GALNT6通过TGF-β/Smad信号通路调控EMT促进乳腺癌转移

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摘要 该文研究了*N*-乙酰半乳糖氨基转移酶6(GALNT6)对乳腺癌上皮–间充质转化(EMT)的影响及其机制。乳腺癌细胞中GALNT6表达被敲低后, 间充质标志物E-cadherin表达增加, N-cadherin、Vimentin等表达减少。同时, 通过恢复shGALNT6细胞中GALNT6的表达, 可以提高间充质标志物的表达。转化生长因子(transforming growth factor, TGF)处理乳腺癌细胞时, Vimentin表达升高, E-cadherin表达降低。然而, 在GALNT6敲低细胞中却得到相反的结果。当使用Smad3的抑制剂SIS3处理时, EMT标记物的表达不受GALNT6敲低的影响, 说明GALNT6主要通过Smad途径诱导EMT。此外, Smad3和TGFBR2可被GALNT6糖基化。研究结果表明, GALNT6通过TGF/Smad信号通路诱导EMT促进乳腺癌转移, 这提示了其在乳腺癌预后和治疗中的潜在作用。

关键词 GALNT6; EMT; 乳腺癌; TGF-β信号通路; 糖基化

GALNT6 Promoted Breast Cancer Metastasis by Regulating EMT via TGF-β/Smad Signaling Pathway

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Abstract This study investigated the effects of GALNT6 on EMT (epithelial-mesenchymal transition) in breast cancer and the underlying mechanism. Results showed that knockdown of GALNT6 in breast cancer cells increased the expression of E-cadherin, and decreased the expression of mesenchymal markers, including N-cadherin and Vimentin. Meanwhile, the expression of mesenchymal markers was increased by restoring the expression of GALNT6 in shGALNT6 cells. When the breast cancer cells were treated with TGF-β1 (transforming growth factor β1), the expression of Vimentin was increased while the expression of E-cadherin was decreased. However, the opposite results were obtained in GALNT6 knockdown cells. While treated with SIS3, an inhibitor of Smad3, the expression of EMT markers were not affected by knockdown of GALNT6, indicating that GALNT6 induced EMT mainly via the Smad pathway. Furthermore, Smad3 and TGFBR2 are glycosylated by GALNT6. Collectively, the findings suggested that GALNT6 induced EMT to promote breast cancer metastasis via TGF-β/Smad signaling

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pathway, supporting its prognostic and therapeutic utility in breast cancer.

Keywords GALNT6; EMT; breast cancer; TGF-β signaling pathway; glycosylation

Breast cancer is the most common malignant tumor with the highest mortality rate and the leading cause of cancer death among women [1]. The lethality of breast cancer is mostly due to the aggressive invasion and uncontrolled metastasis [2]. A lack of effective targeted therapies for breast cancer often results in a rapidly fatal clinical outcome [3]. Therefore, it is important to explore the underlying molecular mechanism of breast cancer, and it may help to improve the therapeutic strategies for breast cancer.

Metastasis of tumor is a complicated process, which was related to complex molecular mechanisms. Reports have shown that the EMT plays a vital role during cancer invasion and metastasis [4-5]. During the progress of EMT, epithelial cells lose their epithelial characteristics and acquire mesenchymal phenotype by increasing the expression of Vimentin, Snail, etc [5-6]. The replacement of E-cadherin with N-cadherin, which is the crucial event in EMT, enable single cells become more motile and aggressive [6]. The TGF-β (transforming growth factor-β) signaling pathway is the most widely studied signal transduction pathway for inducing EMT. It is activated when TGF-β binds to the TGFBR2 (TGF-β type II receptor) and associates with the TGFBR1 (TGF-β type I receptor) and then transmits the signal through recruitment and phosphorylation of the Smad protein [7-8]. TGF-β1 (transforming growth factor-β1) could initiate signals and induce the process of EMT in many cancers, including breast cancer, esophageal cancer, etc [9].

Nowadays, the role of glycosyltransferases has become increasingly important in the pathophysiological processes of tumor development, including tumor cell proliferation, metastasis and immune escape [10-11]. GALNTs (polypeptide *N*-acetylgalactosaminyltransferases) are a family of enzymes catalyzing the initial step of *O*-glycosylation, transfer GalNAc to serine or threonine residues on the target protein [12]. Accumulated evidences have suggested that abnormal expression and function of GALNTs are associated with tumorigenesis and the malignant potential of the tumor cells [13]. It has been demonstrated that ppGalNAc-T2 and GALNT5 can regulate the invasion and metastasis of multiple tumor cells [14-15]. Our previous research showed that GALNT6 played a critical role in biological function of breast cancer cells such as cell adhesion, metastasis and invasion [16]. However, the underlying molecular mechanisms of GALNT6 promoting breast cancer metastasis and invasion is still unclear.

In this study, we found that GALNT6 regulated EMT-related protein and modified the *O*-glycosylation of TGFBR2, and knockdown of GALNT6 inhibited EMT progression in human breast cancer cells. Furthermore, we demonstrated that GALNT6 promoted breast cancer metastasis by regulating EMT mainly through TGF-β/Smad signaling pathway.

1 Materials and methods

1.1 Experimental materials

Human breast cancer cell lines (MDA-MB-231 and MCF-7) were purchased from ATCC (American Type Culture Collection). FBS (fetal bovine serum), DMEM, RPMI-1640, G418 (Geneticine) and penicillin-streptomycin were purchased from Gibco, USA. GALNT6 cDNA expression vector, siRNA oligonucleotides against Smad3 (5′-CCG CAU GAG CUU CGU CAA ATT-3′), negative control siRNA, pGPU6/GFP/Neo-GALNT6 interference vector and pGPU6/GFP/Neo-NC control vector were constructed by Genepharma, China. Lipofectamine 2000 Transfection Reagent was purchased from Thermo, USA. Human recombinant TGF-β1 was purchased from R&D Systems Inc, USA. VVA (Agarose bound Vicia Villosa Lectin) was purchased from Vector, USA. SIS3 was purchased from Selleck, USA. BCA Protein Assay Kit was purchased from Beyotime, China. CCK8 (Cell Counting Kit-8) reagent was purchased from Dojindo, Japan. Prime Script RT Reagent Kit and SYBR® Pre-

mix Ex Taq ™ Perfect Real Time were purchased from TakaRa, Japan. The antibody against E-cadherin was purchased from Santa Cruz (CA, USA). The antibodies against GALNT6, N-cadherin, TGFBR2, Smad3, p-Smad3, Smad2/3 were purchased from Abcam (New Territories, USA). The antibodies against Snail and Vimentin were purchased from Cell Signaling Technology (Danvers, USA).

1.2 Cell culture and transfection

Cells were cultured in medium (MDA-MB-231 cells in DMEM, and MCF-7 cells in RPMI-1640) supplemented with 10% FBS and 1% penicillin-streptomycin. GALNT6 knockdown cells were constructed by shRNA-mediated gene silencing. The PGPU6/GFP/Neo-GALNT6 (or pGPU6/GFP/Neo-NC) vectors were transfected into MDA-MB-231 cells using liposome 2000 transfection reagent according to the manufacturer's instruction. The GALNT6 cDNA expression vector was transfected into shGALNT6 MDA-MB-231 cells by using liposome 2000 transfection reagent.

1.3 Pharmacological drug treatment

MDA-MB-231 cells were trypsinized and placed in six-well plates. One day later, cells were treated with or without 5 ng/mL of TGF-β1 (diluted in sterile 4 mmol/L HCl containing 1 mg/mL bovine serum albumin) for 48 h for WB (Western blot) analysis. In some cases, the cells were pre-treated with Smad3 inhibitor SIS3 (3 μmol/L) for 6 h, then treated with or without TGF-β1 (5 ng/mL) for 24 h for Western blot analysis.

1.4 Western blot

Western blot was performed as described previously [10]. The protein was separated on SDS-PAGE, electro-transferred to PVDF membrane, blocked by 5% non-fat milk and 0.1% Tween-20, then incubated with the primary antibodies. After incubating the second antibodies for 1 h, the bands were visualized by ECL (enhanced chemiluminescence) detection reagents (Beyotime, China). These data were quantified by densitometry. The ImageJ software was used to analyze the relative expression of protein.

1.5 Immunofluorescence

Cells were washed by PBS (phosphate buffered saline) for three times, fixed with 4% paraformaldehyde for 30 min. Repeated the washing steps, cells were permeabilized with 0.1% Triton X-100 at room temperature for 20 min and blocked with 5% bovine serum albumin for 1 h, incubated with rabbit polyclonal antibody E-cadherin (1:200) or N-cadherin (1:200) overnight at 4 ℃, incubated with Cy3-conjugated anti-Rabbit antibody in darkness for 1 h, finally incubated with DAPI (Sigma-Aldrich, USA) for 10 min. The results were analyzed by microscopy.

1.6 Immunoprecipitation

The cell lysate was extracted, centrifuged (4 ℃, 14 800 r/min, 20 min) and the supernatant was added to sepharose-protein A/G beads, pre-cleared for 2 h, then incubated on the rotator overnight at 4 ℃. The protein-antibody complexes were collected by centrifugation at 3 000 r/min for 5 min, washed by PBS, resuspended in SDS sample buffer. Finally, the sample was boiled at 100 ℃ for 10 min, and the complexes were analyzed by Western blot.

1.7 Cell invasion and migration assays

Transwell inserts (8.0 μmol/L pore diameter, Corning, USA) were used to evaluate the cell invasion and migration abilities. Cells were seeded into the upper chamber of 24-well transwell plate coated with serum-free medium, the lower chambers were filled with serum medium (600 μL). After 24 h, cells at the bottom of the membrane surface were fixed with 4% paraformaldehyde and stained with crystal violet for 15 min. The same procedures with the following changes were used to measure invasion ability: the upper chamber coated with a Matrigel® (BD, USA) and cells cultured for 48 h. Cell numbers were counted for each well, and values were presented as $\bar{x}\pm s$.

1.8 Lectin pull down

Lectin pull down is an experiment using Agarose bound Vicia Villosa Lectin (VVA) mixed with cell lysate. Protein concentration was quantified by BCA Protein Assay Kit. Cell lysates (0.6 mg) were added to VVA and rotated at 4 ℃ overnight. The protein was analyzed by Western blot, and incubated with primary antibodies against TGFBR2 or Smad3.

1.9 Small interfering RNA (siRNA) transfection

Cells were cultured in 6-well plate (Corning, USA) and transfected with 100 pmol Smad3 siRNA or control (Ctrl) siRNA using a siRNA transfection reagent (Lipofectamine 2000) according to the manufacturer's instructions. After 48 h of incubation, the total protein of the cells was collected and the expression of Smad3 was detected by Western blot.

1.10 RNA isolation and quantitative RT-PCR validation

Total RNA was isolated using Trizol® (Beyotime, China) according to the manufacturer's protocol, followed by cDNA synthesis using the PrimeScript RT Reagent Kit. Then, the obtained cDNA was amplified by RT-PCR. Primers were used as follows: *TGFBR2*, forward 5′-TGG CGA GGA GTT TCC TGT TT-3′ and reverse 5′-TGT TGG CGA GGA GTT TCC TG-3′; *GAPDH*, forward 5′-GAA CAT CAT CCC TGC CTC TAC T-3′ and reverse 5′-CCT GCT TCA CCA CCT TCT TG-3′.

1.11 Statistical analysis

Statistical analyses were performed using SPSS software version 17.0 (Chicago, Illinois, USA). Comparisons between two groups were performed using a Student's *t*-test. If two groups could not be considered to be of equal variance, Dunnett's multiple comparison test was performed. Data were presented as $\bar{x}\pm s$. *P*-values of less than 0.05 were considered as statistically significant.

2 Results

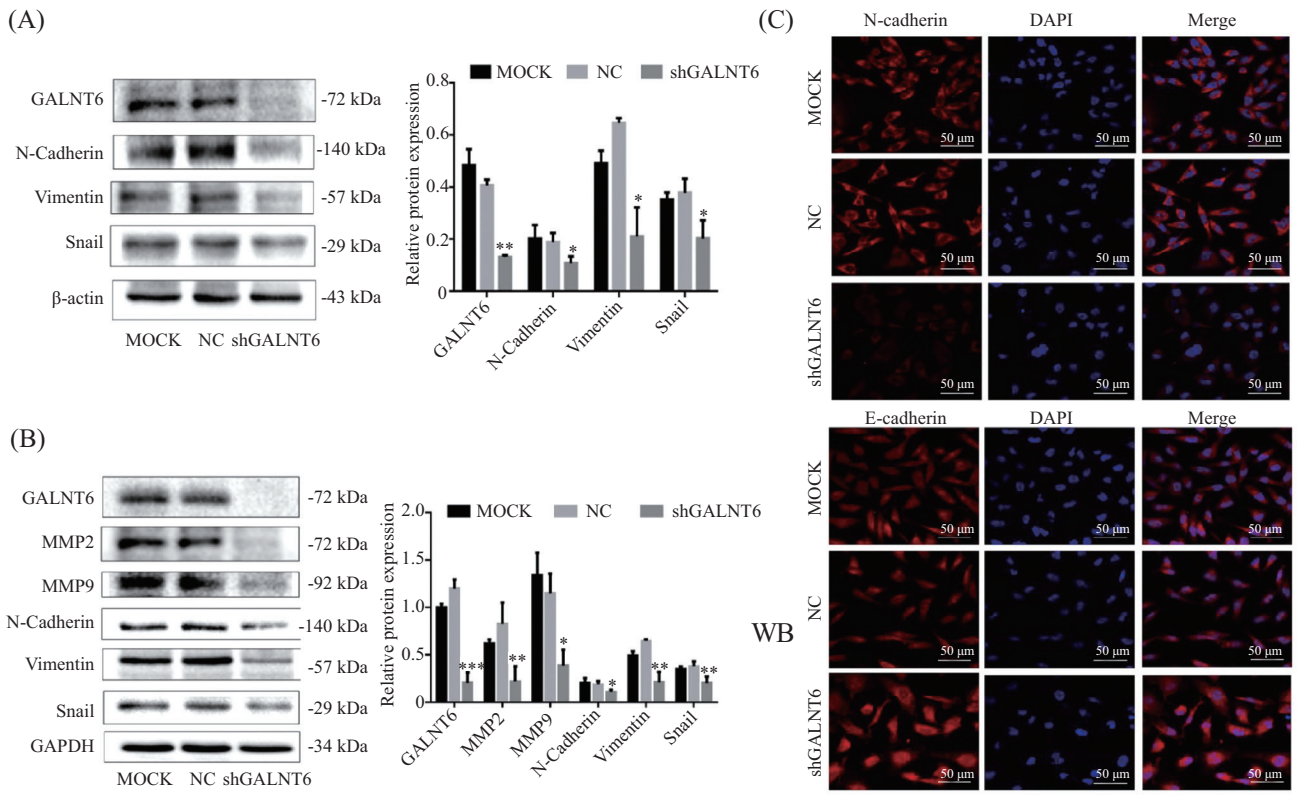
2.1 GALNT6 regulated EMT progress in breast cancer

To determine whether EMT was regulated by GALNT6 in breast cancer, the GALNT6 knockdown MDA-MB-231 and MCF-7 cells were constructed, and the expression of E-cadherin, N-cadherin, Vimentin and Snail was confirmed by Western blot. Down-regulation of GALNT6 significantly decreased the expression of mesenchymal cells markers, such as N-cadherin, Vimentin and the transcription factor Snail, and increased the expression of E-cadherin (Fig.1A). Meanwhile, the expression of E-cadherin and N-cadherin regulated by GALNT6 was further confirmed by immunofluorescence in MDA-MB-231 cells (Fig.1C). In MDA-MB-231 cells, the expression of MMP2 and MMP9 was decreased in shGALNT6 cells, compared with control cells (Fig.1B). These results indicated that GALNT6 regulated the process of EMT in breast cancer cells.

To further confirm the regulatory effect of GALNT6 on the EMT in breast cancer, the GALNT6 cDNA expression vector was transfected into GALNT6 knockdown MDA-MB-231 cells to restore the expression of GALNT6. The expression of GALNT6 was increased in rescue cells, compared with control cells (Fig.2A). The expression of Snail and Vimentin was increased in rescue cells, compared with shGALNT6 cells (Fig.2A). Meanwhile, the migration and invasion abilities of cells were significantly inhibited in shGALNT6 cells, whereas restoring the expression of GALNT6 in shGALNT6 cells significantly enhanced the cell migration and invasion (Fig.2B and Fig.2C). These results suggested that GALNT6 might induce cell migration and invasion through EMT progress in breast cancer.

2.2 GALNT6 enhanced EMT by activating TGF-β signaling pathway

To demonstrate whether GALNT6 affected EMT progress through regulating TGF-β signaling pathway, we detected the expression of related proteins after the cells were treated with TGF-β1. Compared with MOCK and NC cells, the phosphorylation of Smad3 was significantly decreased in shGALNT6 cells, while the expression of Smad3 and Smad2/3 was not affected by knockdown of GALNT6, indicating that GALNT6 could regulate EMT by phosphorylation of Smad3 (Fig.3A). Compared with their corresponding cells without TGF-β1 treated, the expression of Vimentin and p-Smad3 was significantly increased in NC and shGALNT6 cells treated with TGF-β1, while the expression of E-cadherin was significantly decreased (Fig.3B). Meanwhile, knockdown of GALNT6 inhibited the expression of Vimentin and p-Smad3 induced by TGF-β1, and elevated the expression of E-cadherin



A: WB检测MCF-7细胞株中GALNT6、E-cadherin、N-cadherin、Vimentin和Snail的蛋白表达, β-actin为内参。B: WB检测MDA-MB-231细胞株中GALNT6、MMP2、MMP9、N-cadherin、Vimentin和Snail的蛋白表达, GAPDH为内参。C: 免疫荧光检测MDA-MB-231细胞株中E-cadherin和N-cadherin的表达, 红色的荧光标记E-cadherin和N-cadherin, 蓝色的荧光标记细胞核。*P<0.05, **P<0.01, ***P<0.001, 与NC组相比。

A: the expression of GALNT6, E-cadherin, N-cadherin, Vimentin and Snail in MCF-7 cells was detected by Western blot. β-actin was acted as the loading control. B: the expression of GALNT6, MMP2, MMP9, N-cadherin, Vimentin and Snail in MDA-MB-231 cells was detected by Western blot. GAPDH was acted as the loading control. C: the expression of E-cadherin and N-cadherin in MDA-MB-231 cells was detected by immunofluorescence. Representative immunofluorescence staining showed E-cadherin and N-cadherin (red) and nuclear (blue). *P<0.05, **P<0.01, ***P<0.001 compared with NC group.

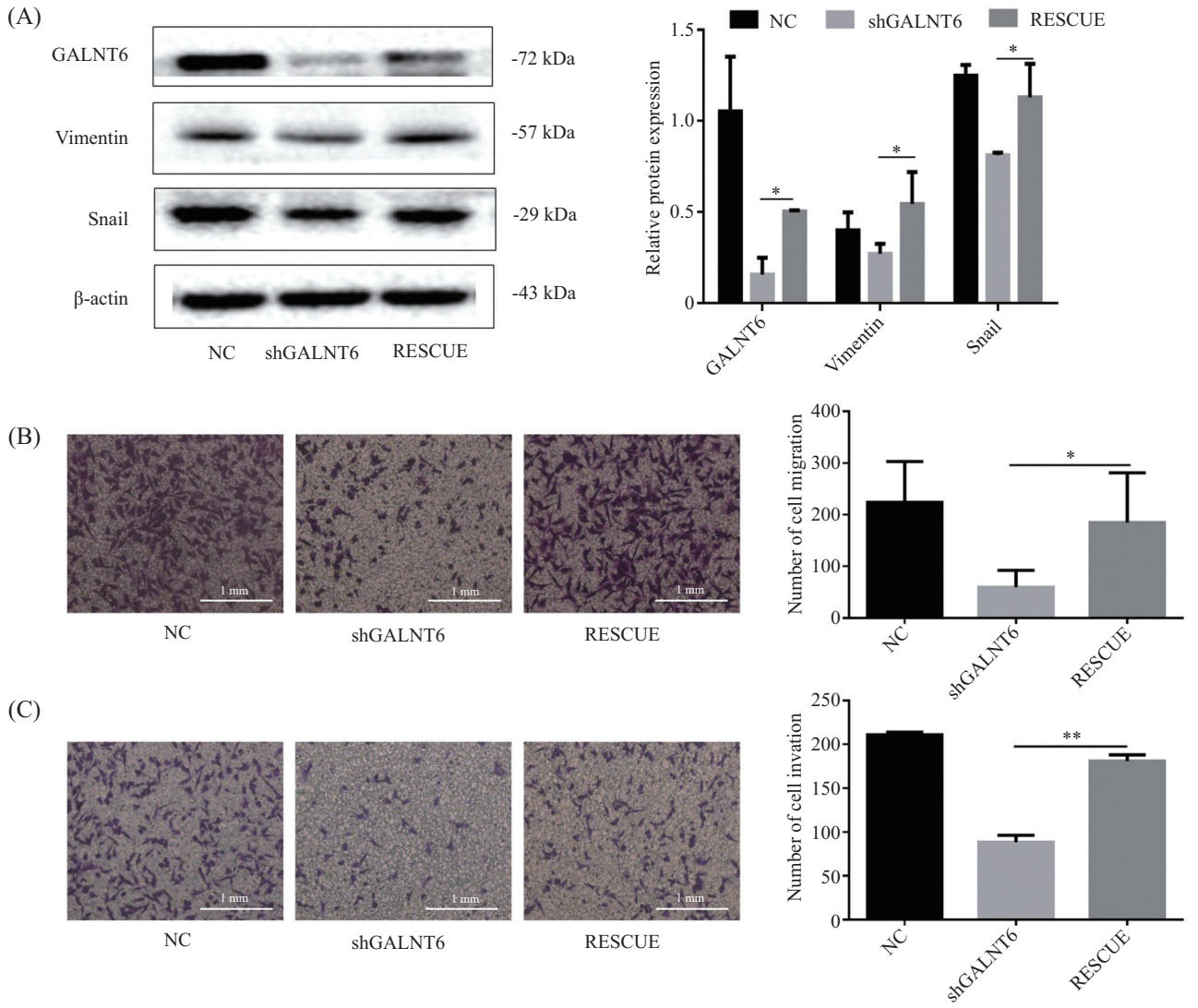
图1 敲低GALNT6抑制乳腺癌的EMT进程

Fig.1 Knockdown of GALNT6 suppressed EMT progress in breast cancer

which was reduced by TGF-β1 (Fig.3B). The cell migration and invasion abilities were enhanced markedly with TGF-β1 treatment, while knockdown of GALNT6 degraded cell migration and invasion with or without TGF-β1 treatment (Fig.4A and Fig.4B), indicating that GALNT6 knockdown reversed the expression of Vimentin and E-cadherin regulated by TGF-β1, and inhibited the cell migration and invasion induced by TGF-β1. The results showed that knockdown of GALNT6 inhibited the EMT progression induced by TGF-β1 in breast cancer cells, and GALNT6 might promote EMT progress through TGF-β signaling pathway.

2.3 GALNT6 promoted EMT progress mainly via classical Smad pathway

In order to examine whether Smad3 is responsible for GALNT6-induced EMT in breast cancer, SIS3, an inhibitor of TGF-β signal pathway, was used to inhibit the classical Smad signal pathway. As shown in Fig.5A, after treated with SIS3, Vimentin and Snail were enhanced in cells with TGF-β1 treatment (with or without GALNT6 knockdown). However, when the cells were treated with SIS3, knockdown GALNT6 had no effect on the levels of Vimentin and Snail in the cells (with or without TGF-β1 treated) (Fig.5A). Meanwhile, siRNA against Smad was used to silence the expression of Smad3. The results showed that the expression of Smad3 was significantly reduced by si-Smad3, and knockdown of GALNT6 suppressed the expression of Vimentin and Snail with or without the si-Smad3, compared with the control group (Fig.5B). These results indicated that GALNT6 regulated EMT in breast cancer



A: WB检测MDA-MB-231细胞株中GALNT6、Vimentin和Snail的蛋白表达, β-actin为内参。B: Transwell法检测MDA-MB-231细胞株中NC细胞、shGALNT6细胞及GALNT6恢复细胞的迁移能力。C: Transwell法(基质包被)检测MDA-MB-231细胞株中NC细胞、shGALNT6细胞及GALNT6恢复细胞的侵袭能力。*P<0.05, **P<0.01。

A: the expression of GALNT6, Vimentin and Snail in MDA-MB-231 cells was detected by Western blot, β-actin was acted as the loading control. B: the cell migration of NC cells, shGALNT6 cells and GALNT6 rescued cells of MDA-MB-231 was evaluated by transwell assay. C: the cell invasion of NC cells, shGALNT6 cells and GALNT6 rescued cells of MDA-MB-231 was evaluated by a matrigel-coated transwell assay. *P<0.05, **P<0.01.

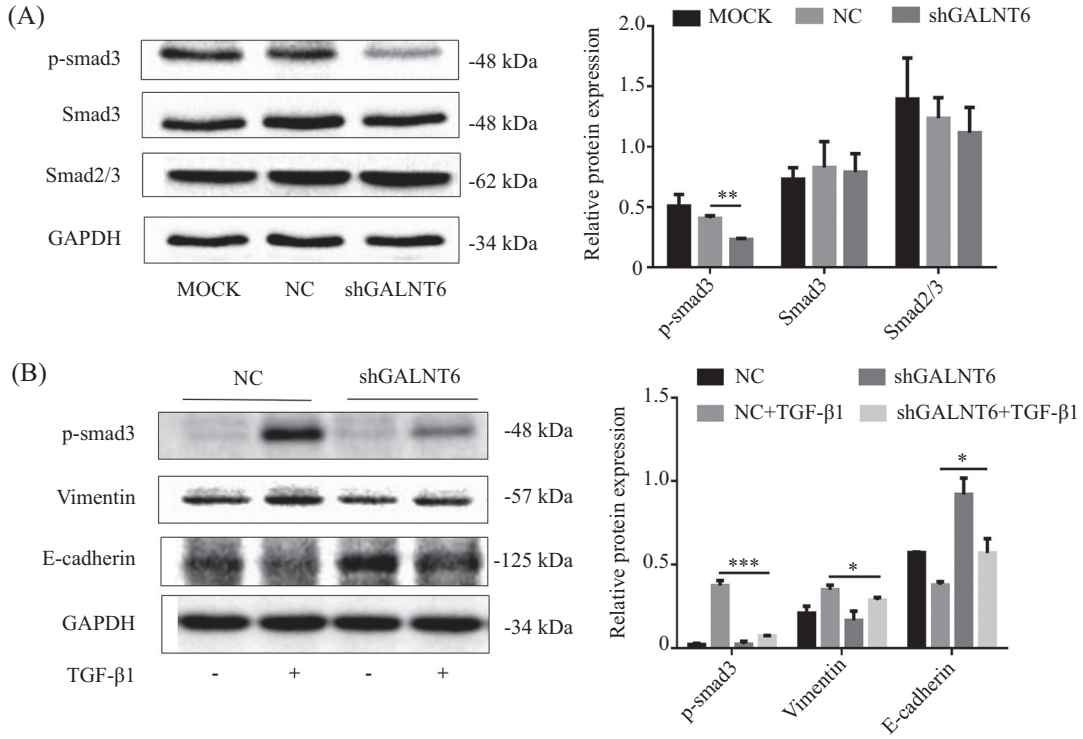
图2 恢复GALNT6表达促进EMT进程

Fig.2 The recovery of GALNT6 reinforced the process of EMT

through TGF-β/Smad signaling pathway.

2.4 GALNT6 *O*-glycosylated Smad3 and TGFBR2

Glycosylation can affect the signal transduction of multiple signal pathways in tumor, including TGF-β signal pathway. To better understand the molecular mechanisms of GALNT6 expression regulating EMT progress through TGF-β signaling, VVA pull down and Co-IP (co-immunoprecipitation) analysis was performed. The results showed that knockdown of GALNT6 did not affect the expression of Smad3 in breast cancer cells, but the *O*-glycosylated Smad3 was significantly reduced (Fig.6A). The interaction between GALNT6 and Smad3 was further confirmed by Co-IP (Fig.6B). These results indicated that Smad3 was glycosylated by GALNT6. In connection with the decreased phosphorylation of Smad3 mentioned above, we speculate that the abnormal glycosylation of Smad3 induced by GALNT6 may affect its phosphorylation modification, which induced the process of EMT in breast cancer.



A: WB检测MDA-MB-231细胞株中GALNT6、p-Smad3、Smad3和Smad2/3的蛋白表达, GAPDH为内参。B: NC及shGALNT6细胞在TGF-β1(5 ng/mL)处理48 h及不处理的对照情况下, WB检测E-cadherin和Vimentin的蛋白表达, GAPDH为内参。*P<0.05, **P<0.01, ***P<0.001。

A: the expression of GALNT6, p-Smad3, Smad3 and Smad2/3 in MDA-MB-231 cells was detected by Western blot. GAPDH was acted as the loading control. B: the expression of E-cadherin and Vimentin in NC and shGALNT6 cells of MDA-MB-231 was detected by Western blot with or without TGF-β1 (5 ng/mL) treated for 48 h. GAPDH was acted as the loading control. *P<0.05, **P<0.01, ***P<0.001.

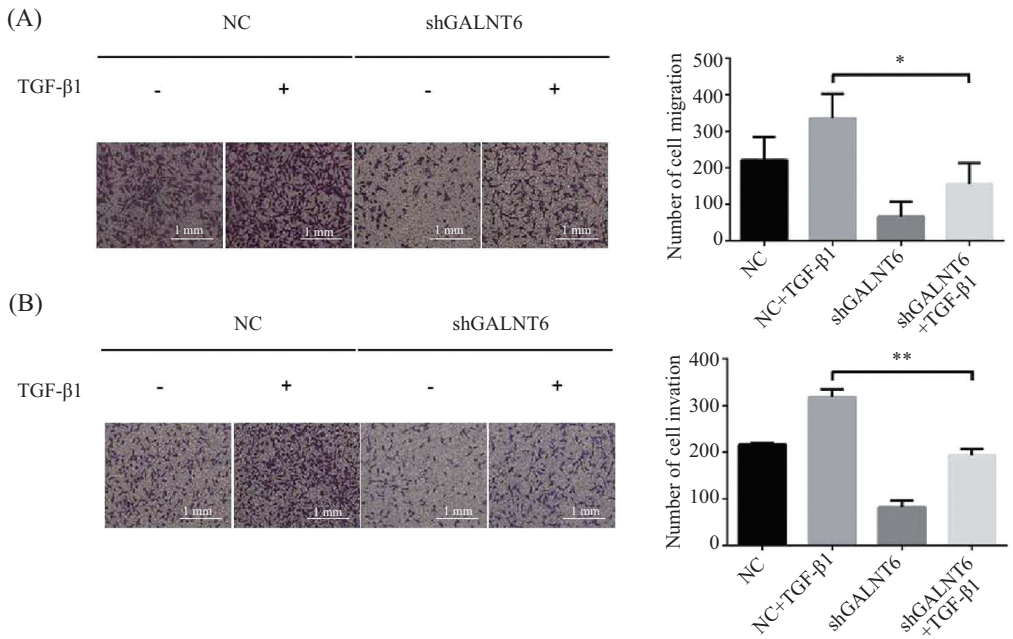
图3 GALNT6通过激活TGF-β信号通路增强EMT

Fig.3 GALNT6 enhanced EMT by activating TGF-β signaling pathway

Reports show that glycosylation of TGFBR2 promotes the combination with TGF-β1 and affects cell surface transportation [30]. The expression of TGFBR2 mRNA and protein was significantly decreased in shGALNT6 cells, compared with NC cells (Fig.7A). Co-IP results showed that GALNT6 was interacted with TGFBR2 (Fig.6D). Furthermore, knockdown of GALNT6 in MDA-MB-231 cells reduced the *O*-glycosylation modification of TGFBR2 (Fig.6C and Fig.7B). These results suggested that TGFBR2 was glycosylated by GALNT6, and the glycosylation of TGFBR2 promoted the TGF-β1-induced EMT, indicating that GALNT6 might regulate TGF-β-induced EMT in breast cancer through glycosylation of TGFBR2.

3 Discussion

Aberrantly *O*-glycosylation, mostly transferred by GALNTs, had been reported to occur in many cancers [17]. GALNT6, as an important number of GALNTs, was involved in multiple biological events of breast cancer, including invasion and metastasis [10,18]. Researches have demonstrated that EMT, a key event in the process of tumor cell metastasis, can be regulated by glycosyltransferases [19-20]. The most common biochemical change associated with EMT is the reduction of E-cadherin expression and the increase of mesenchymal markers Vimentin and N-cadherin [21]. In addition, numerous studies have shown that the EMT process of cancer cells can be activated by MMPs (matrix metalloproteinases), which is a family of zinc-dependent endopeptidases that can reshape ECM (extracellular matrix), affect cell-cell/cell-matrix interactions [22], and further promote cell migration under the regulation of MMPs [23]. MMPs-dependent activation of the EMT program has been observed in a variety of cell types, and involved in tumor development, cell proliferation

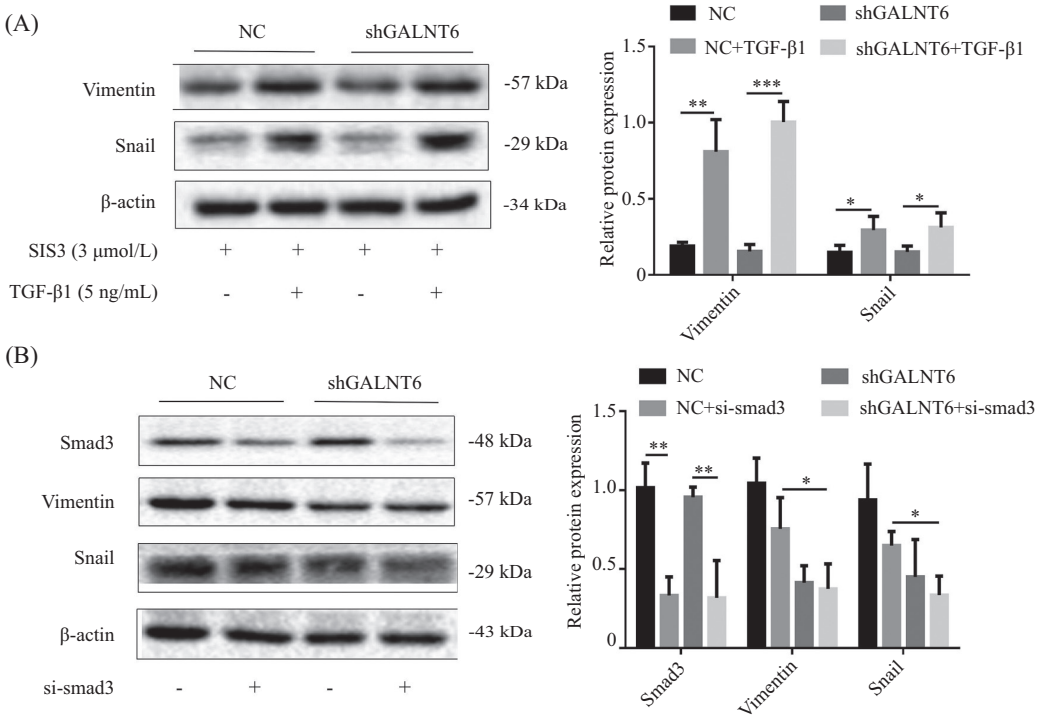


A: NC及shGALNT6细胞在TGF-β1(5 ng/mL)处理48 h及不处理的对照情况下, 用Transwell法检测细胞迁移能力。B: NC及shGALNT6细胞在TGF-β1(5 ng/mL)处理48 h及不处理的对照情况下, Matrigel Transwell法检测细胞侵袭能力。*P<0.05, **P<0.01。

A: cell migration assay was identified by transwell assay with or without TGF-β1 (5 ng/mL) treated for 48 h. B: cell invasion ability was evaluated by matrigel transwell assay with or without TGF-β1 (5 ng/mL) treated for 48 h. *P<0.05, **P<0.01.

图4 GALNT6激活TGF-β信号通路促进EMT进程

Fig.4 GALNT6 promoted EMT by activating TGF-β signaling pathway

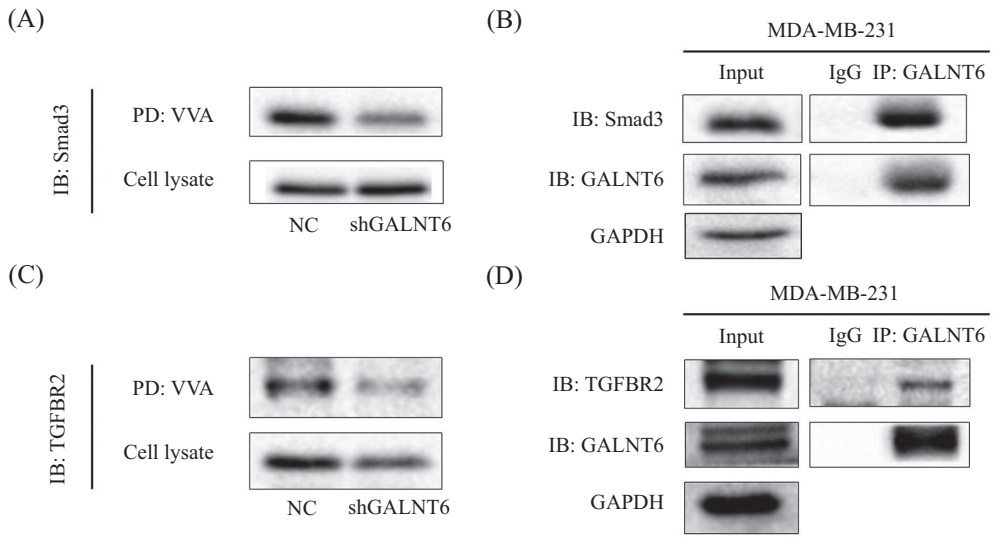


A: NC和shGALNT6细胞经SIS3(3 μmol/L)预处理及TGF-β1(5 ng/mL)处理24 h后, WB检测Vimentin和Snail的蛋白表达, β-actin为内参。B: NC及shGALNT6细胞在si-Smad3处理及不处理的对照情况下, WB检测Smad3、Vimentin和Snail的蛋白表达, β-actin为内参。*P<0.05, **P<0.01, ***P<0.001。

A: the expression of Vimentin and Snail in NC and shGALNT6 cells of MDA-MB-231 was detected by Western blot after pre-treated with 3 μmol/L SIS3 for 6 h and subjected to 5 ng/mL TGF-β1 for 24 h. β-actin was acted as the loading control. B: the expression of Smad3, Vimentin and Snail in NC and shGALNT6 cells of MDA-MB-231 was detected by Western blot with or without si-Smad3 treatment. β-actin was acted as the loading control. *P<0.05, **P<0.01, ***P<0.001.

图5 GALNT6通过TGF-β/Smad信号通路促进EMT进程

Fig.5 GALNT6 promoted EMT progress mainly through TGF-β/Smad pathway



A: NC和shGALNT6细胞裂解蛋白(0.6 mg)与VVA孵育, 用Smad3抗体进行免疫印迹(IB)分析VVA下拉的蛋白, 以来自全细胞裂解液的Smad3(Input)为对照。B: GALNT6抗体对MDA-MB-231细胞裂解产物进行免疫沉淀, 用蛋白A/G琼脂糖珠孵育回收结合蛋白。用抗Smad3或GALNT6的抗体免疫印迹共沉淀Smad3/GALNT6蛋白。C: NC和shGALNT6细胞裂解蛋白与VVA孵育, 用TGFBR2抗体进行免疫印迹(IB)分析VVA下拉的蛋白, 以来自全细胞裂解液的TGFBR2(Input)为对照。D: GALNT6抗体对MDA-MB-231细胞裂解产物进行免疫沉淀, 用蛋白A/G琼脂糖珠孵育回收结合蛋白。用抗TGFBR2或GALNT6的抗体免疫印迹共沉淀TGFBR2/GALNT6蛋白。

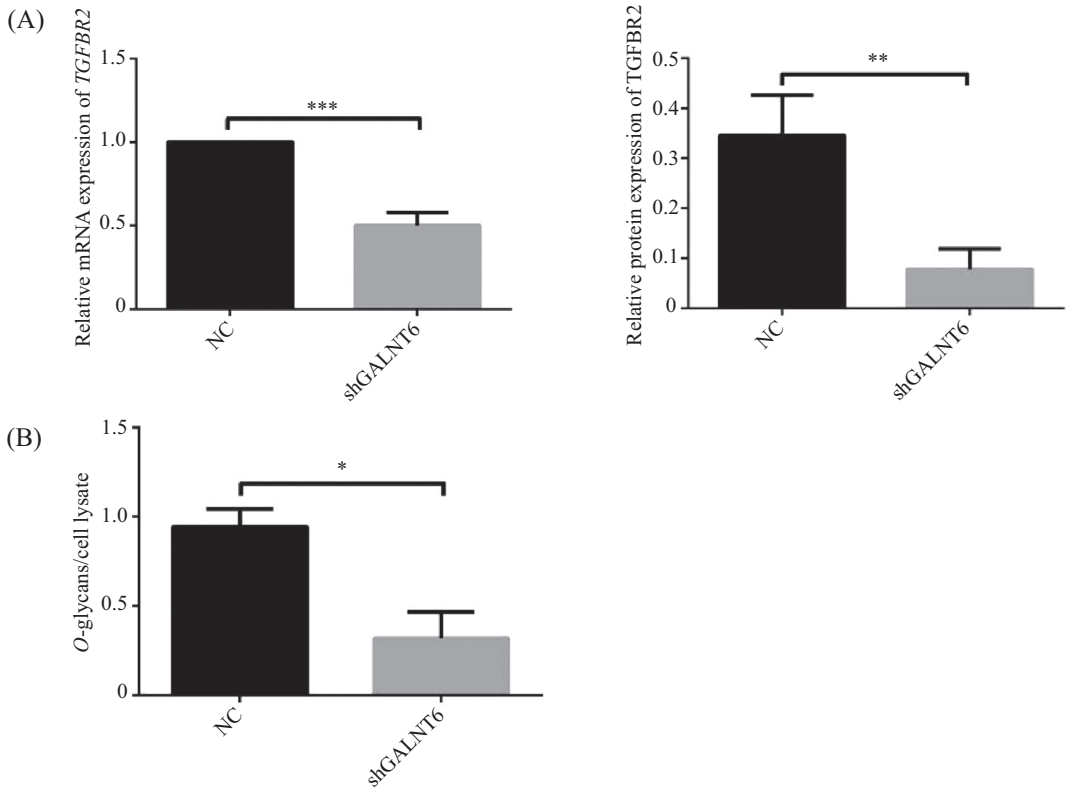
A: the NC and shGALNT6 cells lysates (0.6 mg) were incubated with VVA. Protein pulled down by VVA was analyzed by immunoblotting (IB) with Smad3 antibody. Smad3 (Input) from the whole cell lysates was used as loading control. B: MDA-MB-231 cell lysate was subjected to immunoprecipitation with GALNT6 antibody and bound protein were recovered by incubation with protein A/G Sepharose. The presence of coprecipitated Smad3/GALNT6 was determined by immunoblotting with antibodies against Smad3 or GALNT6. C: the NC and shGALNT6 lysates were incubated with VVA. Protein pulled down by VVA was analyzed by immunoblotting (IB) with antibody against TGFBR2. TGFBR2 (Input) from the whole cell lysates was used as loading control. D: MDA-MB-231 cell lysate was subjected to immunoprecipitation with GALNT6 or TGFBR2 antibody and bound protein was incubated with protein A/G Sepharose. The presence of coprecipitated TGFBR2/GALNT6 was determined by immunoblotting with antibodied against TGFBR2 or GALNT6.

图6 GALNT6糖基化修饰Smad3和TGFBR2

Fig.6 GALNT6 *O*-glycosylated Smad3 and TGFBR2

and metastasis [24]. Among them, MMP2 and MMP9 have been shown to induce the progression of EMT in a variety of tumors. Our findings suggest that GALNT6 may affect cell migration and invasion by regulation of the expression of MMPs and the transformation from E-cadherin to N-cadherin, and promote the EMT process of breast cancer cells.

The abnormal biological function of tumor cells is usually accompanied by abnormal signal transduction between cells, and the regulation of different signal transduction pathways plays a corresponding role. EMT is associated with multiple signaling pathways, such as TGF-β, WNT, and SHH (Sonic Hedgehog) pathways [25], among which TGF-β signal pathway is one of the most characteristic signal pathways in endothelial cell transformation and plays an important role in tumorigenesis and metastasis [26-27]. TGF-β signal can promote the EMT process by directly regulating Smad protein or activating non-Smad signal molecules, while Smad pathway is dominant [28]. TGF-β is mainly related to Smad2 and Smad3 proteins. The phosphorylation of Smad3 is the marker of the activation of Smad signal transduction pathway [29]. When the Smad pathway was blocked by SIS3, the expression of EMT-related proteins and Smad3 were not affected by knockdown of GALNT6. But the interesting thing was the phosphorylation of Smad3, which activated the TGF-β signaling pathway, was reduced by knockdown of GALNT6. Furthermore, when Smad3 was silenced by siRNA oligonucleotides, the knockdown of GALNT6 suppressed the expression of Vimentin and Snail. Lectin pull down and Co-IP results showed that GALNT6 interacted with Smad3, and knockdown of GALNT6 reduced Tn antigen expression on Smad3, indicating that GALNT6 regulated EMT pro-



A: WB检测NC和shGALNT6细胞中TGFBR2的蛋白表达水平, q-PCR检测NC和shGALNT6细胞中*TGFBR2*基因的表达水平。B: NC和shGALNT6裂解产物与VVA孵育, 用抗TGFBR2抗体进行免疫印迹(IB)分析VVA拉下的蛋白。以全细胞裂解液的TGFBR2为对照。*P<0.05, **P<0.01, ***P<0.001。

A: the protein level of TGFBR2 was examined by WB and the gene level of *TGFBR2* was examined by q-PCR in NC and shGALNT6 cells. B: the NC and shGALNT6 lysates were incubated with VVA. Protein was pulled down by VVA and analyzed by immunoblotting (IB) with antibody against TGFBR2. TGFBR2 (Input) from whole cell lysates was used as loading control. *P<0.05, **P<0.01, ***P<0.001.

图7 GALNT6对TGFBR2表达及糖基化水平的影响

Fig.7 Effect of GALNT6 on TGFBR2 expression and glycosylation level

gression by modifying Smad activity.

During the regulation of TGF-β signaling pathway, the TGF-β signal could be activated by TGF-β1, which combined with the TGFBR2 and linked with TGFBR1 to enhance EMT progress [28]. Here, we found that GALNT6 knockdown not only inhibited the expression of TGFBR2 gene and protein, but also reduced the *O*-glycosylation modification of TGFBR2, and Co-IP showed that there was interaction between GALNT6 and TGFBR2. These results indicated that TGFBR2 could be a substrate of GALNT6, and glycosylation of TGFBR2 played a role in TGF-β1-induced EMT. In addition, we found that GALNT6 knockdown reversed the expression of Vimentin and E-cadherin regulated by TGF-β1, and inhibited the cell migration and invasion induced by TGF-β1. To sum up, we knew that knockdown of GALNT6 inhibited the EMT progression induced by TGF-β1 in breast cancer cells and this suggested that GALNT6 enhanced EMT progress through TGF-β/Smad signal pathway.

In conclusion, this study showed that GALNT6 promoted the EMT progression of breast cancer cells. GALNT6 induces EMT by glycosylation of TGFBR2 and Smad3 to promote breast cancer metastasis through TGF-β/Smad signaling pathway. These findings provide insights into the mechanism underlying EMT regulation and support the potential targeted inhibition of GALNT6 as therapeutic strategy for breast cancer.

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