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PRECLINICAL STUDIES

MiR-181a, a new regulator of TGF-β signaling, can promote cell migration and proliferation in gastric cancer

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Summary

Transforming growth factor-beta (TGF-β) signaling pathway plays pivotal roles in various types of cancer. TGF-β receptor 2 (TGFβR2) contains a kinase domain that phosphorylates and activates the downstream of the TGF-β signaling pathway. Our previous microarray analysis revealed marked changes in miR-181a expression in gastric cancers, and the bioinformatics analysis suggested that miR-181a negatively regulated TGFβR2. In order to verify the effect of miR-181a on TGFβR2 and clarify the influence of miR-181a on the migration and proliferation of gastric cancer, studies in gastric cancer cell lines and xenograft mouse models were carried out. We found that a reduced expression of TGFβR2 and an increased expression miR-181a in gastric cancer tissues compared to adjacent noncancerous tissues. A luciferase reporter assay confirmed that TGFβR2 was a target of miR-181a. In addition, we found that miR-181a mimics, which increased the level of miR-181a, downregulated the expression of TGFβR2 in the gastric cancer cell line SGC-7901. Moreover, both the overexpression of miR-181a and the downregulation of TGFβR2 promoted the migration and proliferation of SGC-7901 cells. Conversely, SGC-7901 cell migration and proliferation were inhibited by the downregulation of miR-181a and the overexpression of TGFβR2. Furthermore, the increased expression of miR-181a and the decreased expression of TGFβR2 also enhanced the tumor growth in mice bearing gastric cancer. Our results herein indicated that miR-181a promoted the migration and proliferation of gastric cancer cells by downregulating TGFβR2 at the posttranscriptional level. The present study suggests that miR-181a is a novel negative regulator of TGFβR2 in the TGF-β signaling pathway and thus represents a potential new therapeutic target for gastric cancer.

Keywords TGFβR2 · miR-181a · Gastric cancer · Posttranscriptional regulation · Molecular targeted therapy

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Introduction

TGF-β, a multifunctional growth factor expressed in both normal cells and cancer cells, has important functions, including regulating cell proliferation and development, maintaining stem cell homeostasis, promoting fibrosis and modulating the immune response via the TGF-β/Smads pathway [1, 2]. TGF-β/Smad signaling pathway is also involved in many pathological processes and plays an especially extensive role in cancer [3, 4]. In addition to proliferation, migration and invasion, TGF-β participates in carcinogenesis [5], chemotherapy [6, 7], target therapy [8], angiogenesis, the epithelial-mesenchymal transition [9] and immune escape [10]. Because high TGF-β expression is associated with poor prognoses for gastric cancer patients [11], regulation of the TGF-β pathway is of substantial importance for this disease.

There are three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) and three corresponding TGF-β receptors (TGF-β receptor 1 (TGFβR1), TGFβR2 and TGFβR3). The first two receptors, which possess kinase activity, phosphorylate and activate the downstream of the TGF-β/Smads pathway, while TGFβR3 has no kinase activity. In TGF-β signaling, TGF-β sequentially binds to TGFβR2 and TGFβR1, forming a heteromeric complex. Accordingly, TGFβR2 binds to TGF-β to function crucially and initiates the signal transduction, and aberrant TGFβR2 signaling has been found in many types of cancer, affecting the biological characteristics of tumors and patients' survival. Moreover, N-linked glycosylation of TGFβR2 is required for successful localization at the cell surface and TGF-β signal transduction [12], and defects in this process prevent the transport of TGFβR2 across the plasma membrane. Several alterations may influence TGFβR2 function, including N-linked glycosylation, microsatellite instability [13, 14], and microRNA (miRNA)-mediated regulation [15], with miRNAs being among the most important regulators of TGFβR2 function.

MiRNAs are evolutionarily conserved, small noncoding RNAs that play important roles in cell differentiation and survival, interacting with numerous signaling pathways and serving as protooncogenes or tumor suppressors [16, 17]. MiRNAs function by binding, either individually or in combination, to the 3′-untranslated regions (3'-UTRs) of target mRNAs. Abnormal expressions of miRNAs have been found in numerous types of cancer, including acute myeloid leukemia and solid tumors. In vitro, miRNAs have been linked to cell proliferation [18], the epithelial-mesenchymal transition [19], the immune response [20], and other processes. In vivo, miRNAs have been associated with the diagnosis and survival of patients with malignant cancers [21, 22]. MiR-181 acts as a regulator in some signaling pathways [23–25] and has been associated with pathological types, clinical responses, drug resistances and patients' survival [25–29]. Four members of the miR-181 family have been reported to date: miR-181a, miR-181b, miR-181c and miR-181d. These molecules are encoded by separate loci and function differently in various types of tumors. According to our previous microarray results, miR-181a was changed more often than other members of this family; additionally, bioinformatics analyses predicted that miR-181a was a regulator of TGFβR2. Therefore, the present study focused on the effect of miR-181a on the migration and proliferation of gastric cancer cells and the mechanism underlying the interaction between miR-181a and TGFβR2.

Materials and methods

Human tissues

Human gastric cancer tissues and paired adjacent noncancerous tissues were collected from patients who provided informed consents after undergoing surgery at the Tianjin Medical University Cancer Institute and Hospital. The cancerous tissues were histopathologically confirmed to be adenocarcinoma, and noncancerous tissues were verified to be negative of tumor cells by pathologists. After surgery, all tissues were immediately frozen in liquid nitrogen. Total protein and RNA were extracted and stored in aliquots at −80 °C. This study was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital.

Cell line and culture

The human gastric cancer cell line SGC-7901 was obtained from the Shanghai Institute of Cell Biology of Chinese Academy of Sciences (Shanghai, China). SGC-7901 cells were cultured in culture dishes or plates in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Solarbio, China) in a humidified incubator at 37 °C and 5% CO_2.

Animals and establishment of tumor xenografts

All animal experimental procedures were approved by the Institutional Animal Care and Research Advisory Committee of the Tianjin Medical University Cancer Institute and Hospital and performed according to the protocols described below. Male BALB/c-nu nude mice (5–6 weeks old) were housed in a pathogen-free animal facility and had unlimited access to food and water. Six BALB/c-nu nude mice were divided into three groups and subcutaneously injected with SGC-7901 cells transfected with a control lentivirus, miR-181a mimics or a TGFβR2 overexpression plasmid (1×10^7 cells per mouse). The mice were sacrificed on the 28th day after injection, and the diameters and weights of the tumors were measured and recorded.

Cell transfection

SGC-7901 cells were seeded in 6-, 12- or 24-well plates and transfected with Lipofectamine 2000 (Invitrogen, Life Technologies) in Opti-MEM Reduced Serum Medium (Gibco, Life Technologies) 24 h later. Equal numbers of cells were incubated with equal amounts (100 pmol) of miR-181 mimics, inhibitors or negative controls (RiboBio, Guangzhou, China) for 6 h, after which the medium was replaced to remove remaining liposomes. The miR-181a mimics comprised chemically modified double-stranded RNAs that mimic endogenous miR-181a and enhanced miR-181a activity. The inhibitors, also chemically modified single-stranded RNAs, specifically bound to endogenous miR-181a and inhibited its activity. A TGFβR2 overexpression plasmid without the miR-181a-responsive 3'-UTR was used, and an empty plasmid was used as a negative control. Two siRNAs targeting human TGFβR2 were employed to downregulate TGFβR2 expression, and a scrambled siRNA was used as a negative control (both from RiboBio). Cells were harvested 24 h after transfection for real-time quantitative PCR analysis and 48 h after transfection for Western blot analysis.

Immunohistochemistry

Gastric cancer and noncancerous tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned and stained with anti-TGFβR2 (sc-17,791, Santa Cruz) according to the manufacturer's protocol. The results were confirmed by pathologists.

Western blotting

SGC-7901 cells were plated in 6-well plates and cultured in DMEM containing 10% FBS for 24 h. The cells were then transfected and incubated for an additional 6 h, after which the liposome-containing medium was discarded. Total proteins were isolated and solubilized in lysis buffer at 48 h after transfection, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Roche). The membranes were blocked with 2% bovine serum albumin (BSA) at room temperature for 1 h and then incubated at 4 °C with the primary antibodies rabbit anti-TGFβR2 (1:200, Santa Cruz) and anti-GAPDH (1:3000, Santa Cruz) overnight. The membranes were washed three times and incubated with the appropriate secondary anti-rabbit or anti-mouse IgG (1:2000, Santa Cruz). After washing three times, the membranes were incubated with enhanced chemiluminescence (ECL) reagents and visualized with an ECL system kit (Millipore, Bedford, MA, USA) according to the manufacturer's protocol. Total protein was normalized according to the level of GAPDH.

RNA isolation and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from tissues and cultured cells using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol. cDNA was reverse-transcribed via M-MLV reverse transcriptase (TaKaRa) under the following conditions: 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min. The resulting mRNA was quantified by real-time quantitative PCR (RT-qPCR) using Taqman miRNA probes (Applied Biosystems, Foster City, CA, USA) and a CFX96 instrument (Bio-Rad) as follows: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The cycle threshold (CT) was determined using fixed threshold settings after completion of the reaction, and mean CT values were calculated from triplicates. A comparative CT method was used to compare each condition with the control. U6 snRNA was used as an internal miRNA control, and the housekeeping gene GAPDH was used as a TGFβR2 mRNA control. Relative levels of gene expression compared to the control were calculated using the eq. $2^{-\Delta CT}$, where $\Delta CT = CT\ gene - CT\ control$. All reactions were performed in triplicate. The primers for TGFβR2 and GAPDH were as follows:

5'-GTAGCTCTGATGAGTGCAATGAC-3′ (TGFbR2 sense),
5'-CAGATATGGCAACTCCCAGTG-3′ (TGFbR2 antisense);
5'-AGAAGGCTGGGGCTCATTTG-3′ (GAPDH sense),
5'-AGGGGCCATCCACAGTCTTC-3′ (GAPDH antisense).

Cell proliferation assay

SGC-7901 cells were seeded in 24-well plates and transfected with miR-181a mimics, inhibitors or relevant negative controls. Twenty-four hours after transfection, the proliferation of SGC-7901 cells was measured using the Cell-Light EdU DNA cell kit (Ribobio) according to the manufacturer's instructions. In brief, 50 μM/ml 5-ethynyl-2′-deoxyuridine (EdU) was added to the medium, and the cells were cultured for 5 h, fixed with 4% paraformaldehyde for 30 min and treated with 0.5% Triton X-100 for 15 min. The cells were then incubated with Apollo for 30 min in the dark and in Hoechst 33342 for an additional 30 min. Cells labeled with EdU were manually counted in five randomly selected visual fields from each well. All staining procedures were performed in triplicate.

The viability of SGC-7901 cells carrying TGFβR2 siRNA or the TGFβR2 overexpression plasmid was examined using

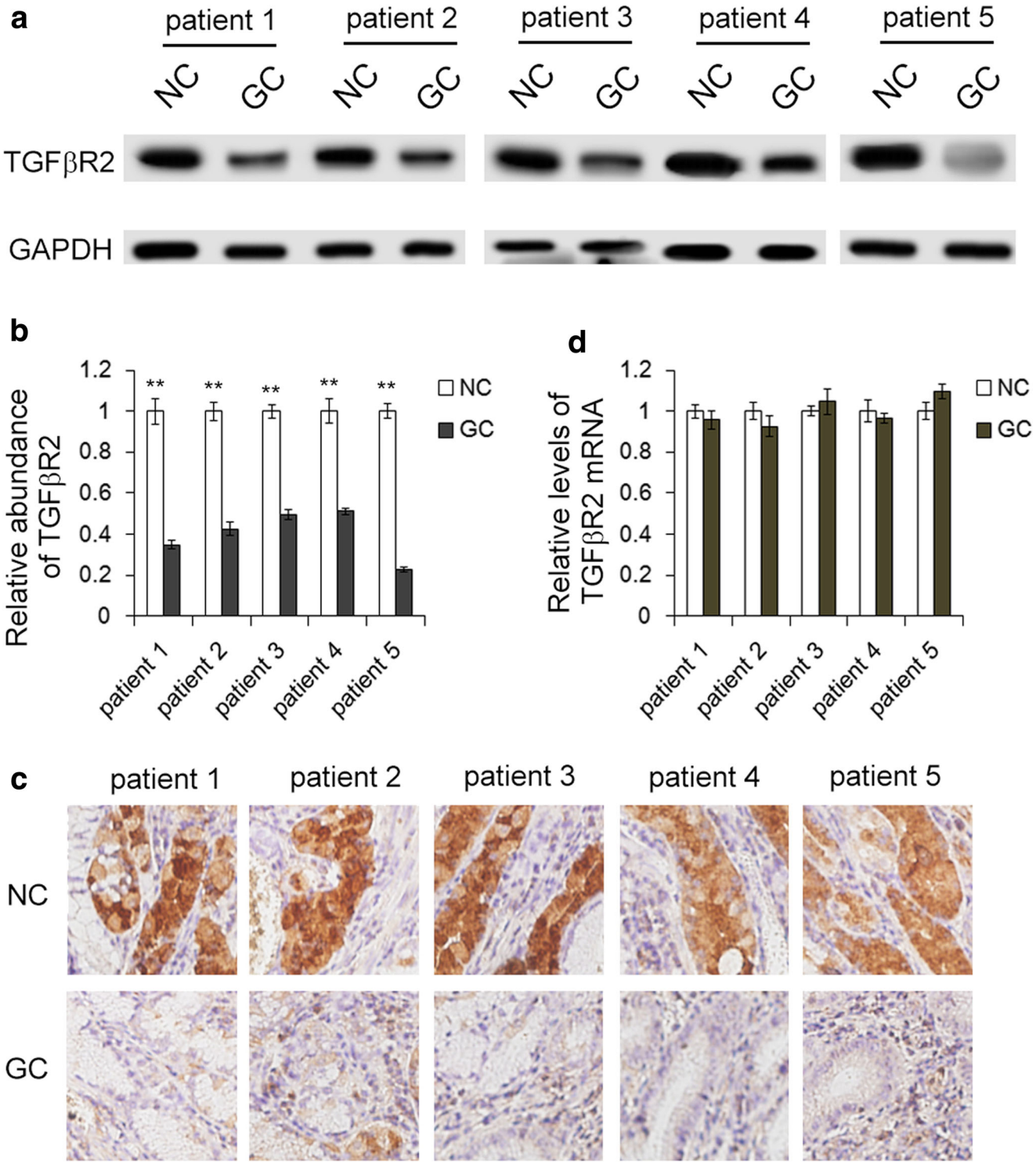


Fig. 1 TGFβR2 expression was decreased in gastric cancer tissues. **a** Western blot analysis of TGFβR2 protein expression in gastric cancer (GC) and adjacent noncancerous tissues (Global Burden of Disease Cancer, #9) ($n = 5$). **b** Quantitative analysis of TGFβR2 protein levels in (**a**). **c** Immunohistochemistry analysis of TGFβR2 expression in GC and NC tissues ($n = 5$). **d** Quantitative RT-PCR analysis of TGFβR2 mRNA levels in GC and NC tissues ($n = 5$)

the Cell Counting Kit (CCK-8) viability assay (MYBiotech, China). Briefly, SGC-7901 cells were cultured in 96-well plates and transfected with TGFβR2 siRNA or plasmid, after which 10 μl of the CCK-8 solution was added to each well; the plates were then incubated at 37 °C for 1 to 2 h. Optical density (OD) values were measured at a wavelength of 450 nm using a microplate reader (Thermo). The assay was performed at least three times.

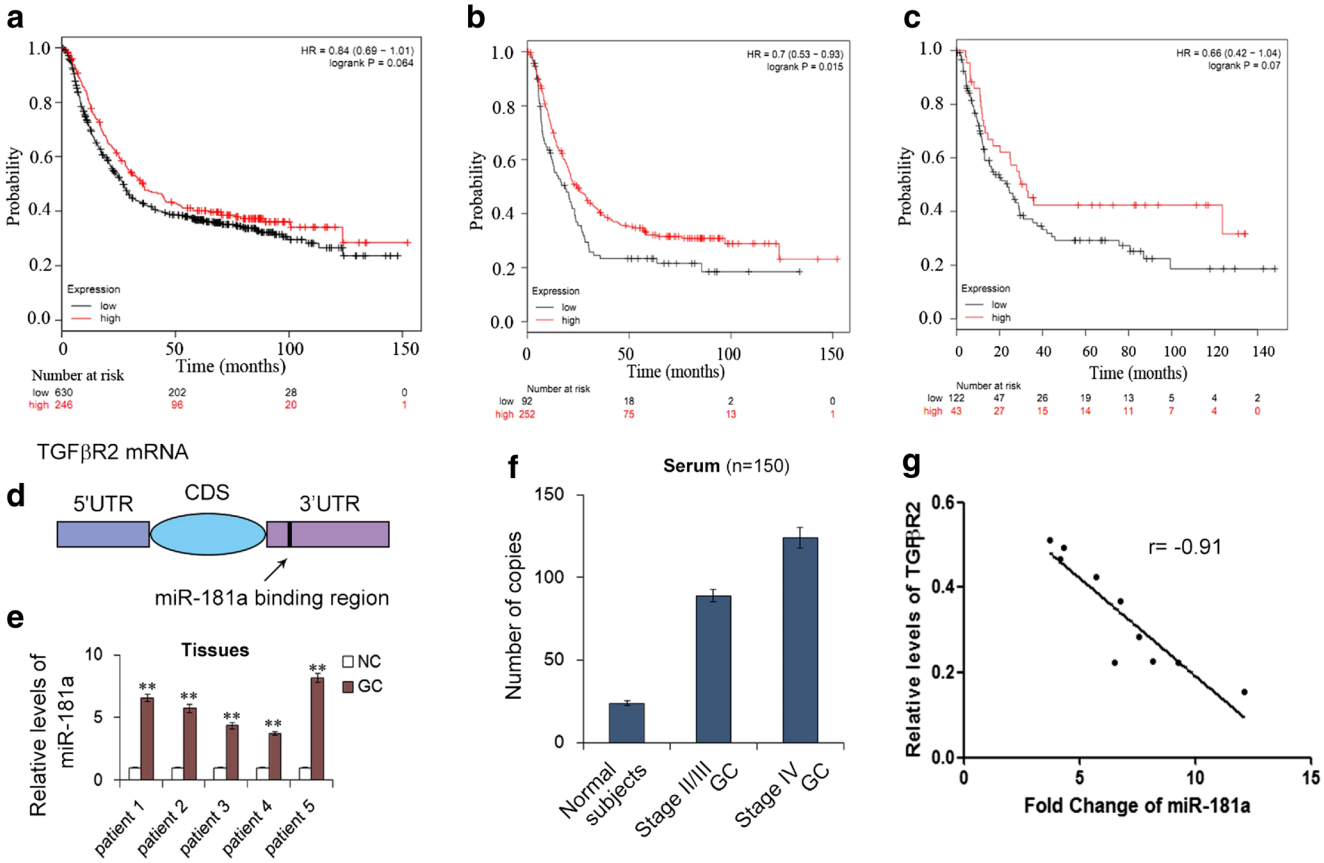


Fig. 2 Survival analysis of gastric cancer patients according to TGFβR2 expression and new regulator prediction. **a** Survival analysis of gastric cancer patients relative to TGFβR2 expression according to a Kaplan-Meier plot ($n = 876$). **b** Survival analysis of gastric cancer patients relative to TGFβR2 and HER-2 expression according to a Kaplan-Meier plot ($n = 344$). **c** Survival analysis of gastric cancer patients relative to TGFβR2 expression and cancer differentiation according to a Kaplan-Meier plot ($n = 165$). **d** Predicted binding site for miR-181a in TGFβR2 mRNA. UTR, untranslated region. CDS, coding sequence. **e** Relative levels of miR-181a in GC and NC tissues ($n = 5$). F, miR-181a copy numbers in GC (stages II/III and IV) and NC tissues ($n = 150$). **g** The relationship between TGFβR2 and miR-181a

Cell migration assay

The migration and invasive capability of SGC-7901 cells was assessed by wound healing and transwell assays. In the wound healing assay, SGC-7901 cells were seeded in 24-well plates; after the cells reached approximately 90% confluence, the surface of the plate was scratched with a 10-μL pipette tip to produce two linear regions without cells. The cells were cultured in DMEM without FBS and photographed every 6 h for 48 h after scraping. The wound zone distances were measured under a phase-contrast microscope in at least three randomly selected fields. The cells were digitally photographed and compared with the control group.

In the transwell assay, SGC-7901 cells were seeded in 6-well plates and transfected 24 h later with miR-181a mimics, inhibitors or relevant negative controls. After another 24 h period, the transfected cells were harvested and counted. Twenty-four-well Boyden chambers covered with an 8-μm-pore size polycarbonate membrane (Corning, NY, USA) were prepared; complete medium containing 10% FBS was added to the lower chamber as a chemoattractant, and approximately 10^5 cells per well were transferred to the upper chamber in 200 μl of serum-free growth medium. After incubation for 24 h at 37 °C, nonmigratory cells in the inner chamber were removed with cotton swabs, and cells migrating to the outside of the chamber were fixed and stained. The number of invading cells was counted under a light microscope. To minimize bias, five randomly selected visual fields were counted at 200× magnification, and the average number of cells was calculated. This experiment was performed at least three times.

Statistical analyses

All data are representative of at least three independent experiments. All statistical analyses were performed with IBM SPSS Statistics (version 20.0). Data were analyzed using Student's t test, and the results are presented as median values ± SEMs. Differences were considered statistically significant

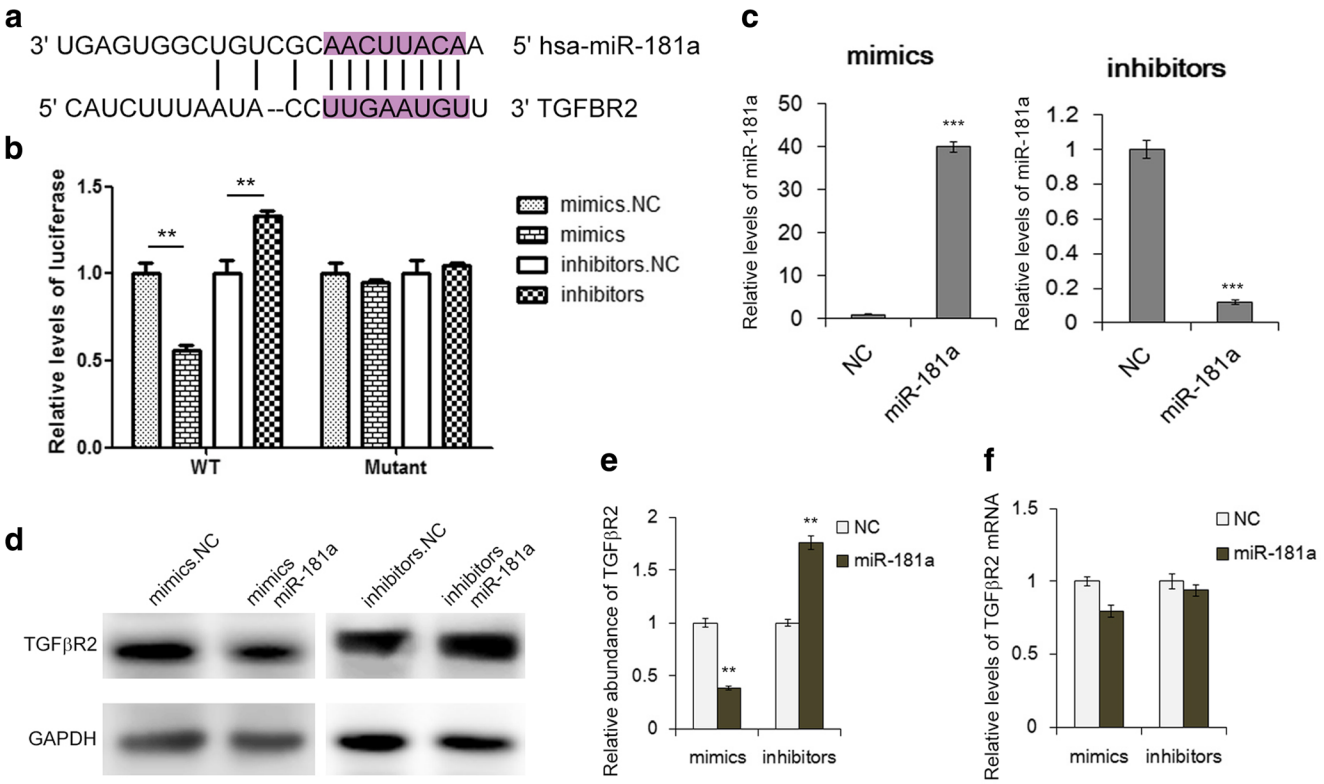


Fig. 3 The regulatory effects of miR-181a on TGFβR2 in gastric cancer. **a** Depiction of the base-pairing interaction between miR-181a and TGFβR2 mRNA. **b** TGFβR2 expression in SGC-7901 cells cotransfected with a firefly luciferase reporter (containing wild-type (WT) or mutant TGFβR2 3'-UTR) and either miR-181a mimics or inhibitors ($n = 3$). mimics. NC, control mimics; inhibitors. NC, control inhibitors. **c** Quantitative RT-PCR analysis of miR-181a expression in SGC-7901 cells transfected with miR-181a mimics or inhibitors ($n = 3$). **d** Western blot analysis of TGFβR2 protein expression in SGC-7901 cells. **e** Quantitative analysis of TGFβR2 protein expression in (**d**). **f**, Quantitative RT-PCR analysis of TGFβR2 mRNA levels in SGC7901 cells transfected with miR-181a mimics or inhibitors ($n = 3$). $^{**}P < .01$; $^{***}P < .001$

when $P < 0.05$. In this study, *, ** and *** indicated $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Results

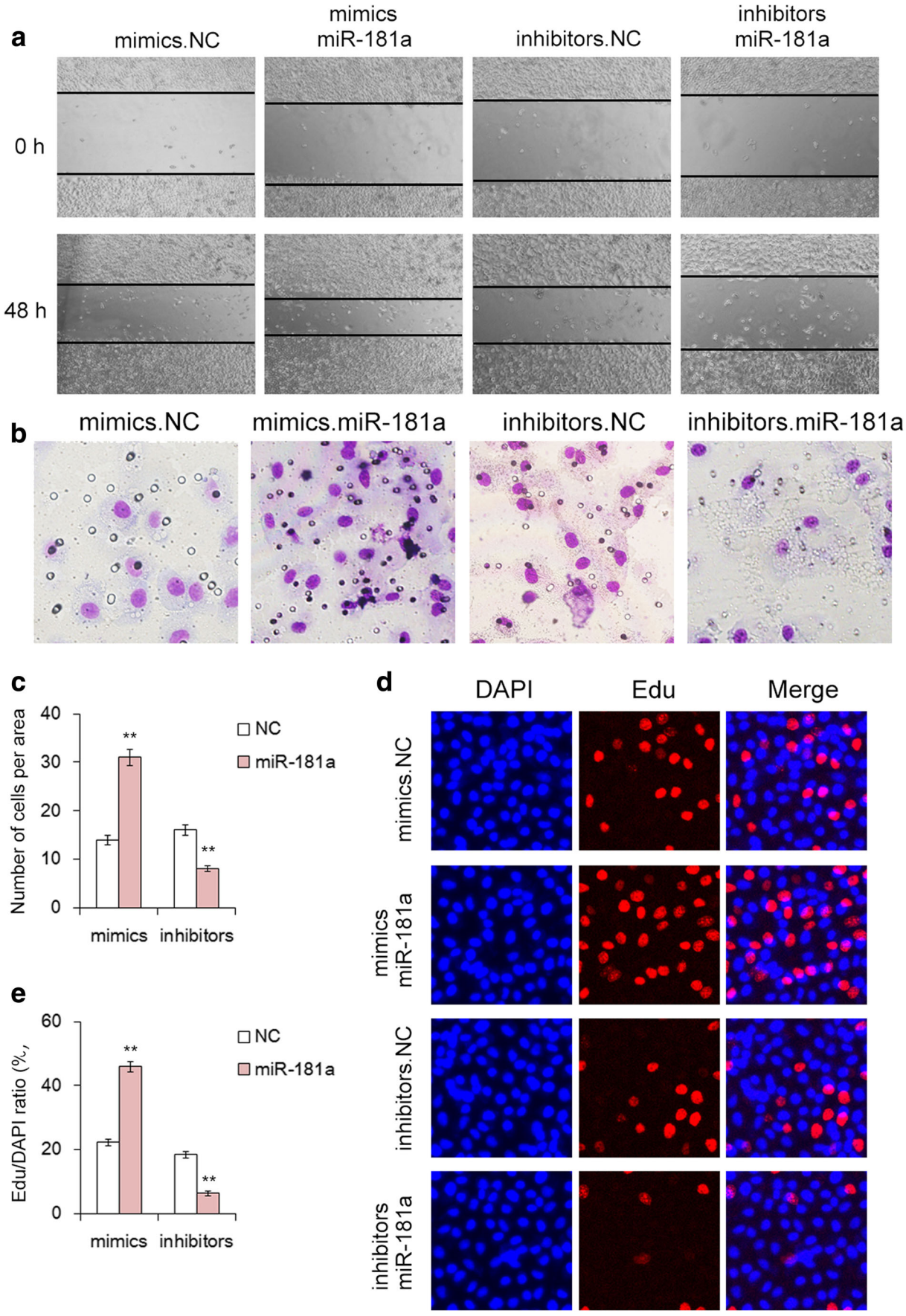
TGFβR2 protein expression was downregulated in tissues from gastric cancer patients

To determine the level of TGFβR2 protein expression in gastric cancer patients, five pairs of cancerous and adjacent noncancerous tissues were collected and examined. Compared with that in noncancerous tissues, the TGFβR2 protein expression in gastric cancer tissues was dramatically downregulated, as revealed by Western blot (Fig. 1a, b). This result was validated by immunohistochemistry, which showed decreased TGFβR2 protein expression in gastric cancer tissues compared to that in adjacent noncancerous tissues (Fig. 1c). To determine the cause of the decreased TGFβR2 protein expression, TGFβR2 mRNA levels in gastric cancer tissues were measured by quantitative real-time PCR. However, no obvious difference in the level of TGFβR2 mRNA expression was found between cancerous and adjacent noncancerous tissues (Fig. 1d). These results suggested that the TGFβR2 gene might act as a tumor suppressor and that the expression of its protein may be posttranscriptionally regulated in gastric cancer.

TGFβR2 expression was associated with the survival of patients with gastric cancer and negatively associated with miR-181a expression in gastric cancer tissues

To investigate the significance of TGFβR2 expression in gastric cancer patients, TGFβR2 protein expression data and gastric cancer prognosis data were analyzed via a Kaplan-Meier

Fig. 4 Effect of miR-181a on the migration and proliferation of gastric cancer cells. **a** The migration of SGC-7901 cells after transfection with miR-181a mimics or inhibitors in wound healing assays at 0 h and 48 h (n = 3). **b**, The migration of SGC-7901 cells after transfection with miR-181a mimics or inhibitors in transwell assays (×20) ($n = 3$). **c** The number of cells per area in (**b**). **d** The proliferation of SGC-7901 cells as determined by 5-ethynyl-2-deoxyuridine (EdU) assays (n = 3). **e** Quantitative analysis of SGC-7901 cells in EdU assays ($n = 3$). $^{**}P < 0.01$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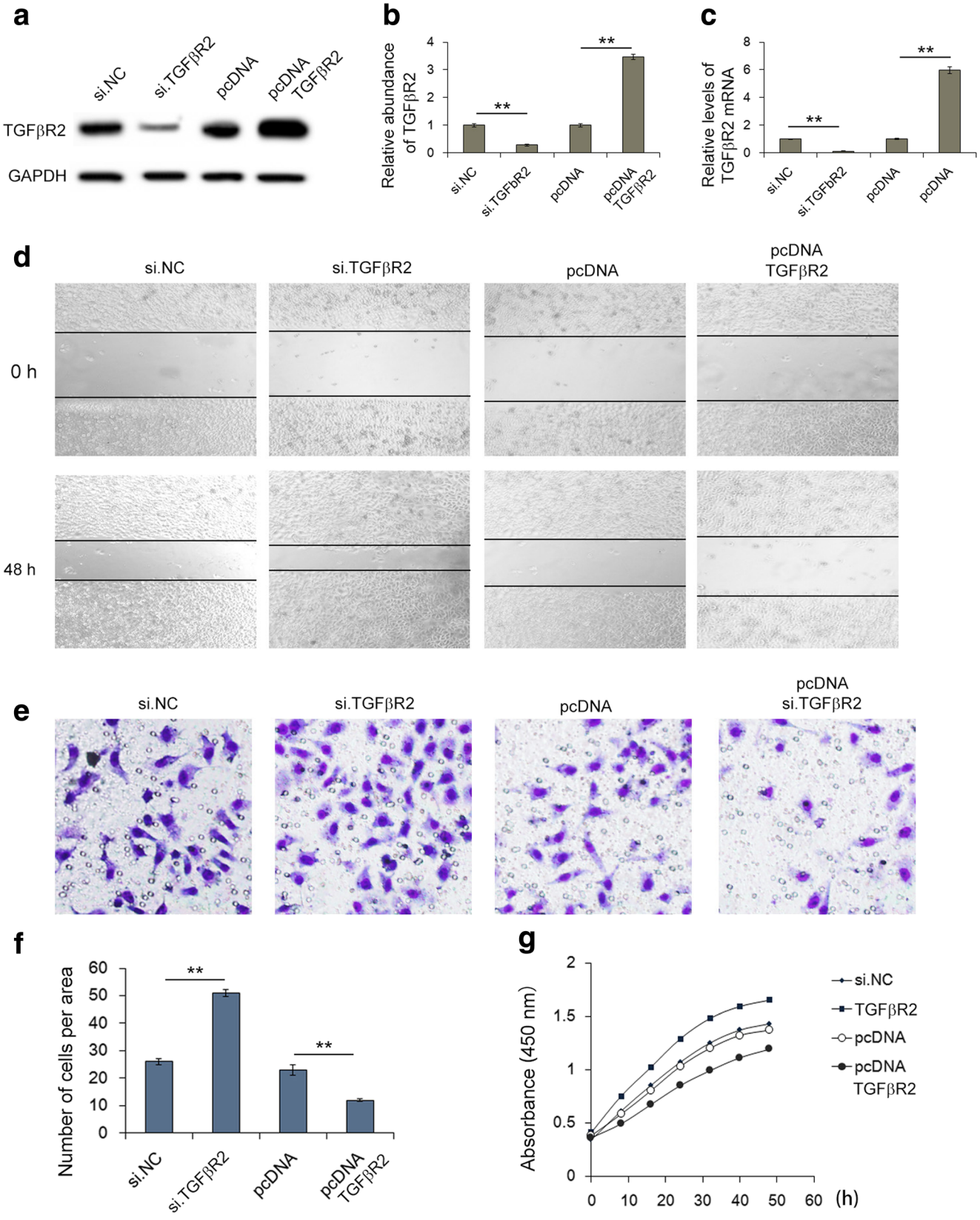

plot (website: kmplot.com/analysis/). Patients with high TGFβR2 expression tended to have a better prognosis than those with low TGFβR2 expression ($P = 0.064$), especially those with HER-2 positivity ($P = 0.015$) and poor

◀ **Fig. 5** TGFβR2 inhibits the migration and proliferation of SGC-7901 cells. **a** Western blot analysis of TGFβR2 protein expression after transfection with TGFβR2 siRNA or plasmid. **b** Quantitative analysis of TGFβR2 protein expression in (**a**). C, Quantitative RT-PCR analysis of TGFβR2 mRNA levels after transfection with TGFβR2 siRNA or plasmid. **d** Changes in the migration of SGC-7901 cells after transfection with TGFβR2 siRNA or plasmid were evaluated by the wound healing assay ($n = 3$). **e** Transwell assays of the migration of SGC-7901 cells transfected with TGFβR2 siRNA or plasmid ($n = 3$). **f** Quantitative analysis of the cell migration transwell assay results shown in (**e**) ($n = 3$). **g** Proliferation of SGC-7901 cells after transfection with TGFβR2 siRNA or plasmid as determined by the CCK-8 assay. $^{**}P < 0.01$

differentiation ($P = 0.07$) (Fig. 2a, b, c). Bioinformatics analyses were utilized to determine the upstream regulator of TGFβR2, revealing that miR-181a might bind to the 3'-UTR of TGFβR2 mRNA and regulate its function (Fig. 2d). Overall, miR-181a expression was significantly increased in cancerous tissues from the five gastric cancer patients (Fig. 2e). Additionally, data from another trial including 150 healthy and gastric cancer patients showed that the miR-181a copy numbers in tissues from gastric cancer patients, especially those in stage IV, were much higher than those in tissues from healthy individuals. These results suggested that the miR-181a copy number increased with the TNM stage (Fig. 2f). Moreover, the association analysis showed that the level of TGFβR2 protein expression was negatively correlated with the level of miR-181a expression ($r = -0.91$) (Fig. 2g). Thus, miR-181a might be a new regulator of TGFβR2 expression and function.

miR-181a inhibited TGFβR2 expression in SGC-7901 gastric cancer cells

According to bioinformatics predictions, a miR-181a binding site is present in the 3'-UTR of TGFβR2 mRNA (Fig. 3a). To investigate the effect of miR-181a on TGFβR2 in vitro, miR-181a mimics, inhibitors and corresponding negative controls were transfected into SGC-7901 cells, and miR-181a, TGFβR2 mRNA and TGFβR2 protein levels were examined. In wild-type gastric cancer cells, transfection of miR-181a mimics decreased TGFβR2 protein expression, whereas transfection of the miR-181a inhibitor elevated TGFβR2 protein expression. In cells expressing a mutated TGFβR2 3'-UTR, no obvious changes in TGFβR2 protein expression were detected after transfection with miR-181a mimics or inhibitors (Fig. 3b). As evaluated by RT-qPCR, miR-181a expression was significantly increased after transfection with miR-181a mimics and inhibited after transfection with miR-181a inhibitors (Fig. 3c). Western blot analyses also revealed that TGFβR2 protein expression was inhibited by treatment with miR-181a mimics and elevated by treatment with miR-181a inhibitors (Fig. 3d, e). Nonetheless, RT-qPCR revealed no significant changes in TGFβR2 mRNA levels after the treatment with either miR-181a mimics or inhibitors. These results suggested that the observed changes in TGFβR2 protein expression did not derive from changes in TGFβR2 mRNA levels and that regulation of TGFβR2 protein expression might occur at the posttranscriptional level without influencing TGFβR2 mRNA levels (Fig. 3f). Therefore, miR-181a might be an important regulator of TGFβR2 protein expression after transcription.

miR-181a promoted the migration and proliferation of SGC-7901 cells

To clarify whether miR-181a affected cell behaviors in gastric cancer, the migration and proliferation of SGC-7901 cells were evaluated using the wound healing, transwell and EdU assays, respectively. In the wound healing assay, SGC-7901 cells migrated faster after the treatment with miR-181a mimics, whereas migration was inhibited after transfection with miR-181a inhibitors (Fig. 4a). In the transwell assay, transfection with miR-181a mimics promoted migration compared to that occurring in the negative control, and transfection with miR-181a inhibitors decreased migration (Fig. 4b, c). According to the EdU assay, cell proliferation was notably increased in SGC-7901 cells after transfection with miR-181a mimics compared to that in the negative control and notably decreased after transfection with miR-181a inhibitors (Fig. 4d, e). Thus, miR-181a altered cell migration and proliferation in gastric cancer cells and might act as an oncogenic miRNA by promoting these processes in gastric cancer.

TGFβR2 might inhibit the migration and proliferation of SGC-7901 cells

To compare the impact of miR-181a with that of TGFβR2, the function of TGFβR2 in cell migration was evaluated using wound healing and transwell assays, and cell proliferation was evaluated with the CCK-8 viability assay. TGFβR2 was silenced with siRNA targeting human TGFβR2 or overexpressed with a plasmid expressing the TGFβR2 open reading frame (ORF). In SGC-7901 cells, TGFβR2 protein expression was markedly inhibited after transfection with TGFβR2 siRNA and dramatically increased after the transfection with the plasmid (Fig. 5a, b). Correspondingly, TGFβR2 mRNA levels were altered in the same manner as its protein levels following TGFβR2 siRNA or plasmid transfection (Fig. 5c). Moreover, the effects of altered TGFβR2 protein levels on gastric cancer cell behaviors were identical to those observed with the miR-181a treatments, and TGFβR2 siRNA promoted the migration in wound healing and transwell assays, in contrast to plasmid treatment (Fig. 5d, e, f). The CCK-8 viability assay showed that proliferation of SGC-7901 cells transfected with the TGFβR2 plasmid was inhibited compared to that of cells transfected with TGFβR2 siRNA or the corresponding negative controls (Fig. 5g).

Therefore, TGFβR2 negatively regulates gastric cancer migration and proliferation.

Effects of miR-181a and TGFβR2 on gastric cancer growth and gene expression in nude mice

To observe the influence of miR-181a and TGFβR2 on gastric cancer in vivo, BALB/c-nu nude mice were divided into three groups and treated individually with gastric cancer SGC-7901 cells, SGC-7901 cells overexpressing miR-181a, or SGC-7901 cells overexpressing TGFβR2 (Fig. 6a). The tumor burdens of mice overexpressing miR-181a were larger than those of mice overexpressing TGFβR2 (Fig. 6b, c). The cancerous tissues were further examined for miR-181a and TGFβR2 expression. Compared with the control, tumors induced by cells transfected with miR-181a showed increased miR-181a levels, whereas those transfected with TGFβR2 exhibited increased levels of TGFβR2 mRNA (Fig. 6d, e). In terms of TGFβR2 protein expression, transfection with miR-181a decreased the level of TGFβR2 protein expression, whereas

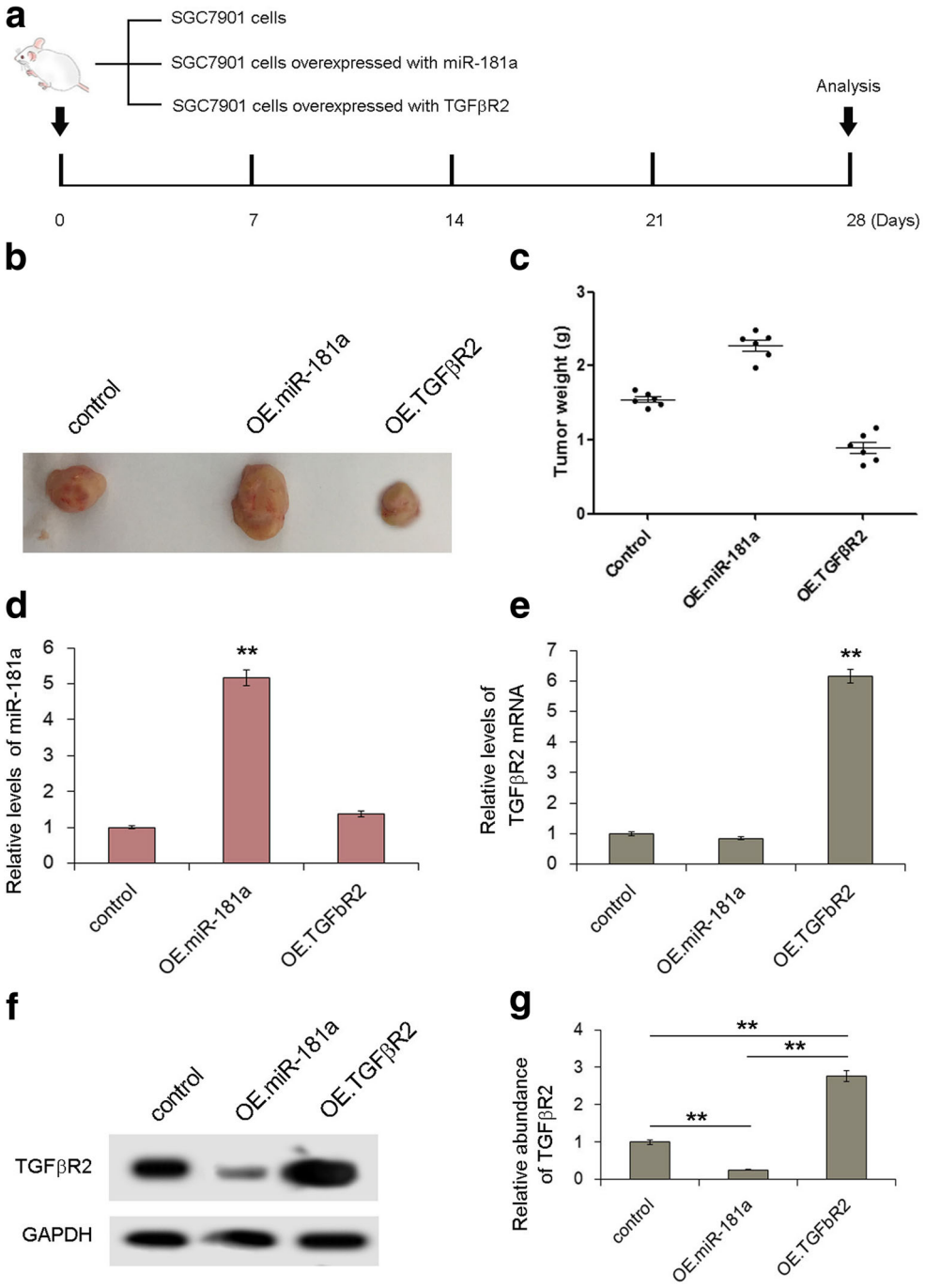

Fig. 6 Changes in tumor growth and gene expression in nude mice treated with miR-181a and TGFβR2. **a** Flow chart of the experiments performed in vivo. **b** Gross morphology of tumor xenografts from nude mice transfected with OE.miR-181a or OE.TGFβR2 ($n = 6$). Scale bar = 1 cm. **c** Weights of tumors in nude mice comprised of SGC-7901 cells transfected with OE.miR-181a or OE.TGFβR2 ($n = 6$). **d** Relative levels of miR-181a in tumors of nude mice ($n = 6$). **e** Relative levels of TGFβR2 mRNA in tumors of nude mice ($n = 6$). **f** Western blot analysis of TGFβR2 protein expression in tumors of nude mice ($n = 6$). **g** Quantitative analysis of TGFβR2 protein expression in (**f**) ($n = 6$). $^{***}P < 0.001$

transfection with the TGFβR2 plasmid obviously increased its protein expression (Fig. 6f, g).

Discussion

Gastric cancer is the fifth most common malignancy and the third most common cause of mortality worldwide. The situation in East Asia is much worse, especially in China. Most cases of gastric cancer have already developed to an advanced stage at diagnosis, and an increasing number of young patients are suffering from advanced gastric cancer. Despite the availability of chemotherapy to prolong the survival, many of these patients will progress to an advanced stage, with hemorrhaging, intestinal obstruction, peritoneal dissemination and ascites, and these complications significantly worsen the prognosis of advanced gastric cancer patients. Thus, a progress in early diagnosis and treatment is urgently needed to improve the survival of patients with advanced gastric cancer. The TGF-β signaling pathway has been reported to play an important role in gastric cancer. The most important receptor in this pathway is TGFβR2, which binds to TGF-β and activates the TGF-β signaling cascade. In our study, we found the TGFβR2 protein expression markedly decreased in gastric cancer tissues. Furthermore, a decreased level of TGFβR2 in gastric cancer was associated with a poor prognosis, especially in HER-2-positive and poorly differentiated cases (according to data from http://kmplot.com/). The prognosis of these patients is overall very poor [30, 31], and because TGFβR2 is a key factor in the TGF-β pathway, understanding and manipulating its regulation will improve the prognoses of these patients.

Apart from epigenetic changes, microsatellite instability and gene polymorphisms, miRNAs are among the most important posttranslational regulators of TGFβR2. Previous microarray and bioinformatics analyses have suggested that miR-181a may regulate TGFβR2. In this work, miR-181a expression was markedly increased in gastric cancer tissues compared to that in noncancerous tissues. Conversely, miR-181a expression has been reported to be clearly downregulated in lung cancer [32]. These varying results demonstrated that miR-181a might have different biological functions in diverse cancers. Next, the function of miR-181a in gastric cancer was investigated. Furthermore, we revealed the important effects of miR-181a-driven suppression of TGFβR2 on the promotion of gastric cancer cells proliferation and migration and on tumor growth in a xenografted nude mouse model. MiR-181a inhibitors promoted TGFβR2 expression and inhibited the migration and proliferation of gastric cancer cells, which resembled the inhibition observed after transfection of the TGFβR2 overexpression plasmid. The functions of miR-181a and TGFβR2 were confirmed by experiments in BALK/c-nu nude mice, showing that the miR-181a inhibitor acts as a tumor suppressor in a manner similar to that of TGFβR2. These findings provided a new treatment target for gastric cancer, especially for patients with HER-2-positive or poorly differentiated cancer.

Because of the relationship among miRNAs, prognosis and chemotherapy, miRNAs may be applied to alter the signal transduction, chemotherapy sensitivity and patients' prognosis for gastric cancer. The basic strategies of miRNA-based interventions include blocking the expression of target oncogene miRNAs and recovering the function of tumor suppressor miRNAs [33]. For highly expressed oncogenic miRNAs, such as miR-181a, a blockade may inhibit tumor migration and proliferation. As such interventions with miRNAs have been carried out mainly in animals [34], a substantial amount of work is still needed before these therapies can be used in patients.

Conclusion

miR-181a plays an important role in regulating the TGFβ pathway by downregulating TGFβR2 at the posttranscriptional level. As a result, miR-181a promotes the migration and proliferation of gastric cancer in vitro and in mice. Thus, miR-181a is a newly identified regulator of TGFβ signaling and may be used as a new treatment target for gastric cancer.

Author contributions SG and HZ performed most of the experiments, analyzed the data, and wrote the manuscript. TD, WS and TN reviewed and edited the manuscript. QF performed some experiments. YB and GY designed the experiments and edited the manuscript. YB is the guarantor of this work and has unlimited access to all of the data in the study and thus takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Data availability The datasets supporting the conclusions of this article are included within the article.

Compliance with ethical standards

Ethics approval and consent to participate Animal care and experiments were performed in accordance with guidelines of the Institutional Animal Use Committee of Tianjin Medical University, and the procedures were approved by the Institutional Animal Care and Use Committee.

Consent for publication Written informed consent for publication was obtained from all participants.

Competing interests The authors have no competing interests to declare.

Abbreviations *3′-UTR,* 3′-untranslated region; *CCK-8,* Cell Counting Kit-8; *cDNA,* complementary deoxyribonucleic acid; *EdU,* 5-ethynyl-2-deoxyuridine; *HER-2,* epidermal growth factor receptor-2; *miRNA,*

microRNA; *RT-qPCR,* real-time quantitative polymerase chain reaction; *siRNA,* small interfering RNA; *TGF-β,* transforming growth factor-beta; *TGFβR2,* TGF-β receptor 2

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