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MiR-141-3p suppresses gastric cancer induced transition of normal fibroblast and BMSC to cancer-associated fibroblasts *via* targeting STAT4

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ABSTRACT

Background: Cancer associated fibroblasts (CAFs) are known to be crucial constituents of cancer microenvironment (CME) and play an important role in initiation, progression and metastasis of various types of cancer, such as oral cancer, pancreatic cancer, and gastric cancer. CAFs are usually derived from normal fibroblasts (NFs), but the mechanism of the transition in gastric cancer has not yet been fully elucidated.
Methods: qRT-PCR and western blot were employed to investigate differences of miR-141 and STAT4 expression respectively. The CAF-like features and wnt/β-catenin pathway related proteins in NF or BMSC were assessed by qRT-PCR or western blot after treated with the conditioned medium from different indicated groups of gastric cancer cells. The invasion and migration ability of AGS cells after transfection were analyzed by Transwell assay and wound healing assay. Dual-luciferase report assay was employed to determine the direct binding of miR-141 to STAT4 3' UTR.
Results: For the first time, the present study found that STAT4 over-expression in gastric cancer cells induced NFs to obtain CAF-like features *via* activating wnt/β-catenin pathway. Further gain-of-function and loss-of-function analysis revealed that miR-141 not only limited the migration and invasion of the gastric cancer cells, but also inhibited the transition of NFs and BMSC to CAFs. The luciferase assay indicated that miR-141 directly targeted the 3'-UTR predictive sequence of STAT4.
Conclusion: Our data showed that miR-141 inhibited migration and invasion of gastric cancer cells and inhibited transition from NFs to CAFs *via* targeting STAT4/wnt/β-catenin pathway.

1. Introduction

Gastric cancer (GC) ranks the fourth most common cancer and the second leading cause of cancer-related death in the world (Kim and Baik, 2014; Bhandari and Crowe, 2012). The recording from National Cancer Institute indicated that East Asia has the highest incidence of GC (Jemal et al., 2010; Torre et al., 2015). Even the therapeutic strategies, such as surgery, chemotherapy, immunotherapy, and gene therapy have significant advances in the last decades, it is still limited for the treatment of advanced stage patients of GC (Catalano et al., 2009; Cui et al., 2013). Thus, further understanding the mechanism of GC development, progression and metastasis is urgently needed.

It has been reported and recognized that the active tumor microenvironment (TME), which contains various types of mesenchymal cells as well as extracellular matrix, plays an important role in tumor metastasis, occurrence and progression (Hanna et al., 2009; Quail and Joyce, 2013; Zhang et al., 2014; Trylcova et al., 2015). Cancer associated fibroblasts (CAFs) are the most abundant component in TME, and play critical role in proliferation, migration and invasion of several different types of tumor cells (Martinez-Outschoorn et al., 2010; Bremnes et al., 2011; Gandellini et al., 2015). CAFs were mainly derived from normal resident fibroblasts, which became more proliferative, aggressive and contractive during the transition triggered by cancer cell (Kalluri, 2016; Quante et al., 2011). In different types of cancer, such as oral cancer (Bello et al., 2011; Vered et al., 2010), breast cancer (Schoppmann et al., 2012), gastric cancer(Kitadai, 2010; Wang et al., 2013) and others (Herrera et al., 2013), the high percentage of CAFs in TME is a potential marker for high-grade malignancies and poor prognoses. Meanwhile, bone mesenchymal stem cells (BMSCs) also shows important function during the tumor development and

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progression (Karnoub et al., 2007) due to their ability of self-renew and differentiation into CAFs (Mishra et al., 2008). However, the mechanism of the transformation from NFs to CAFs in gastric cancer and the relationship between gastric cancer cells and the transformation are still elusive.

In many types of carcinomas, signal transducer and activator of transcription 4 (STAT4) is up-regulated and the high expression level usually correlates with the malignant properties of cancer (Watson and Neoh, 2008; Watson, 2001). Recently, Zhou *et al* reported that STAT4 promoted the metastasis of ovarian cancer *via* inducing normal fibroblasts and bone marrow-derived mesenchymal stem cells to obtain CAF-like features by activating the Wnt7a pathway (Zhao et al., 2017). However, the status of STAT4 in the metastasis of GC has not been yet explored. On the other hand, Zhang *et al* reported that the down-regulation of miR-141, which is a family member of miRNA-200, in GC significantly promoted the migration and invasion of gastric cancer cells by targeting STAT4 (Zhou et al., 2014a). Thus, we hypothesized that the transition from NFs to CAFs in TME might be inhibited by miR-141 over-expression in GC *via* suppressing STAT4 expression.

In present study, we determined the low expression levels of miR-141 and up-regulation of STAT4 in gastric cancer cells, and validated that overexpression of STAT4 promoted the gastric cancer cells migration and invasion ability. Furthermore, our results, for the first time, demonstrated that overexpression of STAT4 in GC cells induced the transformation of NFs and BMSC to CAFs *via* activating the wnt7a/β-catenin pathway. By investigating the function of miR-141 in GC cells migration and invasion, as well as the transformation of NFs to CAFs, we identified that miR-141 suppressed the expression of STAT4 in GC cells *via* directly binding to the 3'-UTR fragments of STAT4 mRNA, which inhibited the transition from NFs to CAFs. We hope our work may shed light on the mechanism of the transition of CAFs in GC and provide a potential therapeutic modality for GC patients.

2. Material and methods

2.1. Patients information and ethics statement

Thirty pairs of gastric cancer samples and adjacent normal tissue were obtained from patients who had the surgery resection at the Fujian Provincial Tumor Hospital from 2015 to 2017. The study was approved by the Research Ethics Committee of the Fujian Provincial Tumor Hospital. Written informed consent was obtained from all patients.

2.2. Cell culture and reagents

SNU-216, AGS, SGC7901, BGC823, and GES-1 were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). A medium of DMEM and F12 medium (1:1) supplemented with 400 ng/mL hydrocortisone, 10% FBS and 1% penicillin/streptomycin was used for all the cell culture. Primary normal human omental fibroblasts were collected from normal omental tissues of people who had surgical resection. Briefly, 2–3 cm^2 piece of omentum was washed with sterile phosphate-buffered saline (PBS), then was broken into small pierce using scissors. Next, the tissues was incubated on an orbital shaker with 10 mL PBS and 10 mL 0.25% trypsin/25 mM EDTA at 37 °C for 30 min. Furthermore, 100 U of hyaluronidase and 1000 U of collagenase type 3 in 100 mL PBS was used to digest the tissue on an orbital shaker at 37 °C for 6 h. Following centrifuging the suspended cells in the solution at 1500 r.p.m. for 5 min, the pellets was washed twice with 20 mL DMEM/F12 medium containing 10% FBS. Then, the fibroblast was purified and confirmed with PDGFRα-based FACS analysis and the purity was 98% (data not shown).

Normal human BMSCs were isolated from patient ilia as described previously (Tang et al., 2015). Briefly, bone marrow was obtained from the iliac crest of voluntary donors from whom informed consent had been obtained, and then was minced into small pieces. The BMSCs were collected by washing the ilia with DMEM with centrifuge at 13000 *g* for 30 min. The cells were then harvested and cultured in DMEM/F12 medium containing 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Plasmids and transfection

miR-141 mimic,miR-141 inhibitor,respective NC and plasmid for human STAT4 were obtained from Gene Copoeia (Guangzhou, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for the plasmids transfection into cells according to the manufacturer's instructions.

2.4. Quantitative RT-PCR (qPCR)

Total RNAs were obtained using Trizol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was reversed transcribed into cDNA using TaqMan Reverse Transcription Reagents (Life Technologies). The expression levels of miR-141, STAT4, ACTA2, FAP, IL6, CXCL12 and VEGFA were analyzed using Taqman MicroRNA Assay Kits (Applied Biosystems, Foster City, CA). Primers were as follows: for miR-141, forward, 5'-CAUCUUCCA GUACAGUGUUGGA-3′; for STAT4, forward, 5'-AGCCATCTCGGAGGA ATA-3'and reverse, 5'-CAGACAACCGGCCTTTAT-3′; for GAPDH, forward, 5'-CGTGGGCCGCCCTAGGCACCA-3'and reverse, 5'-TTGGCTTA GGGTTCAGGGGGG-3′; for α-SMA, forward, 5'-GCAGCCCAGCCAAGC ACTGT-3'and reverse 5'-TGGGAGCATCGTCCCCAGCA-3′; for FAP, forward, 5'-TCTAAGGAAAGAAAGGTGCCAA-3'and reverse 5'-GATCAGT GCGTCCATCATGAAG-3′; for β-actin, forward, 5'-AGCCTCGCCTTTGC CGATCC-3′ and reverse 5'-ACATGCCGGAGCCGTTGTCG-3′; for IL-6, forward, 5'-CCAGCTATGAACTCCTTCTC-3′ and reverse 5'-GCTTGTTC CTCACATCTCTC-3′; for CXCL12, forward, 5'-CTGTGCCCTTCAGATTG TAGCC-3′ and reverse 5'-CAGGTACTCCTGAATCCACTTTAGC-3′; for VEGFA, forward 5'-CTGTGCAGGCTGCTGTAACG-3′ and reverse 5'-GTTCCCGAAACCCTGAGGAG-3′.

2.5. Conditioned medium experiments

The conditioned medium (CM) from indicated treated cancer cells grown in DMEM/F12 supplemented with 10% FBS for 72 h was collected. Normal fibroblasts and BMSC were seed in six-well plates, and then treated with a 1:2 mixture of CM and fresh 10% FBS DMEM/F12 medium for 72 h (Zhou et al., 2014b).

2.6. Western blot analysis

The protein samples from cell lysates were resolved by 8% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (BioRad, Berkeley, CA, USA). Then, a 5% non-fat milk was used to block the membranes at room temperature, followed by the primary antibody incubation at 4 °C for overnight. The primary antibodies used to detect each protein were obtained from Abcam. The peroxidase-conjugated secondary antibodies (Proteintech, Chicago, IL, USA) were incubated, and detected with chemiluminescence (GE, Fairfield, CT, USA). Quantitation of the protein bands was analyzed with Image J software (NIH, Bethesda, MD, USA).

2.7. Invasion and migration assay

The assay was conducted with a Transwell apparatus (Millipore, MA, USA) equipped with an 8-μm-pore polycarbonate membrane Boyden chamber. 1×10^5 transfected cells in log phase was suspended in 0.2 mL serum-free DMEM/F12 medium, followed by seeding in the upper chamber. Then, 600 μLDMEM/F12 medium containing 10% FBS was placed in the lower chamber. The invasion assay was performed following the same procedures, but coating the filters of transwell

chambers with 45 μg Matrigel (BD Biosciences, Sanjose, CA, USA). The transwell apparatus was incubated for 24 h at 37 °C. The cells on the top chamber were removed by wiping with a cotton swab. To count the results, cells on the lower surface were fixed in 4% paraformaldehyde for 15 min, stained with 0.5% crystal violet for 30 min, then rinsed in PBS before microscopic inspection. The cells in five random fields (100 × magnification) per well were counted.

2.8. Wound-healing assay

To assess the tumor cell motility, a wound-healing assay was conducted. Briefly, tumor cells were seeded in six-well plates overnight and transfected with STAT4, miR-141 mimics, inhibitors or respective negative control. The cells layer was scratched with a sterile plastic tip and then washed with culture medium twice and cultured in the fresh medium for 24 h. The status of the gap closure was observed with photo images under a microscope and the data were summarized based on sextuple assays for each experiment.

2.9. Luciferase reporter assay

For miRNA target validation, the luciferase reporter assay was performed. According to the literature research, 3'-UTR sequence of STAT4 might be the target unit to interact with miR-141. To construct the plasmid, the 3'-UTR sequence of STAT4 or a mutant sequence were inserted into pGL3 promoter vector (Invitrogen), which were defined as pGL3-STAT4 and pGL3-STAT4-mut. Then, the AGS cells were seeded in 24-well plates and transfected with either wide-type or mutant pGL3/STAT4 plasmids containing firefly luciferase, and miR-141 mimics (50 nM) or mimic NC by using Lipofectamine 2000 (Invitrogen Corp, CA, USA). Luciferase activities in transfected cells were analyzed using the Dual-luciferase reporter assay kit (Promega, Madison, WI, USA) after 72 h.

2.10. Statistical analysis

All of the experiments were conducted at least three times and data were expressed as Means ± SD. The difference between two groups was compared by Student's *t*-test. Statistically significance was considered as P values < 0.05.

3. Results

3.1. miR-141 was down-regulated in gastric cancer

We firstly analyzed the expression level of miR-141 in gastric cancer tissues samples and four different gastric cancer cell lines, including SNU-216, AGS, SGC7901, and BGC823 cells, and a normal gastric cell line (GES-1) as control. qRT-PCR showed that miR-141 expression was significantly suppressed in tumor tissue compared to adjacent non-tumor tissue (Fig. 1A) and down-regulated in three gastric cancer cells (AGS, SGC-7901, and BGC823) than that in the normal cells GES-1 (Fig. 1C). The expression in SNU-216 only decreased about 15% compared to the GES-1 without significant difference.

3.2. STAT4 was up-regulated in gastric cancer

In previous report, STAT4 was validate as a target of the miR-141 in gastric cancer cells (Zhou et al., 2014a). As a result, we investigated the STAT4 expression in gastric cancer. First, we evaluated the expression of STAT4 in gastric cancer tissues samples and gastric cancer cell lines. STAT4 mRNA was upregulated in the GC tissues compared with the adjacent non-tumor tissues (Fig. 1B). The expression of STAT4 was also inversely correlated with the expression of miR-141 in these cell lines (Fig. 1D). In most gastric cancer cells, the expression of STAT4 was up-regulated, which suggested that the miR-141 and STAT4 may play an important role in the gastric cancer progression and development. Based on these results, we chose the AGS cell line in the following experiments to investigate the functions of miR141 and STAT4 in gastric cancer metastasis and transition of CAFs.

3.3. STAT4 promotes cancer cell migration and invasion

To investigate the function of STAT4 in gastric cancer, we over-expressed STAT4 in AGS cells by transfecting over-expressing plasmid. As shown in Fig. 2A, western blot clearly demonstrated successful over-expression of STAT4 in AGS cells, which was higher than that in the control group transfected with empty plasmid. Transwell assay (Fig. 2B, C) showed that over-expression of STAT4 in AGS cells promoted migration and invasion of cancer cells. Meanwhile, the wound-healing assay (Fig. 2D) also confirmed that the migration ability of AGS cells with over-expressed STAT4 was enhanced.

3.4. STAT4 over-expression in AGS induced CAF-like features in normal fibroblasts and BMSCs

To investigate the role of STAT4 in the interaction between gastric cancer cells and adjacent fibroblasts, we collected the conditioned medium from the STAT4 over-expressed AGS cells (STAT4 CM) and cultured with normal fibroblasts (NF) or BMSC. The medium from the control plasmid expressed AGS cells (NC CM) was used as the negative control. The biomarkers of the CAFs, including smooth muscle actin α (α-SMA), fibroblast activation protein (FAP), and Ki67 were determined using western blot. As shown in Fig. 3A, all the biomarkers of CAFs in the STAT4 CM group were significantly increased compared with the control group, which indicated that over-expression of STAT4 in gastric cancer cells might mediate the conversion of NFs and BMSCs to CAFs. Furthermore, we used qRT-PCR to quantify the expression levels of mRNA of ACTA2 (for α-SMA),FAP, IL-6, CXCL12, and VEGFA to confirm the results from western blot (Fig. 3B & C). The results demonstrated STAT4 CM up-regulated mRNA levels of CAF markers mentioned above in the STAT4 CM treated group, which was consistent with the results of western blot (Fig. 3A). Furthermore, we investigated the pro-tumor capacity of the induced CAF to promote the proliferation and migration of AGS cells. By incubating AGS cells with the CAF CM differentiated from NF or BMSC respectively, we found that the proliferation viability (Fig. S2 A–B) and migration ability (Fig. S2 C–D) of AGS treated by CAF CM were significantly enhanced compared with the respective control group.

3.5. STAT4 expression in AGS activated the wnt/β-catenin pathway in normal fibroblasts

In other tumor types, such as STAT4-overexpressing ovarian cancer, the activation of the wnt/β-catenin pathway was proved to be involved during the transition of CAFs (Zhao et al., 2017). The underlying mechanism of activation of CAFs by STAT4-overexpressing gastric cancer cells was investigated in followed study. We examined the activation of wnt/β-catenin pathway in the normal fibroblasts and BMSCs treated with STAT4 CM or the NC CM. Results showed that the protein levels of both wnt7a and β-catenin significantly enhanced in the STAT4 CM treated group than that of NC CM group (Fig. 3D). The activation of wnt/β-catenin pathway was also confirmed by the enhanced TOP/FOP flash activity in the group treated with STAT4 overexpressed CM (Fig. 3E). Moreover, while blocking the wnt/β-catenin pathway with specific inhibitor ICG-001, the expression level of CAF-like features was significantly compromised incubated with STAT4 CM (Fig. 3F). In summary, the results above demonstrated that CAFs transition induced by STAT4 over-expression in gastric cancer cells was mediated by activation of wnt7a/β-catenin pathway. Cytokines are generally involved in cancer induced CAF transformation. Level of IL-1β, TNF-α and IL-6 were determined in CM, however, our results indicated that there was

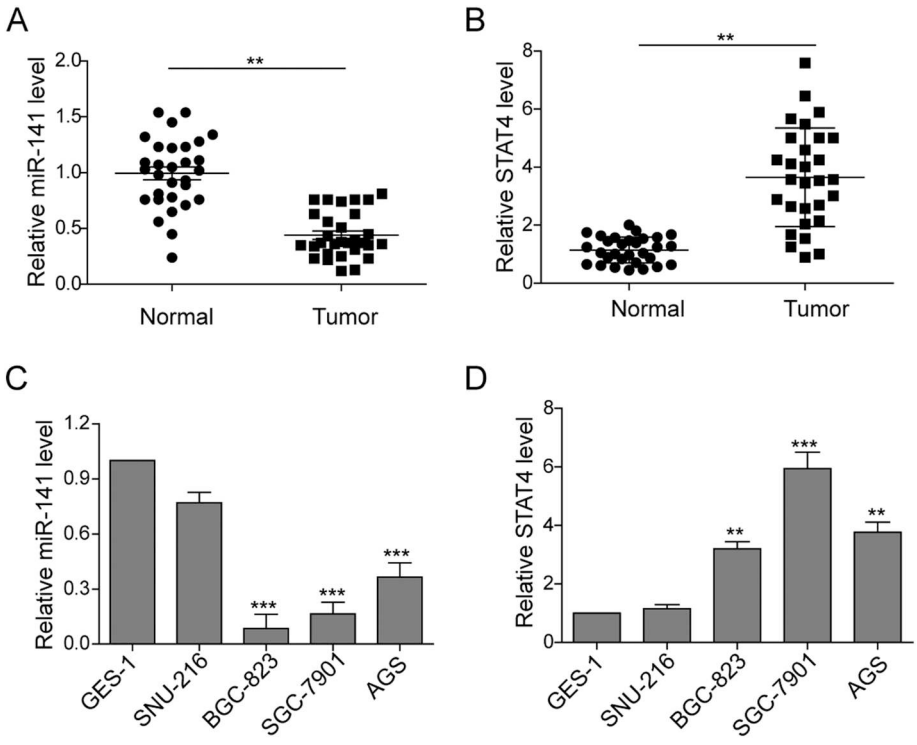

Fig. 1. Down-regulation of miR-141 and over-expression of STAT4 in gastric cancer. (A) The expression level of miR-141 in GC tumor tissues and non-tumor tissues. (B) The expression level of STAT4 mRNA in GC tumor tissues and non-tumor tissues. (C) The expression level of miR-141 in four different gastric cancer cells, including SNU-216, AGS, SGC7901, and BGC823, as well as normal gastric cells (GES-1), was analyzed by qRT-PCR assay. (D) The expression level of STAT4 mRNA in the prementioned gastric cancer cell lines and normal gastric cells. The mean ± SD in the graph presents the relative levels from at least three replications. $^{**}p < 0.01$, $^{***}p < 0.001$.

no significant difference of CM cytokine level between the group with highly expressed STAT4 and control group (Fig. S3).

3.6. miR-141 inhibited the migration and invasion of gastric cancer cells

Next, we confirmed the role of miR-141 in migration and invasion of gastric cancer by transfecting AGS cancer cells with control, mimic NC, miR-141 mimics, inhibitor NC, or miR-141 inhibitors respectively. The transfection efficiency was confirmed by qRT-PCR (Fig. 4A). miR-141 mimic group showed significant higher expression of miR-141 than that of mimic NC. In contrast, the miR-141 inhibitor group showed lower expression than that of inhibitor NC group. Transwell assay was employed to investigate the AGS cells migration and invasion ability after transfection. As shown in Fig. 4B and Fig. 4C, over-expression of miR-141 in the miR-141 mimic group significantly suppressed AGS cell invasion and migration compared to mimic NC group. In contrast, knockdown of miR-141 in the miR-141 inhibitor group showed the enhanced the invasion and migration ability compared with the inhibitor NC group. In wound-healing assay (Fig. 4D), similar trends were observed, indicating that miR-141 inhibited gastric cancer cell migration.

3.7. Conditioned medium from miR-141 mimic or inhibitor transfected AGS cells mediated induction of CAF-like features in NF and BMSC via wnt7a/β-catenin pathway

To investigate the role of miR-141 in the transition of NFs and BMSC to CAFs, we collected the conditioned medium from AGS cell culture system, which were transfected with miR-141 mimic, miR-141 inhibitor, mimic NC, or inhibitor NC, respectively. Normal fibroblasts and BMSCs were cultured in CM from indicated groups for 72 h and the expression of the CAF-related markers was evaluated. As shown in Fig. 5A, western blot results indicated that all the three CAF-related proteins, including α-SMA, FAP, and Ki67, were significantly decreased in miR-141 mimic CM treated group compared to mimic NC group. In contrast, the miR-141 inhibitor CM treatment promoted the expression of these three proteins compared to inhibitor NC group in NF. Furthermore, qRT-PCR results confirmed down-regulation of five CAF-like markers mRNA level in the group of the miR-141 mimic CM compared to mimic NC (Fig. 5B), while up-regulation of the CAF-like markers in the group of the miR-141 inhibitor CM compared to the inhibitor NC CM (Fig. 5C). BMSCs were also treated with CM from the same indicated groups, and the results of qRT-PCR demonstrated that the CM from miR-141 mimic transfected AGS cancer cells significantly suppressed the expression of these five CAF-like markers, indicating the limited transition from BMSC to CAFs (Fig. 5D). Meanwhile, treatment with miR-141 inhibitor CM significantly enhanced the expression of these five CAF-like markers (Fig. 5E).

As we have demonstrated that the wnt/β-catenin pathway was activated when the NFs and BMSCs were cultured in the CM from STAT4 over-expressed gastric cancer cells, we proposed that the CM from miR-141 mimic or inhibitor transfected gastric cancer cell would also regulate wnt/β-catenin pathway in NFs and BMSCs. As shown in Fig. 5F, in the group of NFs and BMSCs cultured in the CM from miR-141 inhibitor transfected AGS cell, both wnt7a and β-catenin were up-regulated compared with the inhibitor NC group. In contrast, in the group of NFs cultured in the CM from miR-141 mimic transfected AGS cell, the expression of these two proteins were suppressed. Meanwhile, the activation of wnt/β-catenin pathway was also proved by the enhanced TOP/FOP flash activity in the group treated the miR-141 inhibitor CM but suppressed in the group of miR-141 mimic CM (Fig. 5G). Moreover, by blocking the wnt/β-catenin pathway using specific inhibitor ICG-001, the up-regulated expression levels of CAF-like features induced by treatment of the miR-141 inhibitor CM was significantly compromised (Fig. 5H).

3.8. miR-141 down-regulated STAT4 expression in gastric cancer cells via direct binding to STAT4 3'UTR

To demonstrate the underlying mechanism between miR-141 and STAT4in gastric cancer, we investigated the expression level of STAT4 in the AGS cells treated with miR-141 mimic, miR-141 inhibitor, inhibitor NC, and mimic NC respectively. The results from western blot and qRT-PCR showed that STAT4 was up-regulated in the miR-141

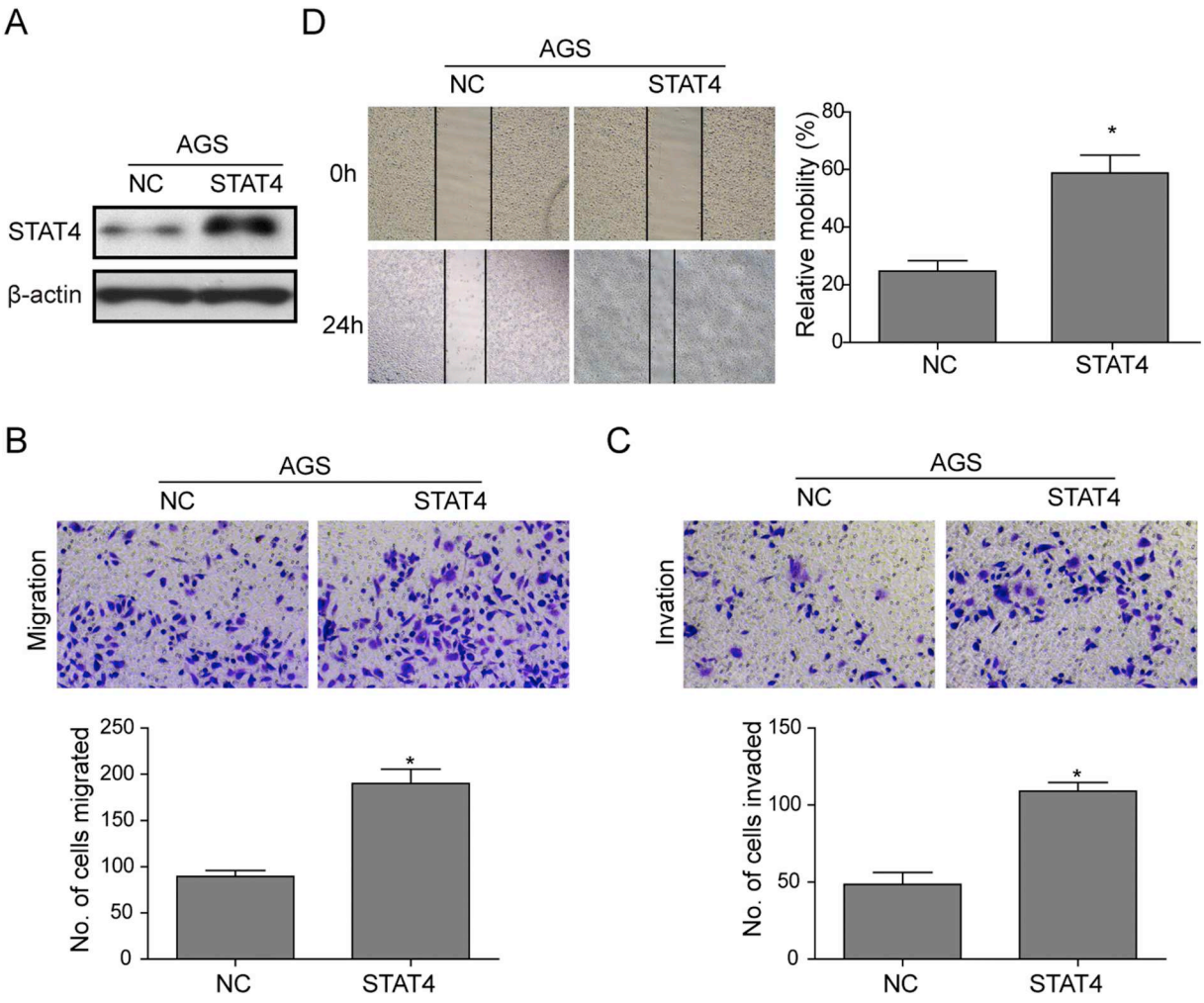

Fig. 2. The over-expression of STAT4 in gastric cancer cells promotes the cancer cells migration and invasion. (A) The STAT4 was up-regulated in AGS cells with transfecting with STAT4 gene and was proved by the western blotting. The β-actin was used as a control. (B) Migration assay and (C) invasion assay were performed as described in Materials and Methods. The migration cells and invasive cells at the bottom of the membrane were stained with crystal violet and imaged. The quantifications of cell migration and invasion were acquired as percentage migrated or invasive cell numbers. (D) The images and quantized histogram of wound-healing assay at 0 h (upper) and 24 h (lower) were taken at magnification of ×200. All experiments were conducted in triplicate and presented as mean ± SD. *$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inhibitor treated cells, and down-regulated in miR-141 mimic treated cells, compared to respective NC group (Fig. 6A and B). Through the bio-informative prediction, we found that the predictive target site of the miR-141 in the 3'-UTR of the STAT4 gene. The 3'-UTR fragment containing binding site was cloned into the pGL3 luciferase reporter vector (pGL3-STAT4) (Fig. 6C). A mutated sequence in 3'-UTR fragment was used as a mutant control (pGL3-STAT4-mut). As shown in Fig. 6D, the luciferase activity in the AGS cells transfected with both pGL3-STAT4 and miR-141 mimic was significantly decreased compared to co-transfected with mimic NC. However, the luciferase activity was not affected by miR-141 mimic compared to mimic NC while co-transfected with pGL3-STAT4-mut vector. These results indicated that the putative site in STAT4 3' UTR was one of the direct targets of miR-141.

3.9. miR-141 and STAT4 regulated migration and induction ability of CAF features in SGC-7901

To prove the function of miR-141/STAT4 axis in other GC cells lines, we repeated key findings of present study in SGC-7901 cells. Similarly, by overexpressing STAT4 in SGC-7901 cells, cell migration was significantly enhanced compared with control group (Fig. S1 A). Treatment of NFs with the CM from STAT4 overexpressed SGC-7901 cells significantly enhanced the expression of the CAF-like feature proteins in normal fibroblasts, indicating the transition to CAFs from NFs (Fig. S1 B and S1 C). Similarly, transfected with miR-141 mimic in SGC-7901 cells decreased the number of migrated cells compared to control group (Fig. S1 D), which demonstrated that miR-141 inhibited migration of SGC-7901 as well. Furthermore, the conditioned medium from miR-141 mimic transfected SGC-7901 reduced CAF-like features in NF both in mRNA and protein level while miR-141 inhibitor treated SGC-7901 CM showed the opposite trends (Fig. S1 E, S1 F and S1 G). In conclusion,the repeat of key experiment in SGC-7901 cell line was consistent with and supported our finding in AGS cell line.

4. Discussion

It has been reported that cancer associate fibroblast (CAF) is important for the tumor development from initiation and progression to metastasis in a variety types of cancers (Hanna et al., 2009). In the tumor microenvironment (TME), CAF is an important component which

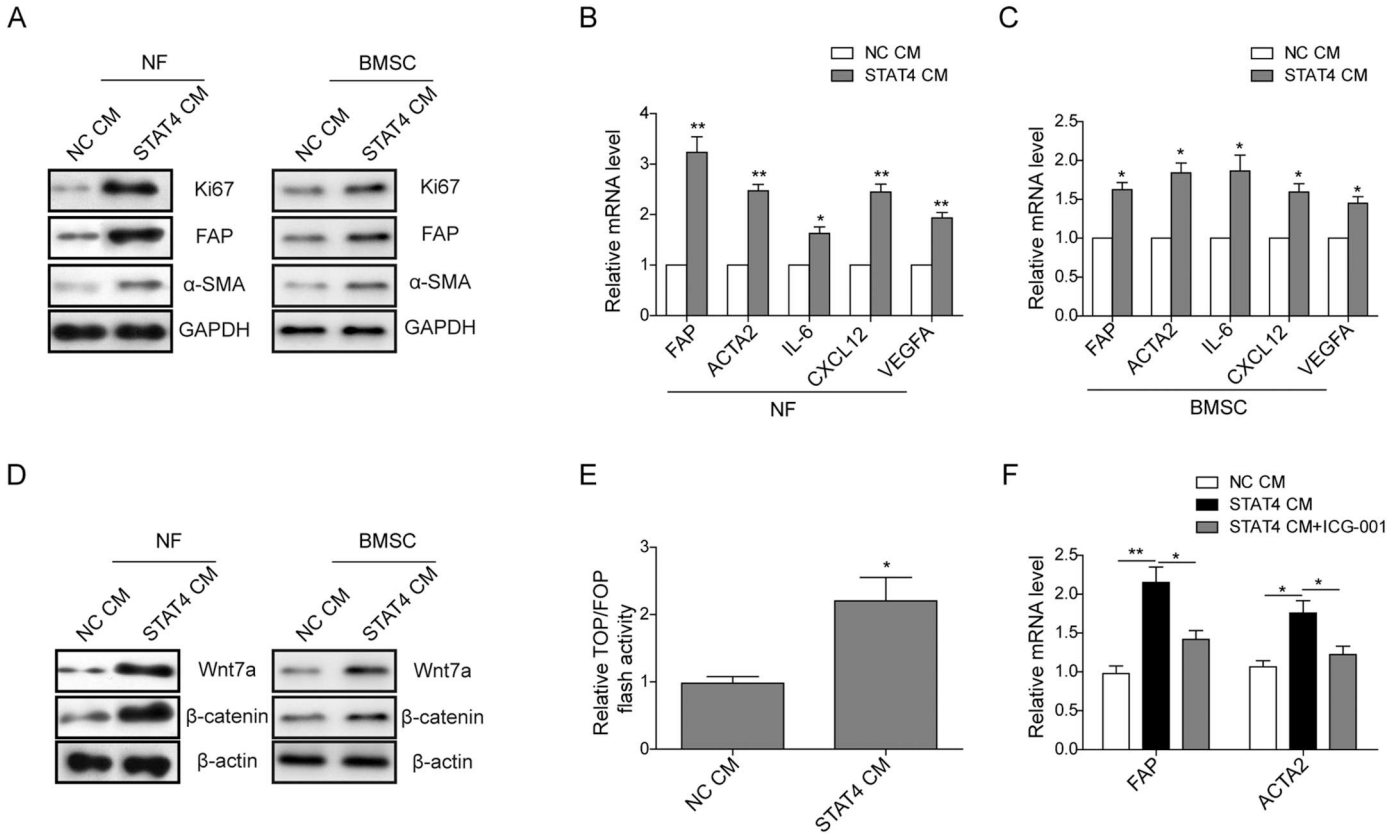

Fig. 3. STAT4 over-expression in AGS induced CAF-like features in normal fibroblasts and BMSC. (A) Western blotting results showed that the level of CAFs features, including FAP, α-SMA, and Ki67, were up-regulated in NFs and BMSC cultured in STAT4 CM. The GAPDH was used as a control. (B) qRT-PCR confirmed the expression levels of ACTA2 (mRNA for α-SMA), FAP, Ki67, IL-6, CXCL12, and VEGFA were up-regulated in NFs cultured with STAT4 CM. (C) qRT-PCR confirmed the expression levels of ACTA2 (mRNA for α-SMA), FAP, Ki67, IL-6, CXCL12, and VEGFA were up-regulated in BMSCs cultured with STAT4 CM. (D) The up-regulation of wnt7a and β-catenin in NFs and BMSCs cultured in STAT4 CM were confirmed by western blotting and indicated the activation of wnt7/β-catenin pathway during the CAF transition. The β-actin was used as a control. (E) STAT4 CM cultured NF were transfected with the TOP/FOP flash reporter plasmid and the reporter activities were detected 48 h after transfection by a luciferase assay. (F) Blocking wnt7/β-catenin pathway by ICG-001 decreased the CAF-like features. All experiments were performed in triplicate and presented as mean ± SD. *$p < 0.05$, **$p < 0.01$.

affects the therapeutic efficiency (Junttila and de Sauvage, 2013; Micke and Tman, 2004; Östman and Augsten, 2009). CAF in the TME is involved in the cancer progression through different pathways, such as immune suppression, secretion of growth factors and cytokines (Marsh et al., 2013; Zhang and Liu, 2013). The high level of CAF in TME might indicate a poor prognosis in several cancers. For instance, Guet al. reported that the CAFs promoted the oral cancer cell tumor growth by regulating CCL2 expression (Wu et al., 2017). In pancreatic cancer, CAF also played a key role in malignant progression, which activated the Hedgehog signal pathway and induced the rapid pancreatic cancer cell growth (Pang et al., 2015). In many cancer types, the CAFs were reported to be converted mainly from NFs. Meanwhile, BMSC cells might be another source of CAFs under certain activation. For example, Yang et al. reported that the CAF differentiation of BMSCs could be induced by exogenous bFGF in breast cancer cells, which facilitated tumor progression (Yang et al., 2016). Compared with NFs, CAFs usually expressed higher FAP, α-SMA, and Ki67 (Cirri and Chiarugi, 2012; Rasanen and Vaheri, 2010). However, in gastric cancer, the mechanism of transformation from NFs to CAFs is still unclear.

In present study, we found that expression of miR-141 was attenuated in the gastric cancer cell lines and tissue whereas the expression of STAT4 was up-regulated. We also demonstrated over-expression of STAT4 significantly enhanced invasion and migration ability of cancer cell lines, which was consistent with the previous studies (Zhou et al., 2014a). As the most frequent type of metastasis of unresectable gastric cancer is peritoneal metastasis, we hypothesized that migrated cancer cells might be able to “educate” the peritoneal stroma to become a preferable metastasis site. As a result, we chose to use the human omental fibroblasts as a normal fibroblast (NF). To the best of our knowledge, this is the first report that demonstrated the conditioned medium from over-expressed STAT4 gastric cancer cells induced the transition of NFs to CAFs, which was proved by the up-regulated CAFs-like features, including FAP, α-SMA, and Ki67. The similar results was also reported in the ovarian cancer cells (Zhao et al., 2017). Interestingly, the CM from STAT4 over-expressed AGS cells also significantly converted BMSC to CAFs as BMSC is also a major source of fibroblast in TME. During the conversion of NFs to CAFs, we found that the wnt7a/β-catenin pathway was activated and we proposed that the expression of STAT4 in the gastric cancer cells activated the wnt7a/β-catenin pathway and thus induced the CAFs transition.

miR-141 has been identified as a potential tumor suppressor miRNA in several cancer types, including gastric cancer (Zhou et al., 2014a; Zuo et al., 2015; Mateescu et al., 2011; van Jaarsveld et al., 2013; Liu et al., 2014). However, the role of the miR-141 for the development of the gastric CAFs, and their relationship with cancer progress has not been demonstrated. Based on the reported relationship between miR-141 and STAT4 (Zhou et al., 2014a),we proposed to investigate whether miR-141 played a role in NF to CAF transition. The gain-of-function and loss-of-function studies suggested that over-expression of miR-141 in AGS and SGC-7901 cells significantly decreased the cell

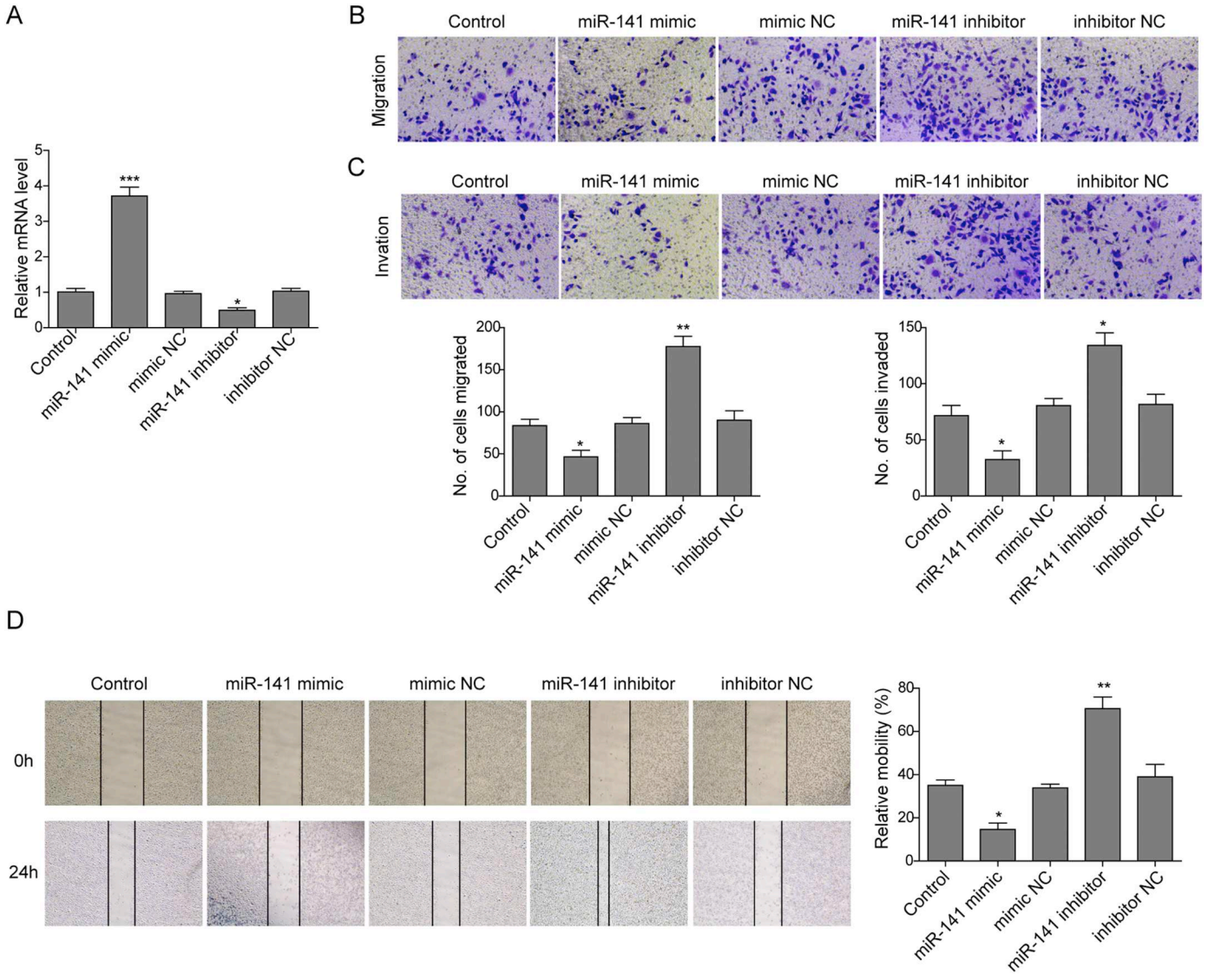

Fig. 4. miR-141 inhibited migration and invasion of AGS cell line. (A) miR-141 expression level in AGS cells transfected with miR-141 mimics, miR-141 inhibitor, negative control (NC), mimic NC, and inhibitor NC were quantified by qRT-PCR. (B) Migration assay and (C) invasive assay indicated the treatment of miR-141 mimic prevented the cancer cell migration and invasion. In contrast, the treatment of miR-141 inhibitor promoted the cancer cell migration and invasion. (D) The images and quantized histogram of wound-healing assay of AGS cells were taken by regular camera at × 200 magnification at 0 h and 24 h. The AGS cells were treated with miR-141 mimics, miR-141 inhibitor, negative control (NC), mimic NC, and inhibitor NC, respectively. All experiments were repeated in triplicate and presented as mean ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$.

migration and invasion ability compared with the control group which was transfected with mimic negative control miRNA. In contrast, the treatment with miR-141 inhibitor promoted migration and invasion of cancer cells.

Moreover, we found that the conditioned medium from miR-141 mimic treated gastric cancer cells inhibited the transition from NFs to CAFs. Similar results were repeated in BMSC to CAF transition. We also discovered that the possible mechanism of CAF transition regulated by miR-141 depended on wnt7a/β-catenin axis.

STAT4 had been identified the functional downstream target of miR-141 in previous study (Zhou et al., 2014a). To figure out the mechanism and the target of miR-141 in this pathway, we analyzed the relationship between miR-141 and STAT4. Western blot and qRT-PCR results showed that over-expression of miR-141 significantly suppressed the expression of STAT4 in gastric cancer cells. Through luciferase assay, we validated that miR-141 could direct target the 3'-UTR fragment of STAT4 to down-regulation expression of STAT4.

5. Conclusions

In conclusion, our data showed that miR-141 was down-regulated, and STAT4 was up-regulated in gastric cancer cells and cancer tissues. To the best of our knowledge, we firstly found out that the over-expression of STAT4 in gastric cancer cells activated the wnt7a/β-catenin pathway in NFs and transformed NFs and BMSC to CAFs. By investigating the relationship between miR-141 and STAT4 expression, we disclosed that miR-141 suppressed the expression of STAT4 and then inhibited wnt/β-catenin mediated transition of NFs to CAFs by targeting the 3'-UTR fragment of STAT4. Our research revealed the tumor suppressive function of miR-141, suggesting that miR-141 may be involved in the transition of CAFs and further *in vivo* study should be employed for further validation.

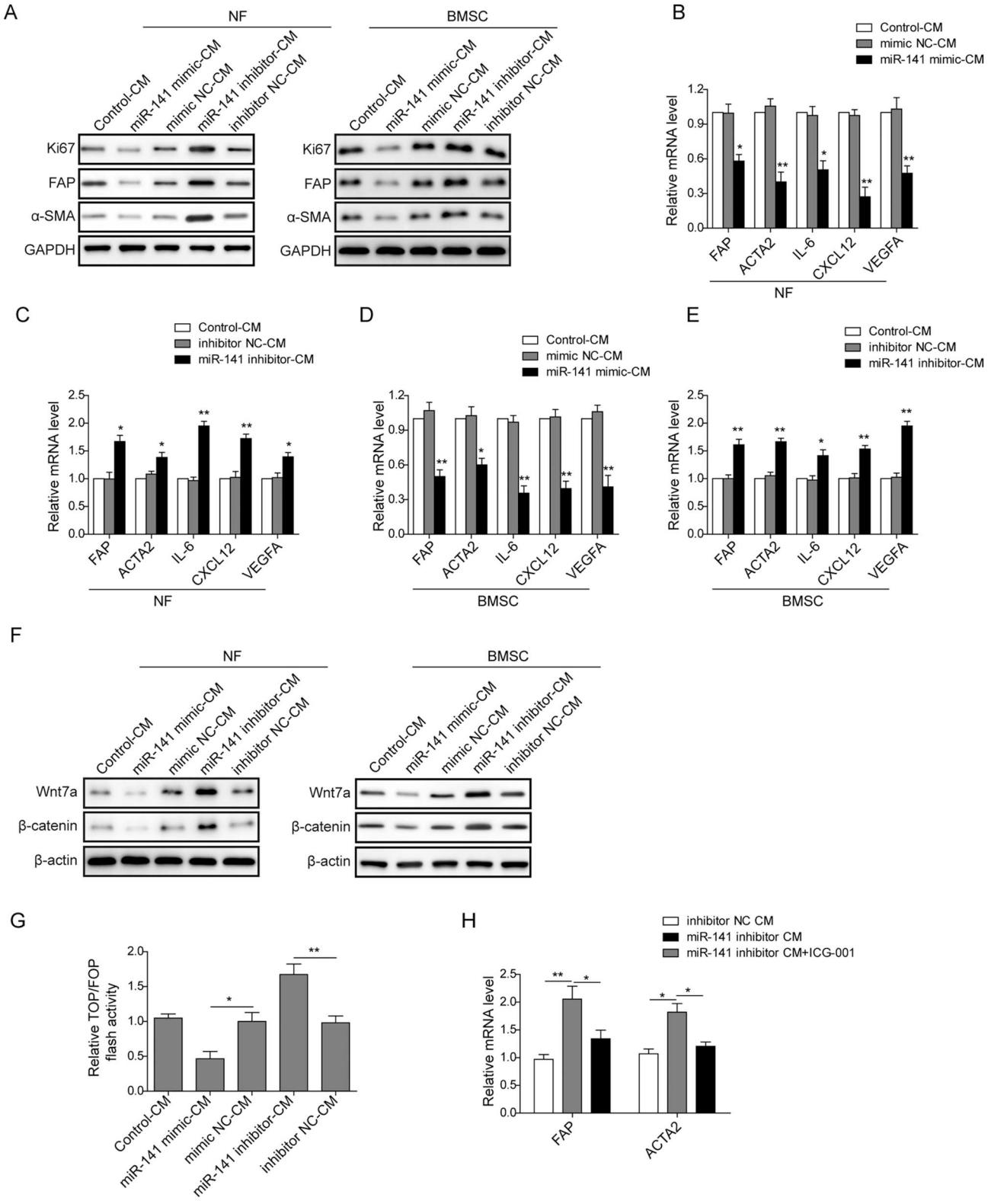

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Fig. 5. miR-141 in AGS inhibited CAFs-like features in normal fibroblasts and BMSC. (A) The expression level of three proteins, including FAP, α-SMA, and Ki67, in NFs and BMSCs cultured in different CM were analyzed by WB. The CM were obtained from AGS cells transfected with control, miR-141 mimics, miR-141 inhibitor, mimic NC, and inhibitor NC, respectively. (B)&(C) The expression level of five mRNA, including FAP, ACTA2 (for α-SMA), Ki67, IL-6, CXCL12, and VEGFA, in NFs cultured in different CM were analyzed by qRT-PCR. The CM were obtained from AGS cells transfected with control, mimic NC, and miR-141 mimics (B), control, inhibitor NC, andmiR-141 inhibitor (C), respectively. (D) and (E) The expression level of five mRNA, including ACTA2, FAP, IL6, CXCL12 and VEGFA in BMSCs cultured in different CM were analyzed by qRT-PCR. The CM were obtained from AGS cells transfected with control, mimic NC, and miR-141 mimics (D), control, inhibitor NC, and miR-141 inhibitor (E), respectively. (F) The expression levels of wnt7a and β-catenin in NFs and BMSCs cultured in different CM were analyzed by WB. The CM were obtained from AGS cells transfected with control, miR-141 mimics, miR-141 inhibitor, mimic NC, and inhibitor NC, respectively. (G) NF cultured in control CM, miR-141 mimic CM, mimic NC CM, miR-141 inhibitor CM, and inhibitor NC CM were transfected with the TOP/FOP flash reporter plasmid and the reporter activities were detected 48 h after transfection by a luciferase assay. (H) Blocking wnt7/β-catenin pathway by ICG-001 decreased the CAF-like features with the transfection of miR-141 inhibitor CM. The β-actin and GAPDH was used as a control. All experiments were performed in triplicate and presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$.

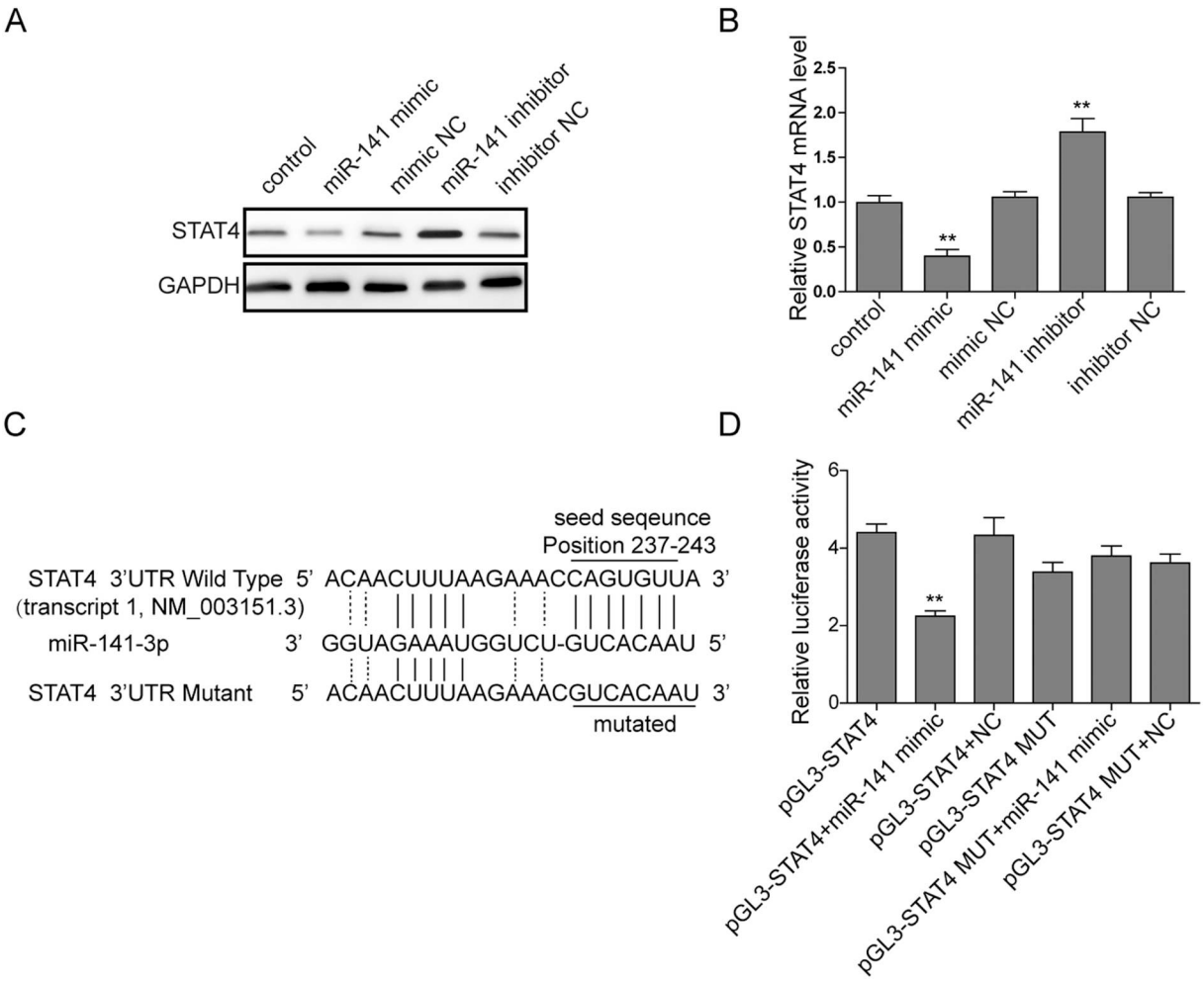

Fig. 6. miR-141 inhibited STAT4 expression *via* direct targeting STAT4 3' UTR. (A) and (B) The expression levels of STAT4 in protein levels (A) and mRNA(B) in AGS cells when they were treated with control, miR-141 mimics, miR-141 inhibitor, mimic NC, and inhibitor NC, respectively. The β-actin was used as a control. (C) The schematics for predictive l miR-141 binding site at 3'-UTR of STAT4 mRNA. (D) The luciferase assay was performed to investigate the effects of miR-141 on STAT4 expression. miR141 mimics (or negative control) with pGL3-STAT4 (or pGL3-STAT4-mut) vector were used to co-transfected AGS cells. Luciferase activity was normalized by the ratio of firefly and Renilla luciferase signals. All data were presented as mean ± SD and repeated three times. $^{**}p < 0.01$.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2018.11.014.

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Conflict of interest

The authors declare that they have no conflict of interest.

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