted cancer therapy by interference with the key mediators of the circuits, for example, miR-200c, ZEB1 and ZNF217, and thus reversal of both the outcome of the regulatory circuit and the malignant phenotypes of advanced breast cancers.

\section*{References}

13. Klymowsky MW, Savagner P. Epithelial-mesen