ADAM17 promotes epithelial-mesenchymal transition via TGF-β/Smad pathway in gastric carcinoma cells

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Abstract. Although a disintegrin and metalloproteinase-17 (ADAM17) overexpression has been demonstrated in numerous human tumors including gastric cancer, its role in gastric cancer development remains to be clarified. In the present study, we identify that ADAM17 activates TGF-β/Smad signaling to promote epithelial-mesenchymal transition (EMT) in gastric cancer cells. We found that ADAM17 promotes proliferation, migration and invasion in gastric carcinoma cells. Subsequently, we revealed that silencing ADAM17 induces the expression of epithelial marker of E-cadherin and downregulates expression of mesenchymal markers including N-cadherin, vimentin and Snail in MGC803 and MKN45 cells, whereas ADAM17 overexpression reverses these changes in BGC823 and HGC27 cells. Furthermore, ADAM17 knockdown significantly inhibits the expression of TGF-β and its downstream signaling molecules p-Smad2 and p-Smad3 in MGC803 and MKN45 cells. Consistently, ADAM17 overexpression reversed these changes in BGC823 and HGC27 cells. These results suggest that ADAM17 promotes epithelial-mesenchymal transition via the TGF-β/Smad pathway. Collectively, the present study demonstrates that ADAM17 plays a critical role in the development of gastric cancer and provides a potential therapeutic target for gastric cancer.

Introduction

Gastric cancer is the fourth most commonly diagnosed cancer and the second leading cause of cancer death worldwide, especially prevalent in developing countries (1). The only potentially curative treatment for gastric cancer is complete resection (2). However, despite aggressive surgical intervention, more than 50% of patients undergoing radical resection will experience disease recurrence, usually in the form of metastatic disease (3). Thus, a better understanding of the underlying mechanisms that promote pathogenesis and progression of gastric cancer is urgently needed.

ADAM17, as a sheddase, releases extracellular domains of transmembrane proteins, which thereby modulate cell-cell and cell environment communication (4). ADAM17 overexpression has been demonstrated in numerous human tumors including gastric cancer (5), and several well-designed studies have shown correlations between the levels of ADAM17 expression and tumor progression (6,7). A previous study found that the expression levels of ADAM17 mRNA and protein in gastric cancer tissues are both significantly higher than those in non-cancerous gastric mucosa (8). It is also identified that the siRNA-targeted ADAM17 transcripts suppress deoxycholate (DC)-induced activation of EGFR and ERK1/2, suggesting that in AGS human gastric cancer cells, DC transactivates EGFR through M-BAR- and ADAM/HB-EGF-dependent mechanisms (9). Another study reported that ADAM17 activated by TGF-β mediates proHB-EGF shedding to promote the proliferation of gastric cancer cells via EGFR transactivation (10). According to current views, the major mechanism by which ADAM17 supports cancer development involves shedding, and thus, activation of growth factors such as TGF-α, HB-EGF, amphiregulin or neuregulins (11,12). In turn, these growth factors stimulate survival, proliferation and migration of tumor cells. Therefore, ADAM17 may be an important molecular marker for predicting carcinogenesis, progression and prognosis of gastric cancer.

It is reported that epithelial-mesenchymal transition (EMT) plays a critical role in the cancer progression and metastasis, including gastric cancer (13,14). EMT is a process characterized by loss of cell-cell adhesion and increase of cell motility (15). During EMT, significant morphological transformation occurs, including reduced expression of epithelial markers, such as E-cadherin, and increased expression of mesenchymal markers, such as N-cadherin and vimentin

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Table I. The primer sequences for the GAPDH, ADAM17, MMP-2 and MMP-9 genes used in real-time PCR experiments.

	Forward primer	Reverse primer
GAPDH	5'-GGTGAAGGTCGGTGTGAACG-3'	5'-CTCGCTCCTGGAAGATGGTG-3'
ADAM17	5'-AGAGCTGACCCAGATCCCAT-3'	5'-TACTCTCTTCCCCTCTGCCC-3'
MMP-2	5'-CACAGGAGGAGAAGGCTGTG-3'	5'-GAGCTTGGGAAAGCCAGGAT-3'
MMP-9	5'-TTCAGGGAGACGCCCATTTC-3'	5'-TGTAGAGTCTCTCGCTGGGG-3'

(16,17). However, there are few reports concerning the association between ADAM17 expression and EMT in gastric cancer.

In the present study, we examined the roles of ADAM17 in EMT of gastric carcinoma cells and elucidated the underlying mechanism. Our data show that ADAM17 promotes the proliferation, migration and invasion of gastric carcinoma cells. Importantly, ADAM17 promotes EMT probably via TGF-β/Smad signaling in gastric carcinoma cells.

Materials and methods

Cell culture. The gastric carcinoma cell lines MGC803, MKN45, HGC27 and BGC823 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured with Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Beijing, China) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), in humidified 5% CO_2 incubator at 37˚C.

Real-time PCR. Total RNA was isolated using RNAiso Plus (Takara Bio, Shiga, Japan). Reverse transcription was performed using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. The SYBR-Green-based real-time PCR was then performed in triplicate using CFX-96 sequence detection system (Bio-Rad Laboratories) and gene expression was normalized by GAPDH. Primers are listed in Table I. The relative fold change in RNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Plasmid construction. The ADAM17 shRNA sequence was obtained from Sigma Company official website, which was produced by Sangon Biotech, Co., Ltd. (Shanghai, China). The oligo sequence of ADAM17 shRNA included: ADAM17 shRNA (F): 5'-CCG GCC TAT GTC GAT GCT GAA CAA ACT CGA GTT TGT TCA GCA TCG ACA TAGG TTT TTG-3' and ADAM17 shRNA (R): 5'-AAT TCA AAA ACC TAT GTC GAT GCT GAA CAA ACT CGA GTT TGT TCA GCA TCG ACA TAG G-3'. The ADAM17 shRNA sequence was inserted into the *Eco*RI and *Age*I site of the pLKO.1-TRC plasmid and ligated into the vector (Sigma-Aldrich, St. Louis, MO, USA).

Lentivirus production and cell transduction. The packaging plasmid psPAX2 and the envelope plasmid pMD2.G were purchased from Sigma-Aldrich. PLKO.1-sh-ADAM17 was cotransfected with psPAX2 and pMD2.G into HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Viruses were harvested 48 h after transfection and viral titers were determined. Cells were infected with $1x10^6$ recombinant lentivirus transduction units in the presence of 8 mg/ml polybrene Sigma-Aldrich. Puromycin (1:10,000 dilutions) was added to cells until the cells in the blank group were non-viable. Cells which survived were stable infected cells.

Transient transfection. Cells were seeded in 6-well plates at a density of $4x10^5$ cells/well. After 24 h of culture, the medium was replaced by Opti-MEM (Invitrogen) and cultured. In total, 2 μg plasmid was transfected using 6 μl Lipofectamine 2000 transfection reagent (Invitrogen). After incubation for another 48 h, the treated cells were used to investigate the effect of gene rescue using western blot analysis or Transwell and Cell Counting kit-8 assay.

Western blotting. The cultured cells were rinsed with cold phosphate-buffered saline (PBS) before treated with RIPA lysis buffer at 4˚C for 10 min. Then the mixture was centrifuged under 4˚C at 12,000 r/min for 15 min. The supernatant was removed and the protein concentration was measured with the BCA method. Approximately 40 μg of protein was loaded in each lane, and separated by 10% SDS-PAGE and then transferred to the PVDF membrane. The membrane was blocked by 5% non-fat milk powder for 1 h at room temperature before overnight incubation with primary antibodies 4˚C, followed by the secondary antibody. The antibodies were rabbit anti-ADAM17 (cat. no. 3976), mouse anti-β-tubulin (cat. no. 6181), rabbit anti-N-cadherin (cat. no. 13116), rabbit anti-E-cadherin (cat. no. 3195), rabbit anti-vimentin (cat. no. 5741), rabbit anti-Snail (cat. no. 3879), rabbit anti-TGF-β (cat. no. 3711), rabbit anti-Smad2 (cat. no. 5339), rabbit anti-p-Smad2 (cat. no. 3108), rabbit anti-Smad3 (cat. no. 9523), rabbit anti-p-Smad3 (cat. no. 9520) (all from Cell Signaling Technology, Danvers, MA, USA).

Cell Counting kit-8 assay. The measurement of viable cell mass was performed with Cell Counting kit-8 (Beyotime Institute of Biotechnology, Shanghai China) according to the manufacturer's instructions. Briefly, 3,000 cells/well were seeded in a 96-well plate, grown in an incubator (5% CO_2, at 37˚C). Respectively in the first, second, third, fourth and fifth day, 10 μl CCK-8 was added to each well, and cells were incubated at 37˚C for 2 h and the absorbance was finally determined at 490 nm.

Colony-forming assay. Transfected MGC803 and MKN45 cells were harvested, resuspended in medium and transferred to the 6-well plate (500, 1,000 and 2,000 cells/well) for 10-14 days until large colonies were visible. Colonies were fixed and

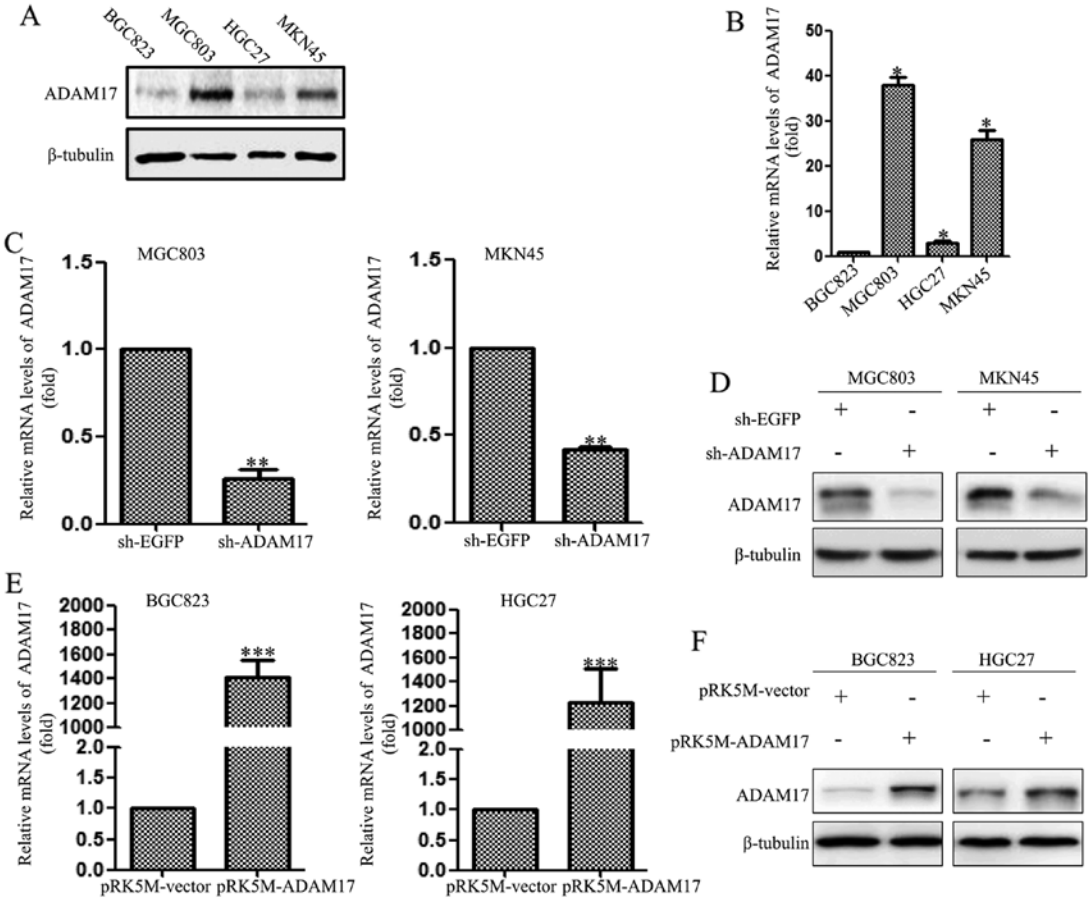

Figure 1. ADAM17 expression in different gastric carcinoma cells. (A and B) Relative expression levels of ADAM17 protein and mRNA were assessed in MGC803, MKN45, BGC823 and HGC27 cells. *P<0.05. (C) Identification of shRNA-mediated knockdown of ADAM17 in MGC803 and MKN45 cells by real-time PCR analysis. GAPDH was used for normalization (Student's t-test, **P<0.001 vs. sh-EGFP). (D) Identification of shRNA-mediated knockdown of ADAM17 in MGC803 and MKN45 cells by western blot analysis. (E) Identification of ADAM17 overexpression in BGC823 and HGC27 cells by real-time PCR analysis. GAPDH was used for normalization (Student's t-test, ***P<0.001 vs. sh-EGFP). (F) Identification of ADAM17 overexpression in BGC823 and HGC27 cells by western blot analysis.

stained with 0.05% crystal violet for 30 min, and the number of colonies was counted or photomicrographs were taken under phase-contrast microscope.

Wound healing assay. Cells have grown to confluence in complete cell culture medium. At time 0 h, a scrape wound was created across the diameter with a 10-μl pipette tip followed by extensive washes with medium to remove dead and floating cells. The distance was recorded at 0 and 48 h. Images were captured using an inverted microscope equipped with a digital camera.

Migration assay and invasion assay. For assessing cell migration, 1x10^5 cells in serum-free media were seeded into the Transwell inserts (Corning) containing 8-μm permeable pores and were allowed to migrate toward 10% FBS-containing medium. Twenty-four to 36 h later, the migrated cells on the bottom of the insert were fixed with 4% paraformaldehyde solution followed by crystal violet (1%) staining. Images were taken after washing the inserts three times with PBS. Five independent fields were counted for each Transwell and the average numbers of cells/field were represented as graphs. For assessing cell invasion, 1x10^5 cells in serum-free medium were seeded in the Transwell inserts which had been covered with a layer of BD Matrigel basement membrane. The cells were later processed similarly to that of cell migration assay. Finally, invaded cells were counted and the relative number was calculated.

Statistical analysis. The data are presented as mean ± SD from at least three independent experiments. All statistical analyses were carried out using SPSS Statistics 19 software. Comparisons between the groups were analyzed using the Student's t-test (two groups) or a one-way ANOVA (multiple groups). P<0.05 was considered statistically significant.

Results

ADAM17 expression in gastric carcinoma cells. First, we analyzed the ADAM17 expression in MGC803, MKN45, HGC27 and BGC823 cells using real-time PCR and western blot analysis. We found that ADAM17 expression, at both mRNA and protein levels, was higher in MGC803 and MKN45 cells than that in HGC27 and BGC823 cells (Fig. 1A and B). Subsequently, we constructed plasmids sh-ADAM17 and pRK5M-ADAM17 to identify the role of ADAM17 in the development of gastric cancer, and then we examined the knockdown effect of sh-ADAM17 at both

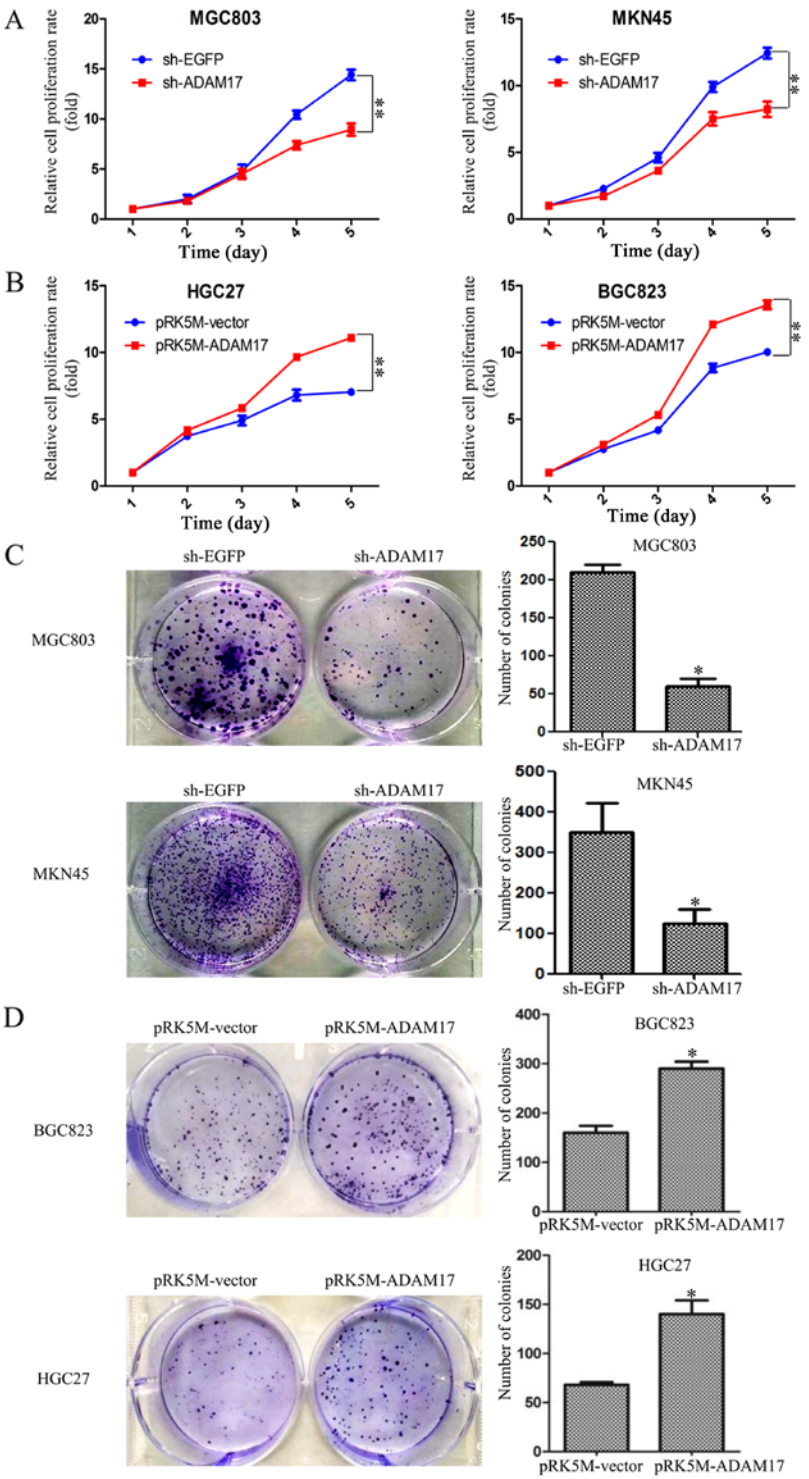

Figure 2. ADAM17 promotes cell proliferation and colony formation in gastric carcinoma cells. (A) CCK-8 assay showed that ADAM17 knockdown inhibited MGC803 and MKN45 cell growth (Student's t-test, $^{**}P<0.001$ vs. sh-EGFP). (B) CCK-8 assay showed that ADAM17 overexpression promoted BGC823 and HGC27 cell growth (Student's t-test,$^{**}P<0.001$ vs. pRK5M-vector). (C) Clone formation assays in MGC803 and MKN45 cells. ADAM17 knockdown inhibited cell clone formation (Student's t-test, $^{*}P<0.05$ vs. sh-EGFP). (D) Clone formation assays in BGC823 and HGC27 cells. ADAM17 overexpression promoted cell clone formation (Student's t-test, $^{*}P<0.05$ vs. pRK5M-vector).

protein and mRNA levels using sh-EGFP as a control. The mRNA and protein levels of ADAM17 were significantly decreased in sh-ADAM17 group compared with sh-EGFP group (Fig. 1C and D). Similarly, pRK5M-vector or pRK5M-ADAM17 were transferred into HGC27 and BGC823 cells, then ADAM17 mRNA and protein levels were examined by real-time PCR and western blot analysis, and the results indicated that ADAM17 expression at both mRNA and protein levels was significantly increased in pRK5M-ADAM17 group compared with pRK5M-vector group (Fig. 1E and F).

ADAM17 promotes proliferation and colony formation in gastric carcinoma cells. To investigate the effect of ADAM17 on cell growth in gastric carcinoma cells, we first used the

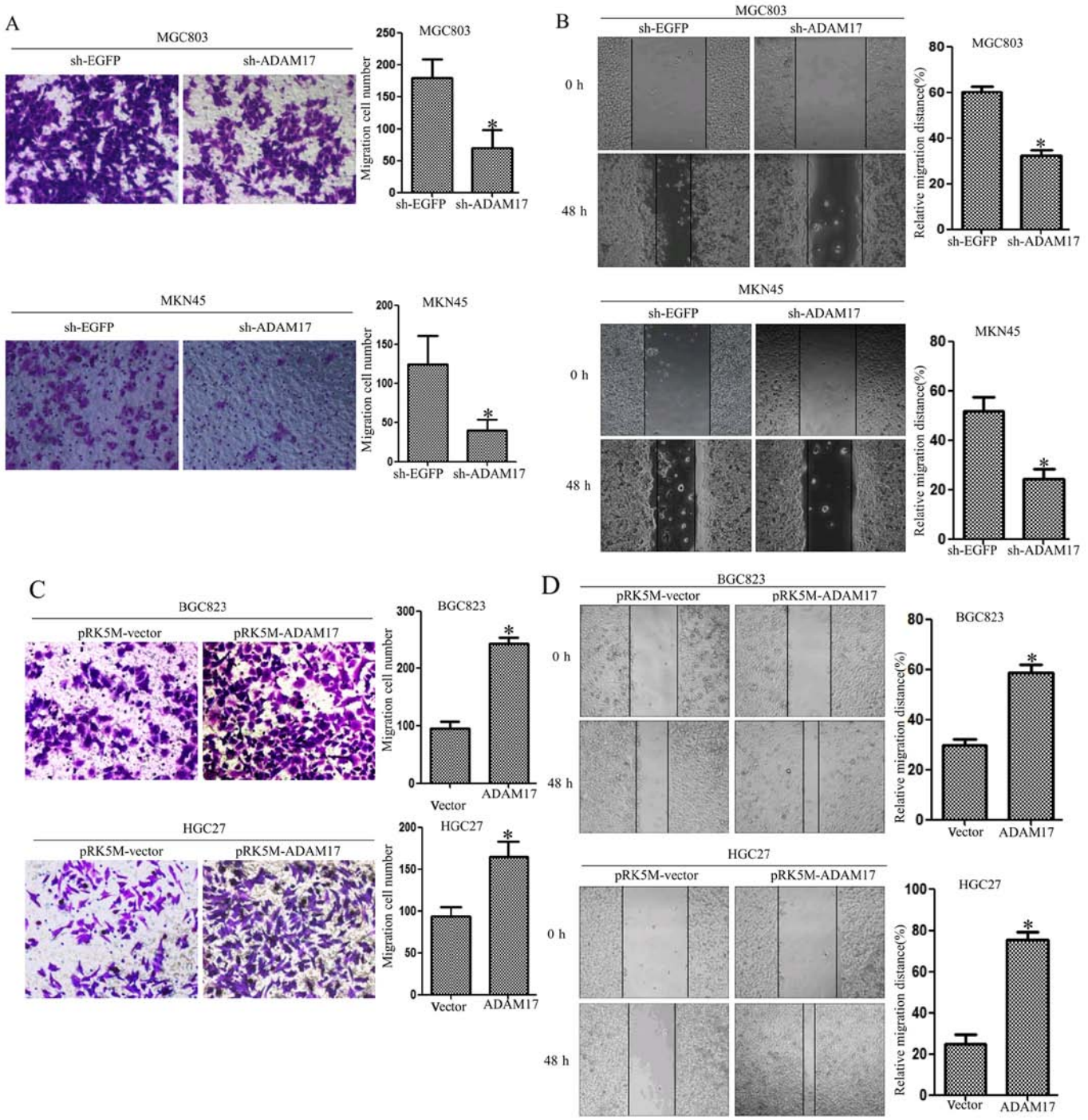

Figure 3. ADAM17 promotes cell migration in gastric carcinoma cells. (A and B) The migration change was measured by Transwell assay and wound scratch assay in MGC803 and MKN45 sh-ADAM17 stably infected cells (Student's t-test, *P<0.05 vs. sh-EGFP). (C and D) After transfected with pRK5M-vector or pRK5M-ADAM17 plasmid in BGC823 and HGC27 cells, the migration change was measured by Transwell assay and wound scratch assay (Student's t-test, *P<0.05 vs. pRK5M-vector).

CCK-8 assay to determine the growth curves. The results indicated that ADAM17 knockdown significantly inhibited the proliferation of MGC803 and MKN45 cells (Fig. 2A). Expectedly, overexpression of ADAM17 promotes the ability of proliferation in HGC27 and BGC823 cells (Fig. 2B). To further confirm the effect of ADAM17 on proliferation of gastric carcinoma cells, we evaluated their ability of colony formation in the above mentioned cells. Colonies with strong, highly dense staining and at least 50 cells per colony were counted. We found that ADAM17 knockdown resulted in smaller colonies and lower colony density compared to the control group in both MGC803 and MKN45 cells. The colony formation rates were 202±11 and 50±9 in sh-EGFP and sh-ADAM17 MGC803 cells, and 331±8 and 114±7 in sh-EGFP and sh-ADAM17 MKN45 cells (Fig. 2C). To confirm the above results, the HGC27 and BGC823 cells were transfected with pRK5M-vector or pRK5M-ADAM17 plasmids, respectively. We found that ADAM17 overexpression increased the colony sizes and densities compare to control group, and the number of colonies (defined as ≥50 cells) was 150±10 and 300±13 in

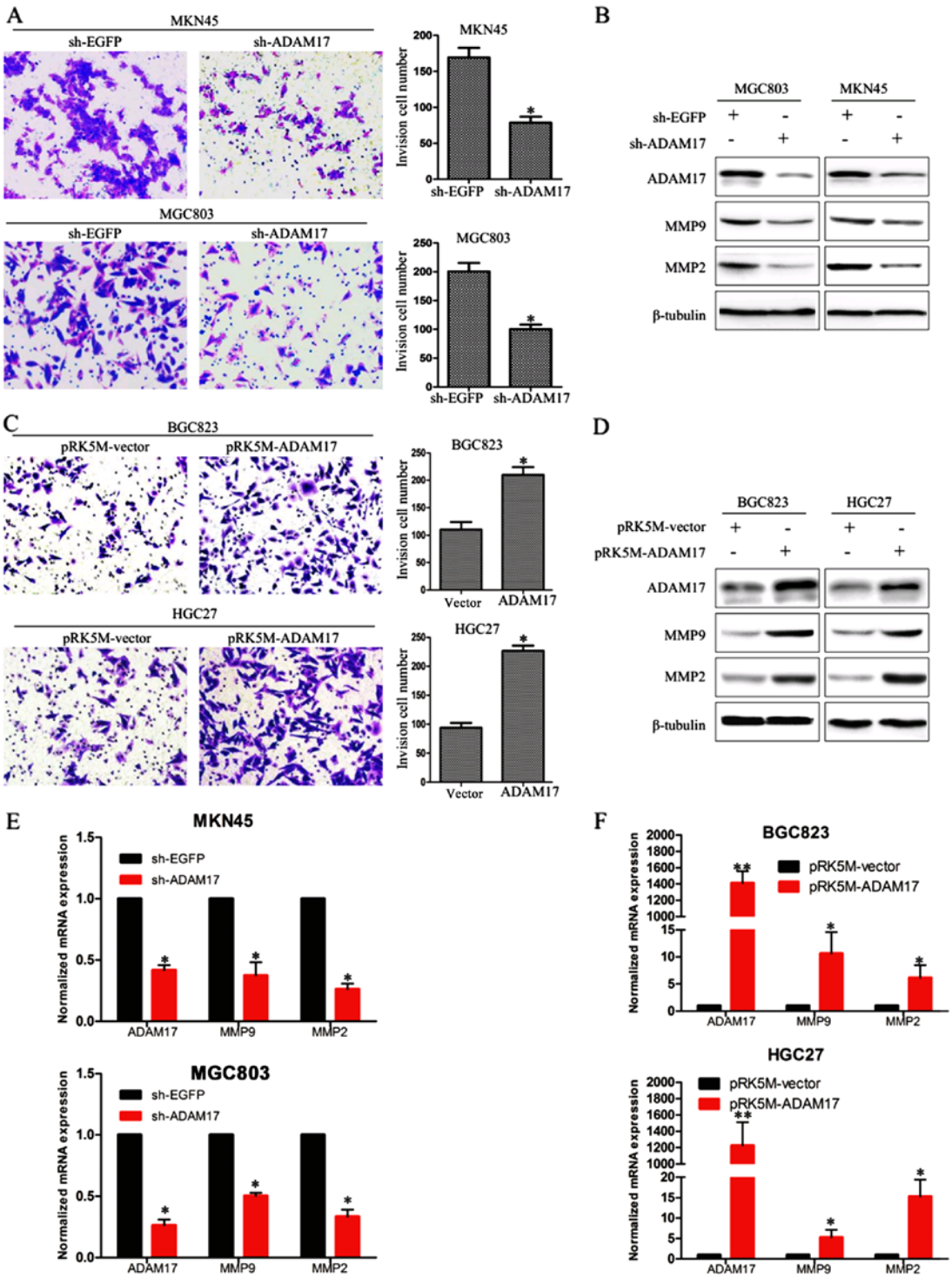

Figure 4. ADAM17 promotes cell invasion in gastric carcinoma cells. (A) The invasion changes were measured by Transwell assay in MGC803 and MKN45 sh-ADAM17 stable infected cells (Student's t-test, $^{*}P<0.05$ vs. sh-EGFP). (C) After transfected with pRK5M-vector or pRK5M-ADAM17 plasmid in BGC823 and HGC27 cells, the invasion change was measured by Transwell assay and wound scratch assay (Student's t-test, $^{*}P<0.05$ vs. pRK5M-vector). (B and D) The protein levels of MMP-2 and MMP-9 were measured by western blot analysis. (E and F) The mRNA levels of MMP-2 and MMP-9 were measured by real-time PCR (Student's t-test, $^{*}P<0.05$, $^{**}P<0.001$).

BGC823 cells, 77±12 and 153±9 in HGC27 cells, respectively (Fig. 2D). It was confirmed that ADAM17 promotes proliferation and colony formation in gastric carcinoma cells.

ADAM17 enhances the migration ability of gastric carcinoma cells. Next, we examined the ability of migration by Transwell assays and wound scratch assays. First, we examined the migratory potential of MGC803 and MKN45 cells using Transwell assays. We found that the migration rates were 180±15 and 60±12 in sh-EGFP and sh-ADAM17 MGC803 cells, and 120±14 and 40±11 in sh-EGFP and sh-ADAM17 MKN45 cells (Fig. 3A). To test the effects of ADAM17 knockdown on cell motility, we performed wound scratch assays. For these assays, a scrape wound was created on confluent cultures of MGC803

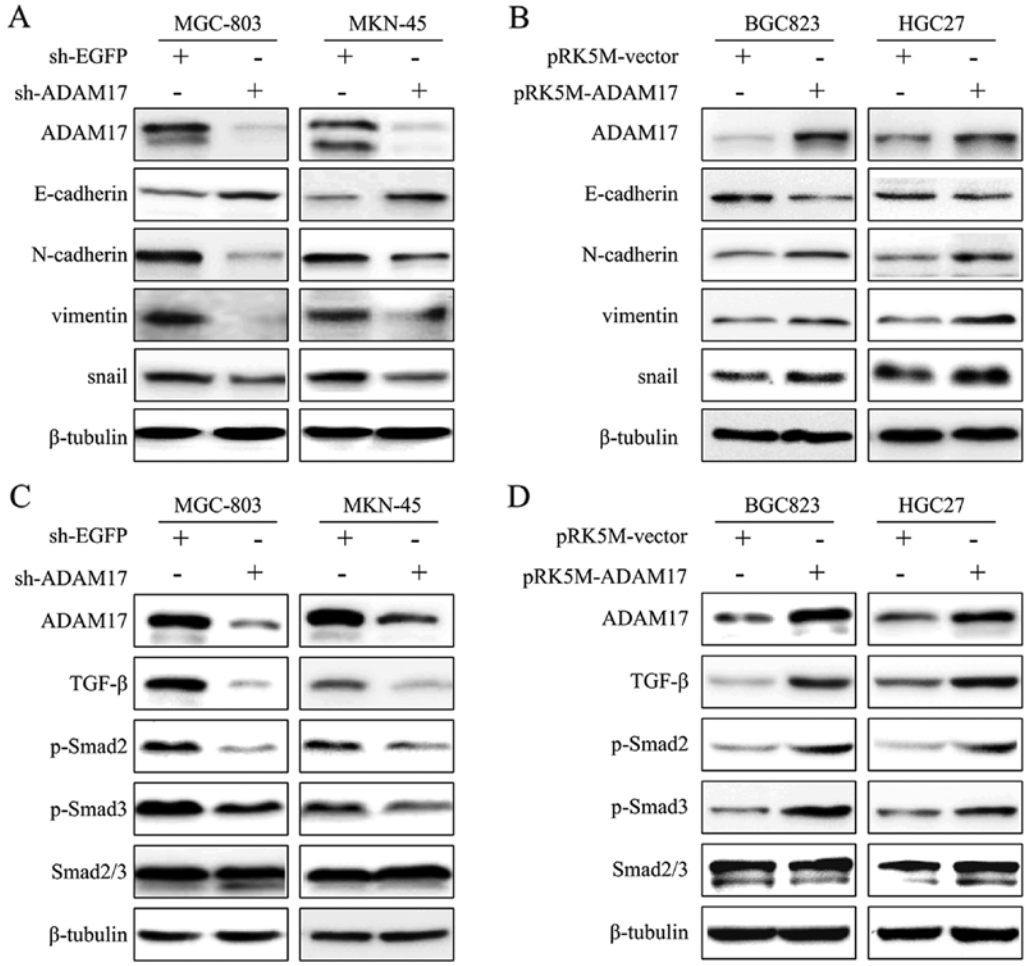

Figure 5. ADAM17 promotes epithelial-mesenchymal transition (EMT) via TGF-β/Smad signaling in gastric carcinoma cells. (A) The protein levels of E-cadherin, N-cadherin, vimentin and Snail were measured by western blot analysis in MGC803 and MKN45 sh-ADAM17 stable infected cells. (B) After transfected with pRK5M-vector or pRK5M-ADAM17 plasmid in BGC823 and HGC27 cells, the protein levels of E-cadherin, N-cadherin, vimentin and Snail were measured by western blot analysis. (C) TGF-β/Smad signaling pathway relative proteins were detected by western blot analysis in MGC803 and MKN45 sh-ADAM17 stable infected cells. (D) After transfected with pRK5M-vector or pRK5M-ADAM17 plasmid in BGC823 and HGC27 cells, TGF-β/Smad signaling pathway relative proteins were detected by western blot analysis.

and MKN45 cells expressing either sh-EGFP or sh-ADAM17. MGC803 and MKN45 cells expressing sh-ADAM17 displayed reduced motility in comparison to MGC803 and MKN45 cells expressing sh-EGFP (Fig. 3B). To confirm the above results, HGC27 cells and BGC823 cells were transfected with pRK5M-vector or pRK5M-ADAM17 plasmids. Conversely, overexpression of ADAM17 promoted the ability of migration in HGC27 and BGC823 cells (Fig. 3C and D). These data suggest that ADAM17 promotes the ability of migration in gastric carcinoma cells.

ADAM17 promotes cell invasion in gastric carcinoma cells. Then, we examined the effect of ADAM17 on the invasion ability of gastric carcinoma cells using BD Matrigel invasion assays. We transfected MGC803 and MKN45 cells with sh-EGFP or sh-ADAM17 plasmids, and HGC27 and BGC823 cells with pRK5M-vector or pRK5M-ADAM17 plasmids for 72 h. The number of invasive cells were 170±12 and 74±9 in sh-EGFP and sh-ADAM17 MKN45 cells, and 200±6 and 98±8 in sh-EGFP and sh-ADAM17 MGC803 cells (Fig. 4A), indicating that knockdown of ADAM17 obviously inhibited the invasion ability of MGC803 and MKN45 cells. Moreover, the number of invasive cells were 105±9 and 200±5 in vector and pRK5M-ADAM17 BGC823 cells, and 98±11 and 210±8 in vector and pRK5M-ADAM17 HGC27 cells, suggesting that upregulation of ADAM17 significantly enhanced the invasion ability of BGC823 and HGC27 cells (Fig. 4C). Also, ADAM17 knockdown was conduced to downregulate the MMP-2 and MMP-9 at both mRNA and protein levels in MGC803 and MKN45 cells (Fig. 4B and E), while ADAM17 overexpression upregulated the expression of MMP-2 and MMP-9 in BGC823 andHGC27 cells (Fig. 4D and F). The above data suggest that ADAM17 promotes the invasion ability of gastric cancer cells.

ADAM17 promotes EMT via TGF-β/Smad signaling in gastric carcinoma cells. The EMT is deemed to be associated with the ability of migration and invasion in cancer cells. Therefore, we detected EMT markers at the protein level by western blotting. Our data suggested that ADAM17 knockdown resulted in downregulation of vimentin, Snail, N-cadherin and upregulation of E-cadherin in MGC803 and MKN45 cells (Fig. 5A). In contrast, ADAM17 overexpression led to upregulation of vimentin, Snail, N-cadherin and downregulation of E-cadherin in BGC823 and HGC27 cells

(Fig. 5B). It is confirmed that ADAM17 promotes EMT in gastric carcinoma cells. As TGF-β/Smad signaling is closely related to EMT in cancer, we investigated the effects of ADAM17 on the classic TGF-β/Smad signaling. We found that ADAM17 knockdown downregulated TGF-β, p-Smad2/3 in MGC803 and MKN45 cells (Fig. 5C), while ADAM17 overexpression resulted in upregulation of TGF-β, p-Smad2/3 in BGC823 and HGC27 cells (Fig. 5D). Knocking down or overexpressing ADAM17 had no influence on total Smad2/3 protein. These data suggest that ADAM17 promotes EMT in gastric carcinoma cells via TGF-β/Smad signaling.

Discussion

It has been reported that ADAM17 may function as an oncogene to promote cancer cell growth (18). ADAM17 expression is significantly increased in different types of cancers, including gastric cancer (19-22). In this study, we identify that ADAM17 promotes proliferation, migration and invasion in gastric cancer cells. Importantly, we found that ADAM17 promotes EMT probably via TGF-β/Smad signaling pathway in gastric cancer. These findings suggest that ADAM17 could represent a novel anticancer strategy.

Epithelial-mesenchymal transition (EMT) is a critical cellular process in cancer metastasis, during which epithelial polarized cells become motile mesenchymal cells (23). The process of EMT consists of three major steps in cancer: i) loss of cell-cell junctions and a decrease in the epithelial marker E-cadherin; ii) acquisition of the mesenchymal marker N-cadherin; and iii) cytoskeleton rearrangement intended for invasive properties. Additionally, these changes are paralleled with secretion of matrix metalloproteinase-2/-9 (MMP-2/-9) and focal adhesion kinase (FAK) (24). MMP-2/-9, proteolytic enzymes that degrade and modify the extracellular matrix (ECM), act directly on cell surface molecules and activate EMT (25). In the present study, we find that ADAM17 overexpression elevates the expression of MMP-2 and MMP-9, while ADAM17 knockdown downregulates the expression of MMP-2 and MMP-9. Therefore, we identified that ADAM17 elevates the expression of MMP-2 and MMP-9 thereby accelerating EMT.

It has been reported firmly that TGF-β signaling pathway plays crucial roles in regulating malignancy initiation, progression and metastasis, including gastric cancer (26). The effects of TGF-β on migration and invasion are associated with changes in ECM components, including collagen (27), fibronectin (28), laminin (29), MMP-2 and MMP-9 (30,31). ADAM17 is also involved in proteolytical digestion of collagen IV of the ECM and the release from the cell surface of several integral proteins, which suggest that ADAM17 affects the invasive activity of a variety of cancers (32,33). Therefore, there may be a possible link between ADAM17 and TGF-β. In this study, we presented clear evidence that ADAM17 induced expression of the TGF-β, and increased phosphorylated Smad2/3 while the total Smad2/3 expression was relatively unchanged. In the TGF-β signaling pathway, TGF-β receptor kinases phosphorylated Smad2 and Smad3 in the C terminal residue, resulting in forming a complex with Smad4, which plays the role of a common mediator, and the nuclear translocation to regulate gene expression leading to the stimulation of EMT (34,35). Hence, it is confirmed that ADAM17 promotes EMT probably via TGF-β/Smad signaling in gastric carcinoma cells.

In conclusion, ADAM17 promotes proliferation, migration and invasion in gastric carcinoma cells. Importantly, the results detail a mechanism of ADAM17-mediated EMT through upregulating TGF-β/Smad signaling pathway. These findings suggest that ADAM17 might be an important therapeutic target candidate in gastric cancer.

Acknowledgements

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