Am J Transl Res 2015;7(5):878-890
www.ajtr.org /ISSN:1943-8141/AJTR0006409

Original Article

Special suppressive role of miR-29b in HER2-positive breast cancer cells by targeting Stat3

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Received January 27, 2015; Accepted May 3, 2015; Epub May 15, 2015; Published May 30, 2015

Abstract: Objectives: MiR-29b has been reported to function as a tumor suppressor in a variety of cancers. However, its role in the regulation of breast cancer is controversial. Materials and methods: In this paper, we explored the expression of miR-29b in a cohort of 67 pairs of formalin-fixed paraffin-embedded specimens with detailed pathological and clinical characteristics, and further analyzed the effects of miR-29b on the malignant phenotype of HER-2-positive breast cancer cells and the relevant mechanisms involved. Results: We found that the miR-29b expression is negatively associated with HER-2 expression in breast cancer tissues. Moreover, overexpression of miR-29b induced a complex phenotype in HER-2-positive breast cancer cells, namely an inhibition of cell proliferation, block of G1/S phase transition, induction of cell apoptosis, suppression of cell invasion *in vitro*, as well as inhibition on tumor growth *in vivo*, indicating that miR-29b functions as a tumor suppressor in HER2-positive breast cancer cells. Further bioinformatic prediction suggested that oncogene Stat3, which is an up-stream regulator of HER-2, was a target gene of miR-29b in breast cancer cells. We have shown that knocking down of Stat3 attenuated the malignant phenotype of breast cancer cells similar to overexpression of miR-29b, while restore expression of Stat3 in HER-2-positive breast cancer cells partially abolished the suppressive effects of miR-29b. Conclusion: Collectively, our data suggest that miR-29b could reverse the malignant phenotype of HER-2-positvie breast cancer through, at least partially, targeting *Stat3* signaling pathway.

Keywords: miR-29b, *HER2*, breast cancer, Stat3 signaling pathway

Introduction

Breast cancer is the most common female malignancy worldwide [1]. With the widespreading of early diagnosis and improvements in treatment, the mortality of breast cancer has been reduced during the past decades, whileit remains the second leading cause of cancer-related death among women. The etiology of this neoplasm is complex, and both genetic and environmental factors contribute to the complicate scenario. This has led to the discovery of 5 distinct subtypes of breast carcinomas, including Luminal A, Luminal B, HER2 overexpression, Basal-like and Normal-like, and each has its own unique recognizable phenotypes and clinical outcomes [2-4]. Consequently, it is difficult to find a unanimous and internationally accepted standard chemotherapy regimen [5]. A comprehensive understanding of differences between these subtypes is definitely necessary to the development of novel therapies.

MicroRNAs (miRNAs) are small non-coding RNA molecules that can regulate gene expression by interacting with multiple mRNAs to either suppress the translation of, or degrade the molecules [6, 7]. Accumulating studies have described the important role of miRNAs in the pathogenesis and development of human cancers including breast cancer [8-10]. By targeting different genes, miRNAs are involved in the regulation of plenty biological processes, including cell cycle, differentiation, development, as well as apoptosis [11-14]. miR-29b is an important member of miR-29 family consisting of three mature members miR-29a, -29b and

-29c [15-17]. Although miR-29b has been reported to function as a tumor suppressor in a variety of cancers [15, 18-20], its exact role in the carcinogenesis of breast cancer is still controversial. Chen et al. reported that miR-29b was up-regulated in MDA-MB-231 cells relative to the MCF-7 cells, and contributed to the migration, invasion and anti-apoptosis of MDA-MB-231 cells [21]. Intriguingly, in a cohort of 70 primary human breast cancers whose clinical classification has been collected in detail, miR-29 was reported to be enriched in these tissues with more differentiated and normal luminal epithelial cells, while, in basal-like and HER2-positive subtypes, its expression is much lower. Moreover, stable overexpression of miR-29b promotes and maintains luminal differentiation, and inhibits metastasis of breast cancer cells through targeting a network of pro-metastatic regulators involved in angiogenesis, collagen remodeling and proteolysis, including *VEGFA*, *ANGPTL4*, *PDGF*, *LOX* and *MMP-9* [22], suggesting that miR-29b expression correlates with more favorable outcomes and reduced metastatic potential. Considering the complexity of phenotypes between different cancer subtypes, we speculate that miR-29b might present different function in different tumor subtypes.

In this study, we examined miR-29b expression and its association with HER-2 expression in a cohort of 67 primary human breast cancers. We also performed functional studies of miR-29b in HER-2 positive breast cancer cell lines. The results support miR-29b as a tumor suppressor in HER2-positive breast cancer cells, possibly through targeting the signal transducer and transition activator 3 (*Stat3*) pathway.

Materials and methods

Tumor tissues and cell culture

For microRNAs detection, study encompassed 67 pairs of formalin-fixed paraffin-embedded specimens and adjacent normal tissues. Study design was revised and approved by TCM-Integrated Hospital of Southern Medical University Institutional Ethics Committee and informed consent was obtained from each participant. The matched normal tissues were obtained from the 5cm distant from the tumor margin, which were further confirmed by pathologists. Pathological and clinical information collected for the cohort included patient age at diagnosis, tumor size, lymph node involvement and hormone receptor status were shown in **Table 1**. All patients did not receive any therapy before recruitment to this research. The human mammary carcinoma cell lines SK-BR-3 and MDA-MB-435 were obtained from the Cell Bank of Chinese Academy of Science. SK-BR-3 cells were maintained in the DMEM supplemented with 10% fetal bovine serum. MDA-MB-435 cells were maintained in the L-15 condition supplemented with 10% fetal bovine serum.

RNA isolation and quantitative real-time RT-PCR

The total RNA of FFPE tissues was extracted by miRNeasy FFPE KIT (QIAGEN, Germany). Total RNA was exacted from cells using TRIzol (Invitrogen, USA). The RNA was quantified by assessing its absorbance at 260 nm. The cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Invitrogen, USA). Stem-loop RT-PCR primers were used for the reverse transcription of miR-29b. Quantitative - RT-PCR was conducted using ABI PRISM 7500 real-time PCR system. For the PCR amplification, the SYBR Premix Ex Taq Kit (Takara, Dalian, China) was utilized according to the manufacturer's instructions. U6 snRNA was used as the endogenous control for miR-29b (the sequences of the primers used for reverse transcription: miR-29b, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACACT G-3'; the sequences of the primers used for quantitative PCR: miR-29b, FW: 5'-CGCGUAG CACCAUUUGAAAUC-3', RV: 5'-CAGTGCAGGGTCCGAGGT-3'). The comparative Ct method was used to quantify target gene relative to their endogenous control. For each individual analysis, one of the samples was designated as the calibrator and given a relative value of 1.0; all of the quantities were then expressed as n-fold relative to the calibrator.

Cell transfection

MiR-29b mimic and scramble mimic were obtained from Shanghai GenePharma Company (Shanghai, China) and were transfected using Lipofectamine 2000 (Invitrogen, Long Island, NY, USA) according to the manufacturer's protocol at a final concentration of 50 nM. The Stat3 siRNA and negative control siRNA were synthesized by Shanghai GenePharma Company

(Shanghai, China) and transfected into SK-BR-3 and MDA-MB-435 cells using Lipofectamine 2000 (Invitrogen, Long island, NY, USA) according to the manufacture's protocol. Cells were cultured for 48 h and harvested for next experiment.

Cell proliferation, apoptosis, and cell cycle assay

Cells were incubated in 10% CCK8 (Dojindo; Kumamoto, Japan) diluted in normal culture medium at 37°C until visual color conversion occurred. Proliferation rates were determined at 0, 24, 48 and 72 hours after transfection. The absorbance of each well was measured with a microplate reader set at 450 nm and 650 nm. The apoptosis assay was performed after 72 hours of transfection using the PE Annexin V apoptosis Detection Kit I (BD Pharmingen; San Diego, CA, USA) and then analyzed by fluorescence-activated cell sorting (FACS). Cell cycle analysis was performed on SK-BR-3 and MDA-MB-435 cell lines 48 h after transfection with miR-29b and scramble mimic, respectively. Cells were harvested, washed twice with cold PBS, fixed in ice-cold 70% ethanol, and then incubated with propidium iodide (PI) and RNase A, then analyzed by FACS. Each sample was run in triplicate.

Cell invasion assays

For the invasion assays, after 24 hours transfection, 2×10^5 cells in serum-free media were seeded onto the transwell migration chambers (8 μm pore size; BD Pharmingen; San Diego, CA, USA) coated with Matrigel (BD Pharmingen; San Diego, CA, USA) on the upper chamber. Media containing 20% FBS was added to the lower chamber. After 24 hours, the non-invading cells were removed by a cotton wool, the invasive cells located on the lower surface of the chamber were stained with cristal violet stain and counted with a microscope. Experiments were repeated three times.

Protein isolation and western blot

At the indicated times, SK-BR-3 cells and MDA-MB-435 cells were harvested in ice-cold PBS and lysed on ice in cold preparation of modified radioimmunoprecipitation buffer supplemented with protease inhibitors. Protein concentration was determined using the BCA Protein Assay Kit (Vigorous Biotechonology; Beijing, China) and equal amounts of proteins were analyzed by SDS-PAGE (10% acrylamide). Gels were electroblotted onto nitrocellulose membranes (BD Pharmingen; San Diego, CA, USA). For immunoblot experiments, membranes were blocked for 2 hours with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20, and incubated at 4°C over night with primary antibody. Detection was performed by peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system. Primary antibodies used were: GAPDH from Zhong-shan JinQiao (Beijing, China); Stat3 and phosphor-Stat3 (rabbit anti-Stat3/p-Stat3, Cell signal technology, CST, MA, USA); HER2, CCND2 and MMP-2 (rabbitanti-HER2/CCND2/MMP-2, Cell signal technology, CST, MA, USA).

Dual luciferase reporter assay

The reverse complementary sequence of miR-29b was inserted into pRL-TK downstream of the Renilla luciferase gene (Promega, WI, USA) to generate a reporter system (pRL-TK-miR-29b). The 3'UTR of human Stat3 gene was amplified by PCR and cloned into pRL-TK to generate the Stat3 reporter (Stat3_WT). Mutations in Stat3 mRNA were created using the QuickChange Site-Directed Mutagenesis kit (Stratagene, CA, USA). The two cell lines were co-transfected with 0.4 μg of the reporter construct, 0.2 μg of the pGL-3 control vector, and miR-29b mimic or scramble. The cells were harvested at 24 hours post-transfection and assayed using the Dual Luciferase Assay (Promega, WI, USA) according to the manufacture's instructions. All of the transfection assays were conducted in triplicate.

Xenograft experiments

For animal xenograft model assays, 3×10^6 SK-BR-3 cells were subcutaneously injected into the posterior flanks of 6 weeks old nude mice, four mice per group. When tumors reached 50 mm^3, miR-29b or scramble mimic (100 nmol) was suspended in Lipofectamine 2000 (100 μl). The diluted miRNA was injected into the tumors directly. The injections were performed every 3 days for 6 times totally. Tumor diameters were measured after 7 days from injection and then every 3 days. At 28 days after injection, mice were killed and tumors were weighted after necropsy. Tumor volume was calculated as follows: length × width2 × 1/2. The mice xenograft model assays

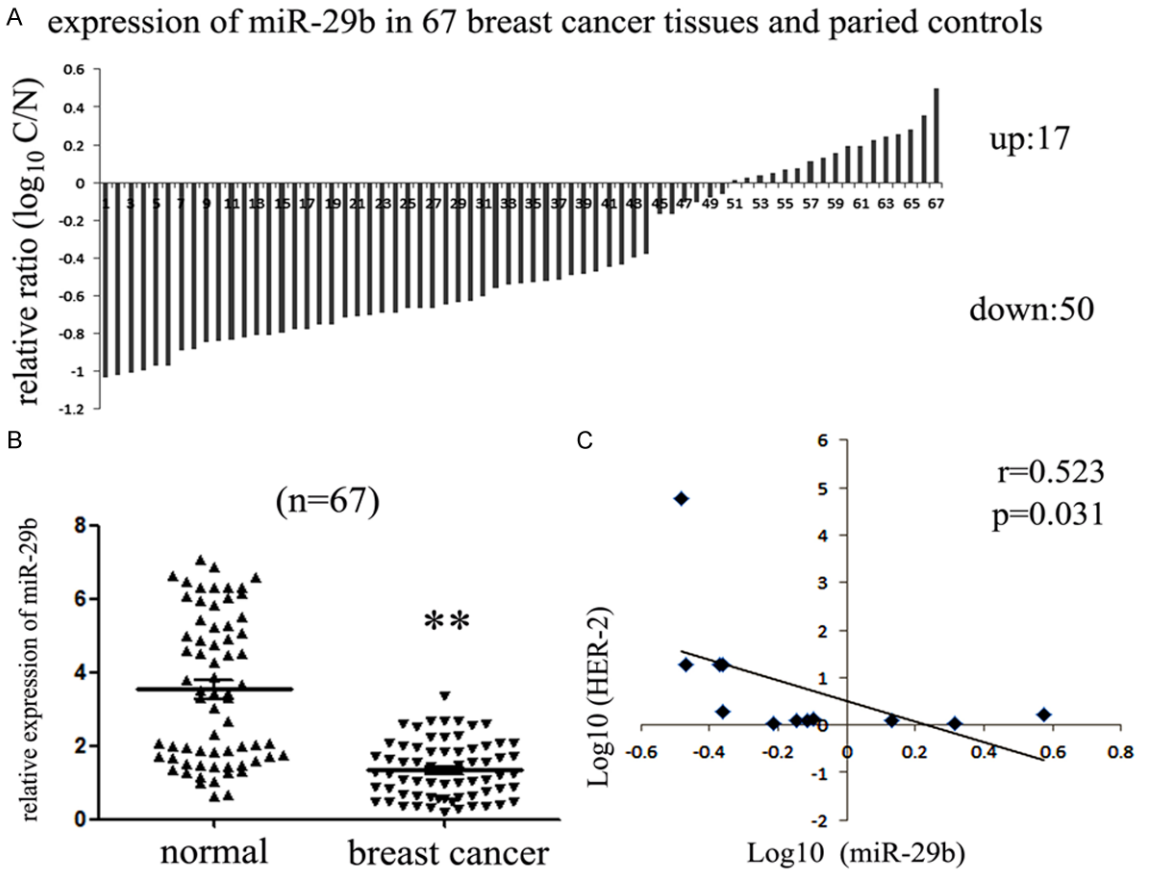

Figure 1. The expression of miR-29b is down-regulated in breast cancer tissues with HER-2-positive. The level of miR-29b was quantified by quantitative RT-PCR assays. Panel A provides the statistical results of miR-29b expression in breast cancer tissues and matched controls; Panel B illustrates the expression of miR-29b in an independently validated set of 67 breast cancer tissues with HER2-positive relative to the paired normal tissues. Panel C showed the negative relationship between miR-29b expression and HER-2 expression in another 12 paired fresh breast tissues with HER-2 positive. **indicates $P < 0.01$.

were performed according to institutional guidelines.

Statistical analysis

Data were expressed as the mean ± standard deviation of at least three independent experiments. Statistical analysis was carried out using the Student's t-test for comparisons of two groups, unless otherwise indicated (χ^2 test). Statistical analysis was also carried out using SPSS 15.0 software. *P*-values < 0.05 were considered significant.

Results

MiR-29b was suppressed in HER2-positive breast cancer tissues

To determine the relationship between miR-29b expression and clinical and pathological features of breast cancer, we assessed miR-29b expression in 67 paired of human breast cancers and adjacent normal tissues. As compared with normal tissue samples, more than half of the tumor tissues exhibited low levels of miR-29b (74%, 50 out of 67; **Figure 1B**). Further statistical analysis showed that the expression of miR-29b was consistently suppressed in breast cancer tissues compared with the matched normal tissues (**Figure 1A**). Thus, we further explored whether its suppression is related with its pathological characteristics. The pathological and clinical characteristics including patient age at diagnosis, tumor size, lymph node involvement and hormone receptor status was collected in **Table 1**. Although the results showed that no statistically significant correlations were observed between the miR-29b expression and age, lymph node involvement, ER status and PR status, interestingly,

Table 1. Relationship between miR-29b expression and their clinicopathological parameters in 67 breast cancer patients

Clinicolpathological parameters	Number of cases	Expression of miR-29b	*P*-value
Age (years)			
<50	25	1.2708±0.7696	0.5903
≥50	42	1.3774±0.7857	
Histology			
Infiltrating duct	41	1.2178±0.7089	0.8447
Infiltrating lobular	26	1.5265±0.8505	0.1130
Tumor size (cm)			
<5	36	1.5314±0.7271	0.0264*
≥5	31	1.1126±0.7804	
Nodal status			
Positive	23	1.4643±0.7078	0.3373
Negative	44	1.2714±0.8086	
ER status			
Positive	46	1.2754±0.7934	0.3352
Negative	21	1.4738±0.7350	
PR status			
Positive	32	1.2919±0.8036	0.6479
Negative	35	1.3794±0.7583	
HER2 status			
Positive	30	1.0783±0.6163	0.0126*
Negative	37	1.5478±0.8339	

Abbreviations: *P*-value represents the probability from a Student's t-test for miR-29b expression between variable subgroups. *$P < 0.05$ and **$P < 0.01$ were considered to have a significant difference.

a statistically significant association was observed between the expression of miR-29b and tumor size as well as HER-2 status. Tumors with low miR-29b expression tended to be large ($P < 0.05$), and those tumors with HER2-positive expression also expressed low levels of miR-29b ($P < 0.05$), which means miR-29b might function as a tumor suppressor in breast cancer, especially the subtypes with HER2-positive expressing subtypes. As a first step toward elucidating the molecular mechanisms underpinning the relationship between miR-29b and HER-2 expression, another twelve pairs of fresh primary breast cancer tissues with HER-2-positive were picked out. A Pearson correlation analysis was performed to explore the relationship among these genes. As shown in **Figure 1C**, miR-29b expression is inversely correlated with the expression of HER-2 in breast cancer tissues. However, the underlying mechanism of this co-expression was not identified yet.

Overexpression of miR-29b suppresses the malignant phenotype of HER2-positive breast cancer cell lines

The prominent reduction of miR-29b in breast cancer tissues with HER2-expressing promoted us to evaluate the potential biological significance between miR-29b and HER2-expressing breast cancer cells. Accordingly, two breast cancer cell lines with HER2-positive, SK-BR-3 and MDA-MB-435, were picked up for further experiments. Firstly, we restored the expression of miR-29b in both cell lines upon exogenous transfection with miR-29b mimic. Upon transfection, the expression of miR-29b was significantly up-regulated in both cell lines (**Figure 2A**).

Next, we further explored the effects of miR-29b on the malignant phenotypes of SK-BR-3 and MDA-MB-435 cells. As shown in **Figure 2B**, up-regulated expression of miR-29b significantly suppressed the cell growth rate of both cell lines. Since cell growth was closely related with the cell cycle progression, we next explored the effects of miR-29b on the cell cycle progression of SK-BR-3 and MDA-MB-435 cells. As expected, a remarkable reduction in the number of cells in the S-phase of the cell cycle, as well as a marked increase in the number of cells in the G1-phase were detected in both cell lines transfected with miR-29b (**Figure 2C**). Moreover, to further characterize miR-29b-mediated inhibition of cell proliferation, we next analyzed the cells undergoing apoptosis upon transfection. As shown in **Figure 2D**, transfection with miR-29b mimic induces cell apoptosis of both cell lines. Thus, these results indicated that miR-29b could efficiently inhibit the cell proliferation and arrest cell cycle progression of SK-BR-3 and MDA-MB-435 cells *in vitro*. Lastly, considering the important role of tumor invasion in the metastasis and recurrence of breast cancer, we also analyzed the effects of miR-29b on the invasive capacity of SK-BR-3 and MDA-MB-435 cells. As expected, treatment with miR-29b significantly suppressed the invasion of both breast cancer cells. Taken together, the above results highlight the ability of miR-29b in suppressing malignant phenotypes of HER2-positive breast cancer cells.

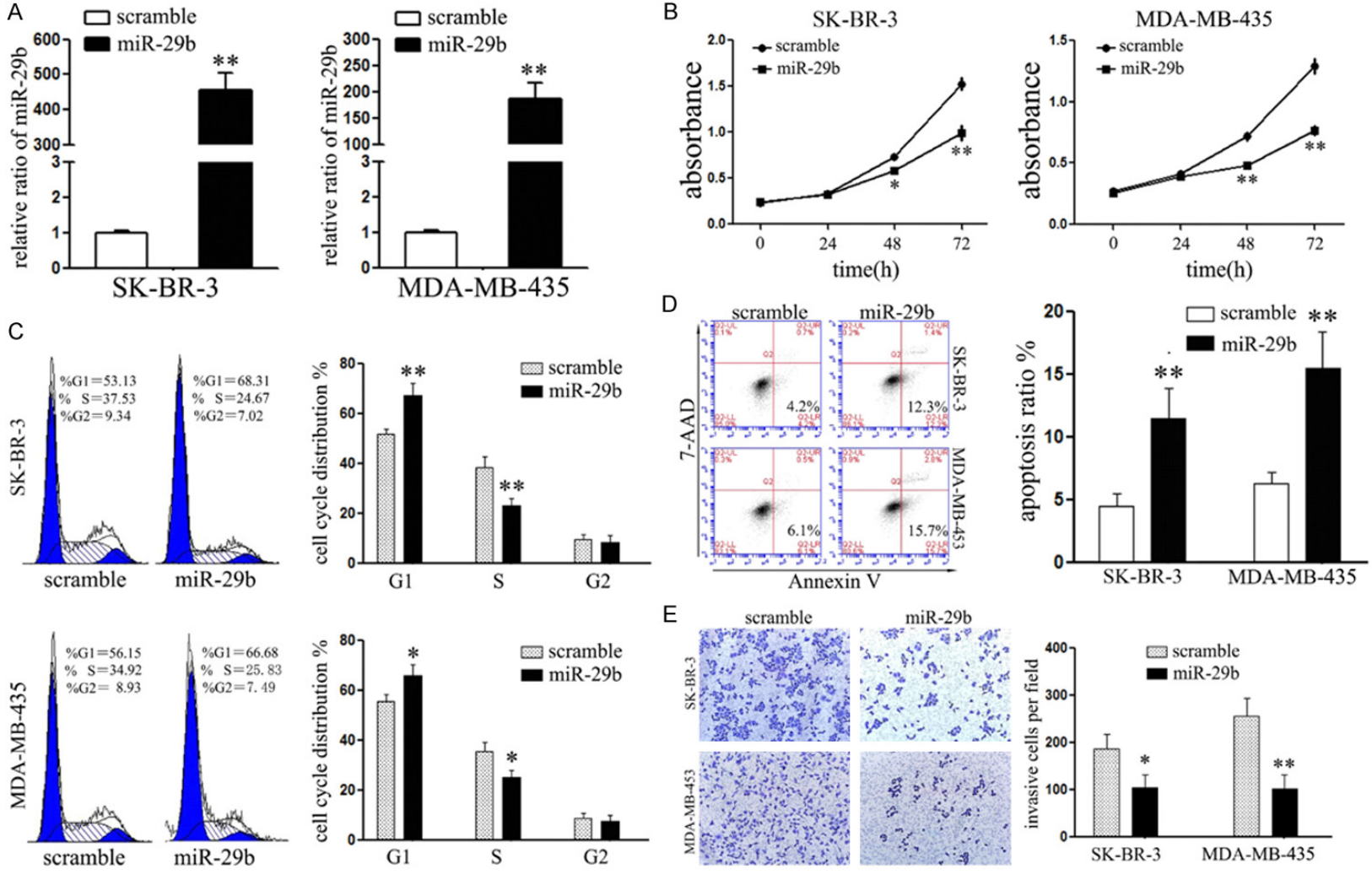

Figure 2. Overexpression of miR-29b suppresses the malignant phenotype of HER2-positive breast cancer cell lines. A. Expression level of miR-29b was examined by quantitative RT-PCR upon transfection with miR-29b mimic or scramble control. B. The CCK8 assays were used to evaluate the proliferative rates of SK-BR-3 and MDA-MB-435 cells upon transfection with miR-29b mimic or scrambled oligonucleotide. C. The cell cycle analysis of two cell lines treated with either miR-29b mimic or scrambled oligonucleotide and cultured for 24 hours after cell transfection. D. SK-BR-3 and MDA-MB-435 cells were stained with 7-AAD and PE Annexin V 72 hours after treatment with miR-29b mimic or scrambled control. Early apoptotic cells were shown in the right quadrant. E. Transwell invasion assays were performed to analyze the effects of miR-29b overexpression on the invasive capacity. The left panel showed the representative photos of cells passing through the chamber in each group. The right panel showed the relative ratio of invasive cells per field was shown. Magnification for identification of invasion was × 100. *indicates $P < 0.05$; **indicates $P < 0.01$.

Stat3 is the direct target of miR-29b and involved in miR-29b-mediated suppressing on HER2 expression

To further identify the putative mechanisms of miR-29b-mediated regulating of HER-2 expression, the algorithms for target gene prediction, including TargetScan, PicTar, and miRanda, were performed. Although HER-2 did not show putative binding sites with miR-29b, signal transducer and activator of transcription 3 (Stat3), an up-stream of HER-2 gene, was predicted to be a putative target gene of miR-29b. Stat3 is an important member of signal transducer and activator of transcription family, and plays a critical role in the development of breast malignancies and tumorigenesis [23]. To investigate whether it was involved in miR-29b-mediated regulation of HER-2, Dual-luciferase reporter assays were performed. As shown in **Figure 3B**, miR-29b overexpression inhibited the transcriptional activity of a luciferase reporter containing the Stat3 3′UTR, but did not affect the activity of a mutated reporter construct lacking the miR-29b binding site (**Figure 3B**). These data suggest that Stat3 is physically associated with the ectopically expressed miR-29b in breast cancer cells.

By partially complementing with the 3'-untranslated region (3'UTR) of specific messenger RNAs (mRNAs), miRNAs can modulate gene expression by regulating translational efficiency or cleavage of target mRNAs. We, therefore, speculated that miR-29b might regulate Stat3 expression in a similar fashion in breast tumor cells. Indeed, we observed a consistent decrease in the levels of endogenous Stat3 mRNA and protein in both SK-BR-3 and MDA-MB-435 breast cancer cells transfected with miR-29b mimic, as compared with cells transfected scramble control (**Figure 3C** and **3D**). Next, we further analyzed the effects on the expression of down-stream gene, HER-2. Consistent with the suppressed expression of Stat3, HER-2 expression was significantly decreased when transfected with miR-29b mimic. Moreover, another down-stream genes of Stat3, CCND2 and MMP2, which respectively plays important role in the regulation of cell cycle progression and metastasis, were consistently suppressed upon transfection. Collectively, these results indicated that the expression of miR-29b could suppress the expression of HER-2 through targeting its upstream gene Stat3 signaling in breast cancer cells.

Downregulation of Stat3 suppresses the malignant phenotype of HER2-positive breast cancer cells

Stat3 is a latent cytoplasmic transcription factor that conveys signals from the cell surface to the nucleus by cytokines or growth factors. It was constitutively activated in about 50-60% of the primary breast tumors [24]. To test whether Stat3 drives the malignant phenotype in HER2-overexpressing breast cancer cells, we knocked down Stat3 gene by RNA interferences and analyzed its effects on cell proliferation. Targeted Stat3 knockdown was confirmed by western blot analysis (**Figure 4A**). Interestingly, when Stat3 and its downstream genes were knocked down, the growth rate of both breast cancer cell lines were inhibited (**Figure 4B**). In agreement with cell proliferation, knockdown of Stat3 also promoted cell apoptosis and arrested the cell cycle progression. As shown in **Figure 4C**, the percentage of cells in G0/G1 phase increased and cells in S phase decreased significantly. Furthermore, to varying degrees, knockdown of Stat3 impaired the invasive capability of both cell lines (**Figure 4E**). These results suggest that Stat3 plays an important role as an oncoprotein in HER2-positve breast cancer cells. The tumorigenic role of Stat3 combined with our substantial evidence that miR-29b inversely regulates Stat3 expression indicates that the antitumor effects of miR-29b achieved, at least in part, through downregulating of Stat3 mRNA.

Stat3 might be involved in the suppressive effects of miR-29b in vivo

Our above findings strongly suggest the potential therapeutic target of miR-29b in HER-2-positive breast cancer. Thus, we further explored the effects of miR-29b on the tumor growth of HER-2-positive breast cancer cells *in vivo*. MiR-29b significantly inhibited tumor growth of SK-BR-3 cells xenografted mice relative to the scramble mimic injected mice (**Figure 5A**). Consistent with the tumor volume, the average tumor weight was also significantly reduced (**Figure 5B**). Based on these observations, we speculate that overexpression of miR-29b in tumor tissues upon local injection might result in the suppression of tumor growth. To

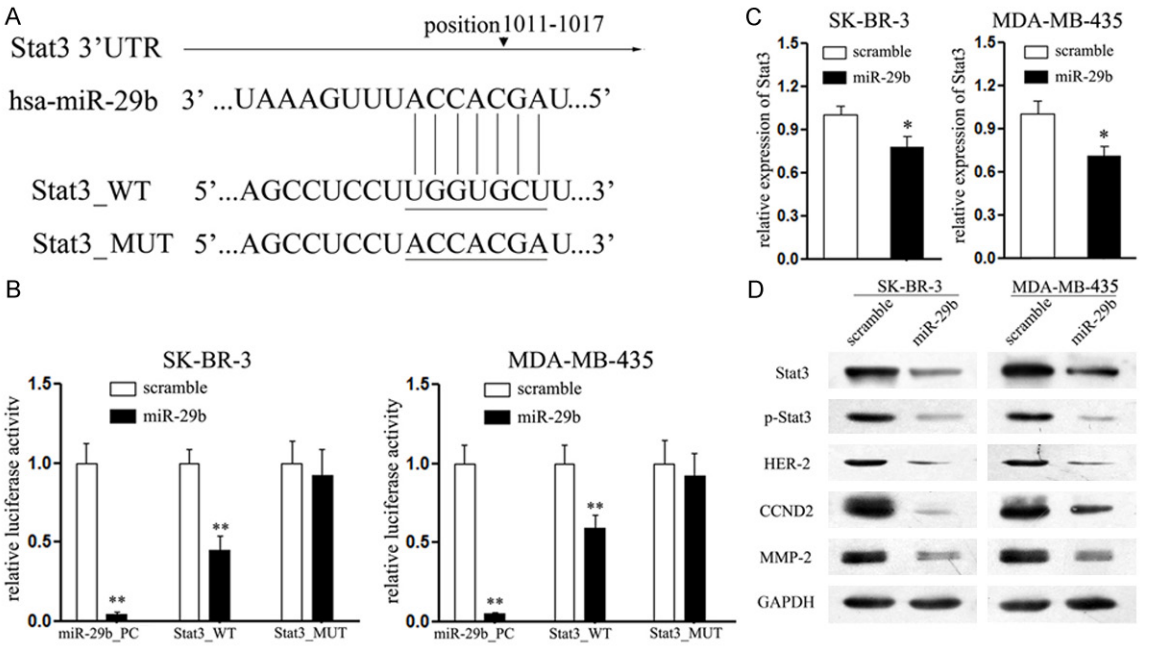

Figure 3. Stat3 is a direct target gene of miR-29b. A. Schematic sequence of putative binding sites of miR-29b within the 3'UTR of Stat3 mRNA. Stat3_WT represents the reporter constructs containing the entire sequence of Stat3. Stat3_MUT represents the reporter constructs containing mutated nucleotides. B. The analysis of the relative luciferase activity in SK-BR-3 and MDA-MB-435 cells upon co-transfection with miR-29b and Stat3_WT/MUT contrasts. C. Relative expression of Stat3 mRNA in SK-BR-3 and MDA-MB-435 cells transfected with miR-29b mimic and scrambled control. D. The Western blotting results for Stat3 and its downstream effectors in extracts from SK-BR-3 and MDA-MB-435 cells transfected with either miR-29b mimic or scrambled mimic. The error bars are derived from triplicate experiments, and *indicates $P < 0.05$, **indicates $P < 0.01$.

address this, we measured the expression of miR-29b, Stat3 and HER-2 in the harvested tumor tissues. Consistent with our *in vitro* results, the expression of miR-29b was significantly increased (**Figure 5C**), and the expression of Stat3 and HER-2 were consistently decreased in mice treated with miR-29b (**Figure 5D**). Thus, introduction of miR-29b mimic may suppress the carcinogenesis of HER-2-positive breast cancer through targeting Stat3-mediated activation of HER-2. Taken together, we interpret these results to indicate that miR-29b induced downregulation of Stat3, resulting in the subsequent modulation of the expression of HER-2, suppressed the malignant phenotype of HER-2-positve breast cancer cells.

Discussion

Recently, accumulating evidence led oncologists to speculate that unrevealed molecular factors, particularly non-coding RNAs, play important roles in the initiation and progression of various tumors. Depending on their mRNA targets, miRNAs can function as either tumor-suppressors or oncogenes in various tumor microenvironments. MiR-29b, which was reported to function as a tumor suppressor in a variety of cancers is controversial in breast cancers [15, 18-20]. Chen et al. found that through targeting tumor suppressor gene, *PTEN*, miR-29b might function as an oncogene in breast MDA-MB-231 cells [21]. While, it this paper, we found that miR-29b was suppressed in breast tumors, and its low level is negatively related with large tumor size and HER-2-positive status, which means its might function as a tumor suppressor in breast cancer, especially the ones with HER-2-expression. This is partially consistent with Chou *et al*. who proposed that the level of miR-29b expression is highest in good prognostic, well-differentiated, luminal-type cancers and inhibits tumor metastasis, which is the main cause of cancer-related deaths [22]. The specific reason for this discrepancy in response has not been identified. However, one possible explanation is that the cell lines and tissues have different background characteristics. For example, MCF-7 and MDA-MB-231 fell to Luminal A subtype which was

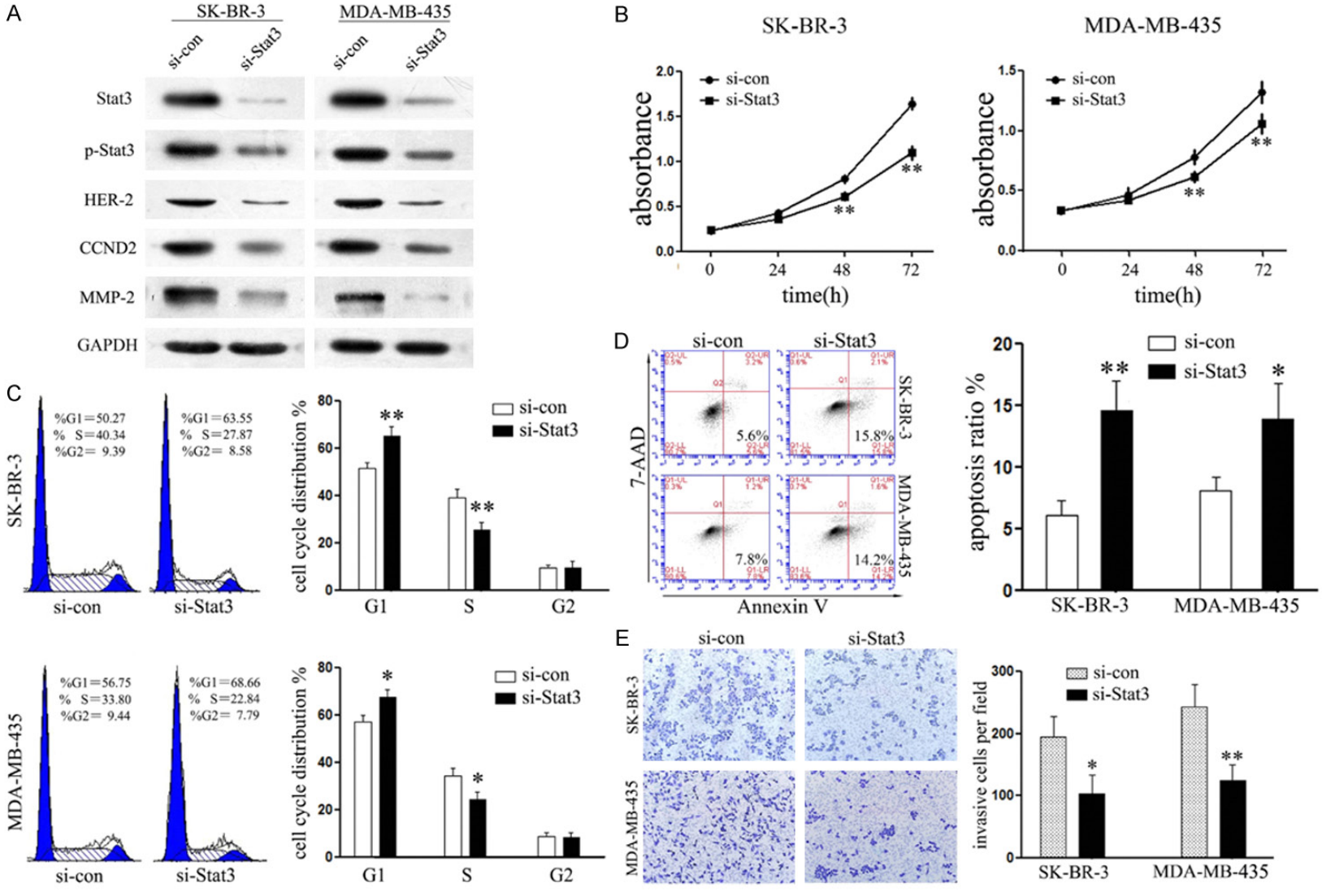

Figure 4. Downregulation of Stat3 inhibits cell proliferation and invasion. A. Expression levels of Stat3, p-Stat3, cyclin D2, MMP2 and HER-2 were analyzed using western blot assays upon transfection with si-Stat3. GAPDH was detected as the loading control. B. Results of the CCK8 assay for the proliferation in SK-BR-3 and MDA-MB-435 cells after their transfection with either si-Stat3. C. The cell cycle analysis of two cell lines treated either si-Stat3 or si-con and cultured for 24 hours after cell transfection. D. FACS analysis was performed to analyze the effects of Stat3 inhibition on the cell apoptosis of SK-BR-3 and MDA-MB-435 cells. Early apoptotic cells is shown in the right quadrant. E. Transwell invasion assay was performed upon cells were transfected with si-Stat3 or si-con. The relative ratio of invasive cells per field was shown. Magnification for identification of invasion was × 100. *, $P < 0.05$; **, $P < 0.01$.

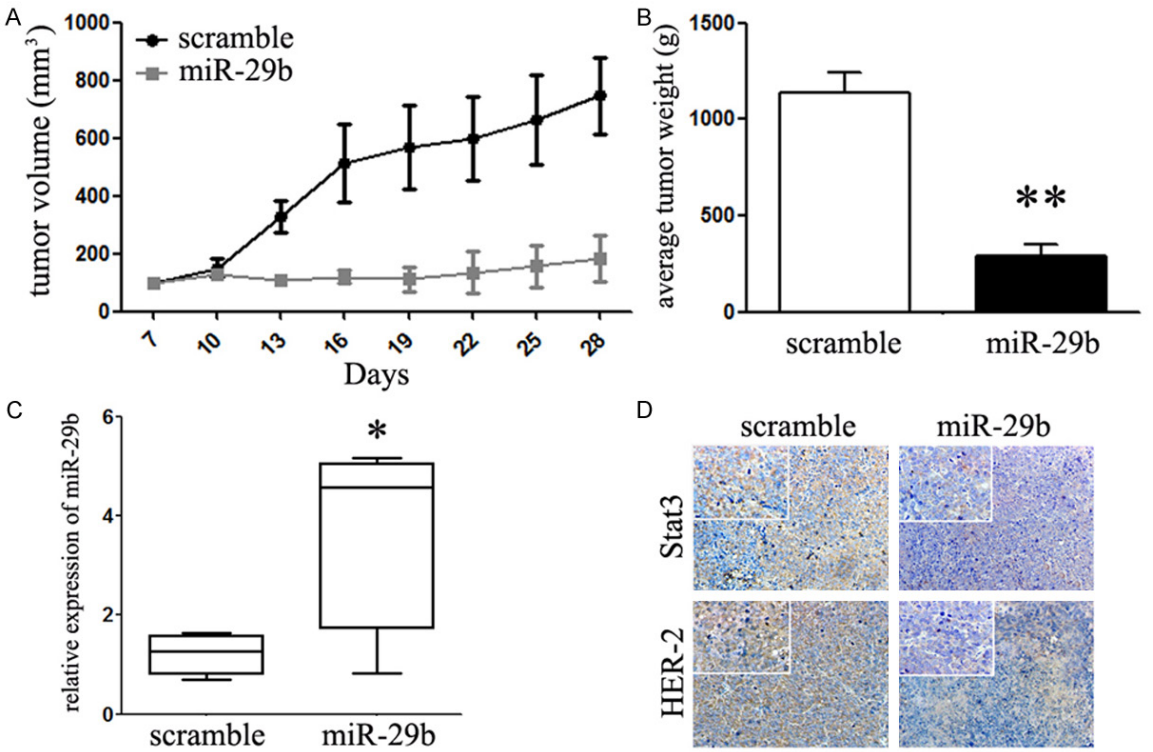

Figure 5. MiR-29b inhibits cancer growth of HER-2-positvie breast cancer cell line *in vivo*. A. Tumor volume averages between scramble and miR-29b mimic treated mice groups at the indicated days during the experiment. Four mice per group; B. Tumor weight averages between scramble and miR-29b mimic treated mice groups at the end of the experiment (28 days); C. Quantitative RT-PCR analysis showed the relative expression of miR-29b in injected tumor tissues (normalized to U6); D. Representative images of the immunohistochemistry analysis of Stat3 and HER-2 in tumors from xenograft mice. Data are presented as means ± SD. $^{**}P < 0.01$.

HER2-negatvie, while the cells used in this paper were SK-BR-3 and MDA-MB-435 which were HER2-positive. Moreover, we also found that miR-29b is negatively related with the expression of HER2 in breast cancer tissues, further promotes us to speculate that HER-2 is related with the discrepancy.

Breast cancer encompasses a group of very heterogeneous diseases, which can be demonstrated at the molecular, histopathologic and clinical levels. Due to different molecular classification, the prognosis is definitely different. Numerous studies have shown that Luminal A subtype presents better differentiated tumors, while HER-2-positive subtype is often associated with nodal metastasis [25]. Thus, it is particularly urgent to take the molecular subtype into consideration in research in the future.

HER-2 is a part of the epidermal growth factor (EGF) family. It is located on the long arm of chromosome 17 and encodes a 185-kDa transmembrane protein. It is notably overexpressed in about 20-30% of breast cancer tumors, and is associated with poor prognosis, with a lower disease-free survival rate and a shorter time to relapse [26, 27]. Herein, to further identify the relationship between miR-29b and HER-2 in breast cancer, we explored its effects on the malignant phenotype of HER2-positive breast cancer cell lines. Overexpression of miR-29b in both cell lines induced a complex phenotype, namely an inhibition of cell proliferation, block of G1/S phase transition, induction of cell apoptosis, and suppression of cell invasion, indicating that miR-29b functions as a tumor suppressor in HER2-positive breast cancer cells, which is also consistent with Chou et al. who proposed that miR-29b promoted luminal differentiation, while conversely, loss of miR-29b promoted mesenchymal traits and metastasis [22]. Our further *in vivo* assays showed that treatment with miR-29b mimic significantly suppressed the HER2-positive breast cancer cells xenografted tumor growth. It might provide new strategy in the treatment of breast cancer in the future.

To further identify the mechanisms involved in the suppressive effects and regulation of HER-2, the putative target genes of miR-29b were searched. Among these genes, Stat3 attracted our attention mostly. *Stat3* is an critical member of signal transducer and activator of transition family which have been recognized as integrators of cytokine and growth factor receptor signaling required for cell growth, survival, differentiation and motility [23, 28]. Most importantly, it could positively regulate the expression of HER-2 through a novel cis-acting element in HER-2 promoter [31]. Its potential oncogene role has been widely revealed in breast cancer. It was shown that Stat3-RANTES autocrine signaling is essential for tamoxifen resistance in breast cancer [29]. Another group has demonstrated that the Stat3 signaling was specifically required for the growth of stem cell-like breast cancer cells [30]. Herein, we further identified that *Stat3* is the direct target gene of miR-29b in breast cancer cells, and overexpression of miR-29b suppressed the expression of *Stat3* as well as HER-2 at the same time. Consistently, we also found that suppression of Stat3 through transfection with siRNA, showed suppressive effects on the expression HER-2 in both cell lines. Moreover, knockdown of *Stat3* present inhibitory effects on cell growth, cell cycle progression and invasion of HER-2-positive breast cancer cells, which suggested that *Stat3* might be involved in miR-29b-mediated tumor suppression of HER-2-positive breast cancer cells. Moreover, it is also reported that HER2 overexpression elicited IL-6 secretion and activated *Stat3*, enforcing an autocrine loop of HER2-IL6-*Stat3* expression [29]. However, more experiments are warranted to identify this.

Since HER-2-driven mechanism has been widely linked to tumor growth, resistance to chemotherapy and metastasis, its specific antibody, Herceptin, is being used as a first-line drug treatment for HER-2-positive breast cancer [32, 33]. While, about 52% of breast cancer patients fail to respond to the initial Herceptin treatment or develop resistance to the antibody therapy within one year [34]. A recent study proposed that *Stat3* signaling pathway might play a crucial role for the chemotherapy resistance in human breast cancer, as *Stat3* activation is responsible for the stem cell marker expression in HER-2-expressing breast cancer cells, and suppression of *Stat3* could delay or even reverse incurred Herceptin-associated resistance with HER-2-overexpression [30]. Thus, more experiments are needed to further identify whether miR-29b/*Stat3*/HER-2 interaction is also involved in the chemotherapy resistance. It will uncover a novel mechanism of regulation of HER-2 expression and provide new strategy in the future.

Acknowledgements

This research was supported by the Southern Medical University.

Disclosure of conflict of interest

The authors declare no conflict of interest.

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