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Trefoil factor 3 peptide regulates migration via a Twist-dependent pathway in gastric cell

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ABSTRACT

Trefoil factor 3 (TFF3) is a member of the TFF-domain peptide family and essential in regulating cell migration and maintaining mucosal integrity in gastrointestinal tract. However, the role of TFF3 and its downstream regulating mechanisms in cancer cell migration remain unclear. We previously reported that TFF3 prolonged the up-regulation of Twist protein to modulate IL-8 secretion in intestinal epithelial cells. In this study, we investigated the role of Twist protein in TFF3-induced migration of SGC7901 cells. While Twist was activated by TFF3, siRNA-mediated knockdown of Twist abolished TFF3-induced cell migration. Furthermore, the migration related marker CK-8 as well as ZO-1 and MMP-9 was also regulated by TFF3 via a Twist-dependent mechanism. Our study suggests that Twist, as an important potential downstream effector, plays a key role in TFF3-modulated metastasis in gastric cancer and can be a promising therapeutic target against intestinal-type gastric cancer.

1. Introduction

It is estimated about 80–90% gastric cancer (GC) patients being diagnosed at the advanced stage when the tumor is no longer operable due to extensive invasiveness and metastasis [1]. Metastasis remains a major concerning threat to GC patients. Cancer cell migration is an early event of metastasis and the underlying mechanism has drawn increased research effort and clinical attention. Recently, emerging studies report the serum level of TFF3 as a better marker for the GC risk evaluation than pepsinogen (PG), meanwhile, the combination of serum TFF3 and PG suggested as a new marker [2,3]. Several studies demonstrate that TFF3 was involved in cell migration [4,5]. TFF3 reduced cell–cell [6,7] and cell–matrix [8] interactions and enhanced cell scattering in bronchiole or other epithelia cells. However, the underlying molecular mechanism involved in the TFF3-related GC cell migration is largely unknown.

The trefoil factor family (TFF) peptides are small regulatory proteins consisting of three members. TFF3 is a major constituent in the goblet cells in small and large intestines [9], especially a typical secretary peptide of the normal human antral and pyloric gastric mucosa [10]. TFF3 is essential in regulating cell migration and maintaining normal GI mucosal integrity [10–12]. However, its role in GI cancer metastasis remains poorly defined. Accumulating evidence indicates that TFF3 may be a causal link between local inflammation and occurrence of GI tumors [13–16]. Recent reports have also shown that TFF3 is involved in tumor cell scattering, angiogenesis, and invasion [17–20]. Our previous study demonstrated that TFF3 prolonged up-regulation of Twist protein in intestinal epithelial cells to regulate IL-8 secretion via an ERK pathway [21]. The result was specially reviewed by Dr. Barrett (2005) [22] and agreed by Dr. Dafna in his paper of Cancer Cell (2010) [23].

Twist, a transcription factor of the basic helix-loop-helix class, has been shown to regulate cancer metastasis and induce epithelial-mesenchymal transition (EMT) [24,25]. Meanwhile, it can be used as a useful marker for the development, progression and metastasis of GC [26–28]. In this work, we study the effect of TFF3 on gastric cancer cell migration and its underlying mechanism. Here, we report for the first time that the TFF3-Twist pathway modulated the SGC7901 cell migration. Therefore, our study

Abbreviations: TFF3, trefoil factor 3; ITF, intestinal trefoil factor; ZO-1, zonula occludens protein 1; CK-8, cytokeratin 8; siRNA, small interfering RNA; HRP, Horseradish peroxidase.

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suggests that Twist may serve as the potential therapeutic target in TFF3-upregulated gastric cancer.

2. Materials and methods

2.1. Antibodies and Cell culture

Mouse anti-Twist, rabbit anti-CK-8 and mouse anti-MMP-9 were obtained from Abcam (MA, USA). Mouse anti-ZO-1 and mouse anti-ZO-1-FITC were purchased from Invitrogen (CA, USA). Rabbit anti-ITF (FL-80) and mouse anti-GAPDH were acquired from Santa Cruz (CA, USA). Horseradish peroxidase- conjugated goat anti mouse/anti rabbit IgG antibodies were obtained from Cell signaling technology (CA, USA). 4′,-6-Diamidino-2-phenylindole-dihydrochloride (DAPI) was purchased from Sigma (MO, USA). Transwell migration chambers were acquired from Corning (NY, USA).

The gastric cancer cell lines SGC-7901, BGC823 and MKN-1 were kept in our Cell Biology Laboratory in China Medical University. The cells were cultured in dulbecco′s modified eagle′s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified chamber with 5% CO^2. They were digested using 0.25% trypsin and 0.02 mol/L EDTA in PBS and washed using DMEM medium containing 1% FBS for subculture. The cells were seeded in 12- and 24-well plates for the experiments.

2.2. Twist knockdown assay

Three shRNA plasmids pSIREN-RetroQ-Twist-420/423/460 were gifts from Dr. Jun Ninomiya-Tsuji. To knockdown Twist expression, the cells at 50–70% confluence were transiently transfected with either Twist siRNA or scramble as negative control using Lipofectamine™ 2000 (Invitrogen, CA, USA) according to the manufacturer′s instructions. After the plasmids being transfected for 60 h, the total cell protein was extracted. The protein level of Twist expression in the knockdown group along with the control was determined by Western blotting assay.

2.3. Wound scratch assays

SGC7901 cells were seeded at a 5×10^5 cells/per well concentration in 12-well culture plates and transfected with FLAG-*hTFF3* and Twist shRNA plasmids. 24 h after treatment, the SGC7901 cells were washed with PBS, and then scratches were made on the subconfluent monolayer cells using a sterile P200 pipette tip to mimic the wound process. After removal of cell debris, the cells were observed under microscope to confirm the uniform width of scratches in each single group. The cells in the plate-well were washed with PBS, and were incubated in DMEM containing 1% FBS. For the image-analysis, five different zones of each well were chosen for the digital image capture using a Phase-Contrast Leica DMI3000B microscope (German) with 20× objective. The digital images were captured continuously from the same field at 0, 6, 12 and 24 h after scratching. This wound scratch assay was carried out in triplicate per treatment group.

2.4. Transwell migration assay and image analysis

Double-chamber migration system was used for the transwell migration assay. SGC7901 cells were seeded at 10^5 cells/per upper chamber of a 24-well plate. The upper chamber and lower chamber were separated by a 8 μm pore-size permeable polycarbonate membrane. The cells were allowed to migrate from upper to lower chamber for 12 h. Then the migration was stopped. The chamber membranes with cells adhering to the lower surface were fixed with cold 4% paraformaldehyde (PFA) for 30 min. All cells were stained with 0.1% viola crystalline for 30 min, followed by washed three times with PBS and mounted on glass slides. Ten different fields of each membrane were selected randomly for the images capture. The number of migration cells were counted to determine the index of cell migration. This transwell migration assay was carried out in triplicate.

2.5. Western blotting analysis

After treatment in each cell experiment, the cells were washed with ice-cold PBS and lysed in a modified RIPA buffer containing 1 mM DTT, 1 mM PMSF, complete protease inhibitor cocktail (1 tablet/10 ml, Invitrogen, USA). The cell lysate was sonicated and followed by centrifugation at 12,000*g* for 20 min at 4 °C. Supernatants were mixed with an equal volume of 2× Laemmli buffer and boiled for 5 min. The isolated protein samples were loaded at 30 μg on 12% SDS–PAGE gel to perform electrophoresis. The separated proteins by SDS–PAGE were then transferred to the PVDF blotting membranes. For immunoblotting, the membranes were blocked in 5% defatted milk in Tris-buffered saline (TBST, 10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20) for 2 h. The membrane was then incubated with the antibodies mentioned above at 4 °C overnight. After incubation, the blot was washed three times with 0.05% Tween-20 TBS (TBST), and then incubated with 1:5,000 diluted goat anti-rabbit or mouse IgG conjugated with HRP for 2 h at room temperature. After additional washing with TBST, the target proteins on the blot membrane were visualized using the ECL system. The MF-ChemiBIS 3.2 imaging system (DNR Bio-Imaging Systems, Israel) was used for image capture. The optical density (OD) of each band was analyzed using the Image-J software.

2.6. Immunofluorescence and confocal imaging

After treatment of each experiment, the cells on the slides were fixed in cold 4% PFA for 20 min, and then washed three times in PBST (PBS with 0.2% Triton X-100). The cells were then incubated within normal goat serum for 1 h at room temperature. After blocking of the non-specific staining, the cells were incubated with the anti-ZO-1-FITC antibody at 4 °C overnight. The DAPI was used to detect the nuclei as counter staining. Cell samples were imaged on confocal microscope.

2.7. Statistical analysis

All experiments were obtained at least in three replicates. In addition, assays producing quantitative data were run in triplicate. SPSS 13.0 software was employed. All results presented in this study are expressed as mean values ± S.D. Statistical significance was determined by one-way ANOVA or two-tailed Student′s *t*-test, as appropriate $p < 0.05$ is considered significant.

3. Result

3.1. Twist mediated TFF3 expression in gastric cell

We have demonstrated that TFF3 triggered transient activation of NF-κB and prolonged up-regulation of Twist protein in intestinal epithelial cells [21]. In this experiment, we aimed to analyze whether the expression of Twist is induced by TFF3 in gastric cancer cells. Overexpression of TFF3 in SGC7901 cell was confirmed by immunoblotting using anti-hTFF3 antibody (Fig. 1A). Interestingly, Twist protein was elevated in TFF3-expressing cells (Fig. 1B), while its expression was knocked down by Twist shRNA460 (Fig. 1C).

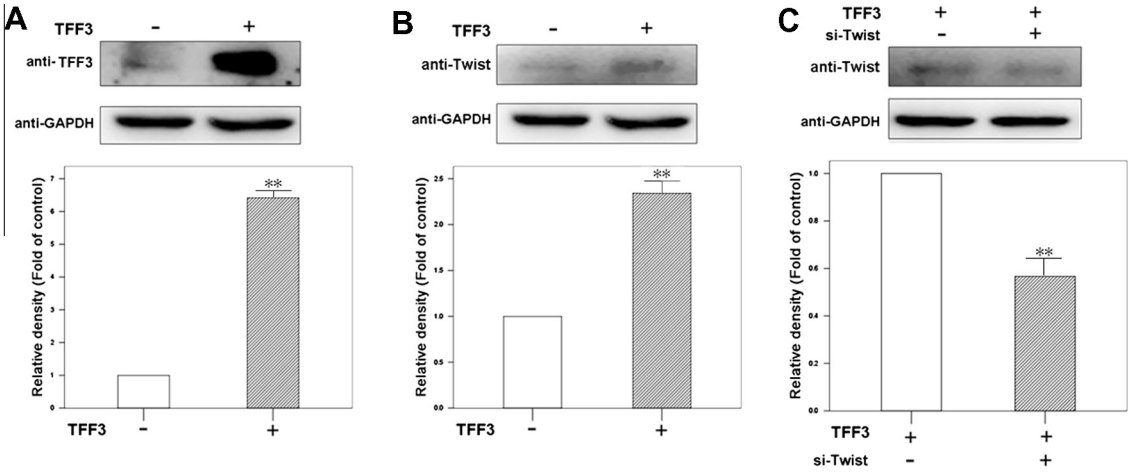

Fig. 1. Analysis of Twist expression induced by TFF3 and Twist silenced effect. (A) Western blotting analysis of TFF3 expression in control- and FLAG-hTFF3-transfected cells ($p < 0.01$). (B) Western blotting analysis of Twist expression up-regulated by TFF3 in SGC7901 cells with anti-Twist antibody ($p < 0.01$). (C) Knockdown of Twist expression by Twist siRNA. Western blotting analysis of Twist protein level in TFF3-overxpression SGC7901 cells ($p < 0.01$).

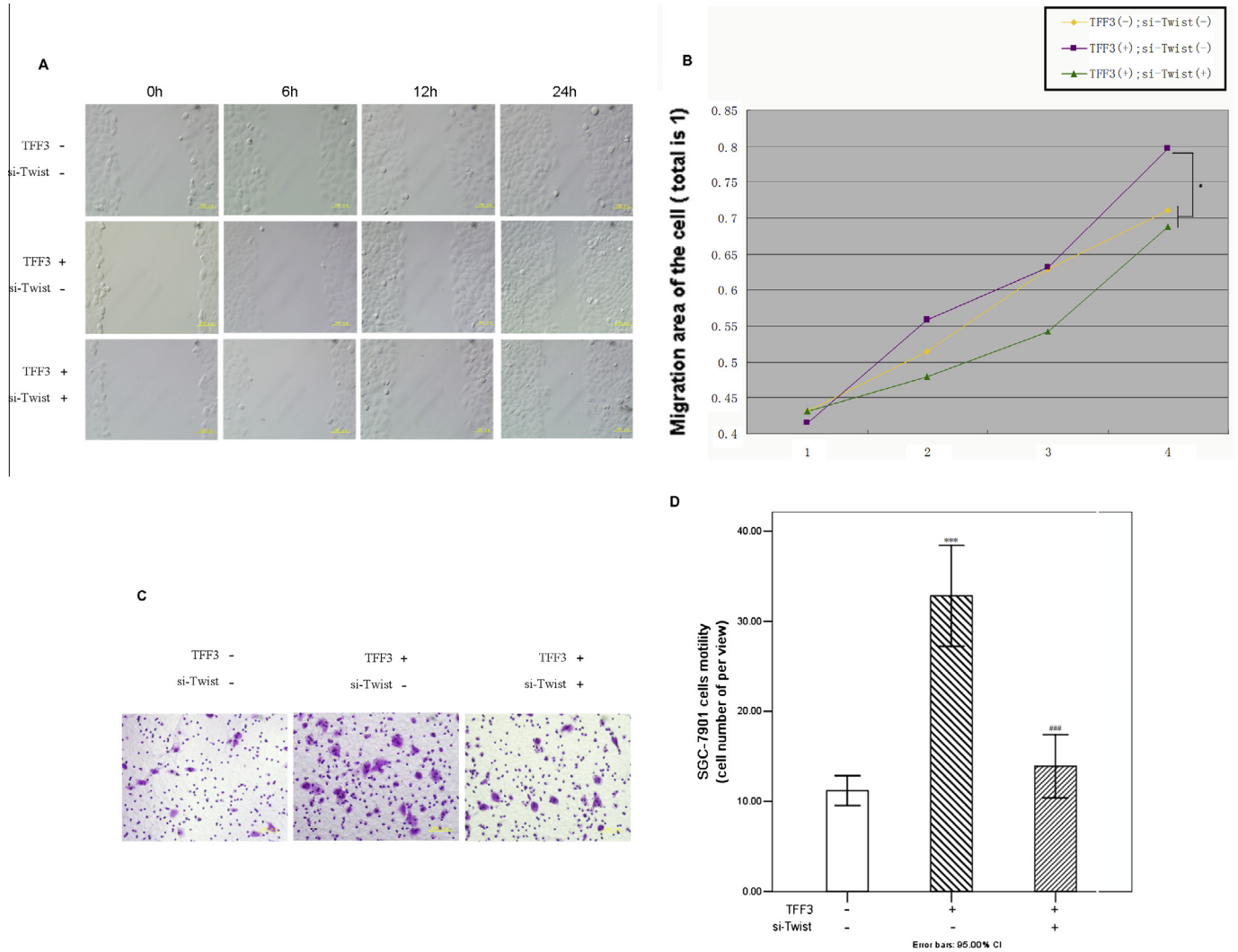

Fig. 2. Twist was required for TFF3-enhanced migration in SGC7901 cells. (A) Cell migration of FLAG-hTFF3- and Twist shRNA460-transfected SGC7901 cells was analyzed in wound scratch assays. (B) Quantitative presentation of cell motility was analyzed in wound scratch assays. $^{*}p < 0.05$, comparison among the Row2 and the Row1/Row3, respectively. (C) Cell migration of FLAG-hTFF3- and Twist shRNA460-transfected SGC7901 cells was analyzed in transwell migration assays. (D) Quantitative presentation of cell motility was analyzed in transwell migration assays. $^{***}p < 0.001$, comparison between the first and the second group. $^{\#\#\#}p < 0.001$, comparison between the second and the third group.

3.2. Twist is required for TFF3-induced SGC7901 cell migration

TFF3 was previously reported to promote cell migration of epithelial cells [11,29,30]. To verify if Twist took part in this stimulatory effect, wound scratch assays and Transwell migration assays were performed. As shown in Fig. 2A and B, the results showed that TFF3 improved cell migration (Row 2) compared to vector control (Row 1) ($p < 0.05$). To further study if Twist played a role as an essential effector, it was silenced in Row 3. The results showed that siRNA-mediated knockdown of Twist abolished the migration induced by TFF3 (Row 2 and 3,$p < 0.05$). These results suggested that Twist might be a key downstream mediator in TFF3-regulated migration.

Furthermore, Transwell migration assays showed that TFF3 promoted the cell migration by 2.2-fold ($p < 0.001$) as compared to the control. In contrast, the motility was inhibited by Twist siRNA

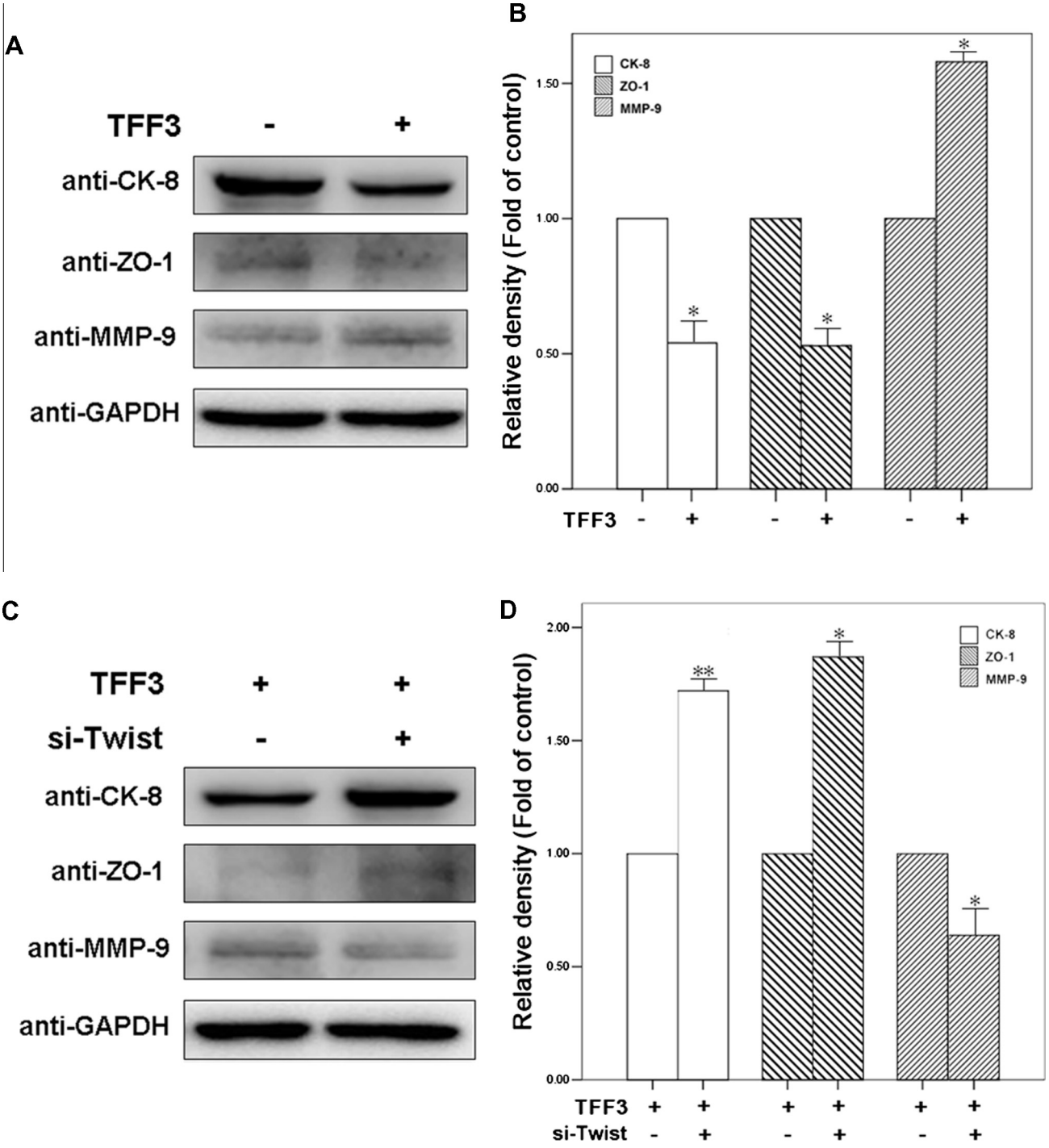

Fig. 3. Migration-related markers expression induced by TFF3 was Twist-dependent. (A) Expressions of CK-8, ZO-1 and MMP-9 protein were analyzed by Western blotting. (B) Quantitative analysis of the protein expressions. CK-8 and ZO-1 were decreased by TFF3 ($p < 0.05$), while MMP-9 was increased ($p < 0.05$) as compared to control. (C) Protein expressions modulated by Twist knockdown in TFF3 expression-enhanced SGC7901 cells were analyzed by Western blotting assay. (D) Quantitative analysis of the protein expressions by Twist knockdown. Compared with the scramble, expressions of CK-8 ($p < 0.01$) and ZO-1 ($p < 0.05$) were increased by TFF3 with Twist knockdown. In contrast, MMP-9 expression was decreased ($p < 0.05$) as compared to the control.

($p < 0.001$) as compared to the scramble (Fig. 2C and D).This result disclosed that the migration induced by TFF3 was dramatically abolished because of Twist knockdown.

3.3. Migration-related markers were regulated by TFF3-Twist pathway

To further examine the molecular changes in migration in theT-FF3-enhanced expression cells, migration-related markers were detected. As shown in Fig. 3A and B, the expression of MMP-9 was up-regulated ($p < 0.05$) while CK-8 was down-regulated ($p < 0.05$) induced by TFF3 as compared to control. In contrast to other reports [7], the expression of ZO-1 was decreased by TFF3 ($p < 0.05$). We further investigated how Twist mediated TFF3-regulated migration. To this end, si-Twist interference test was carried out. Results showed that expression of CK-8 and ZO-1 was significantly re-raised ($p < 0.01$ and $p < 0.05$)while MMP-9 was decreased ($p < 0.05$) by si-Twist compared with the scramble (Fig. 4A and B).

3.4. Tight junction marker ZO-1 modulated by TFF3 was Twist-dependent in gastric cancer cell lines

To illustrate the expression of ZO-1 regulated by TFF3-Twist pathway, three gastric cancer cell lines with different metastasis potential ability were used. The results further confirmed the decreased expression of ZO-1 was modulated by TFF3 (Fig. 4A, Row 1 and 2) and the mean intensity of the fluorescence was 30.1%, 35.2% and 28.5% decreased in three different cell lines as shown in Fig. 4B($p < 0.01$, $p < 0.01$ and $p < 0.05$). Furthermore, the analysis

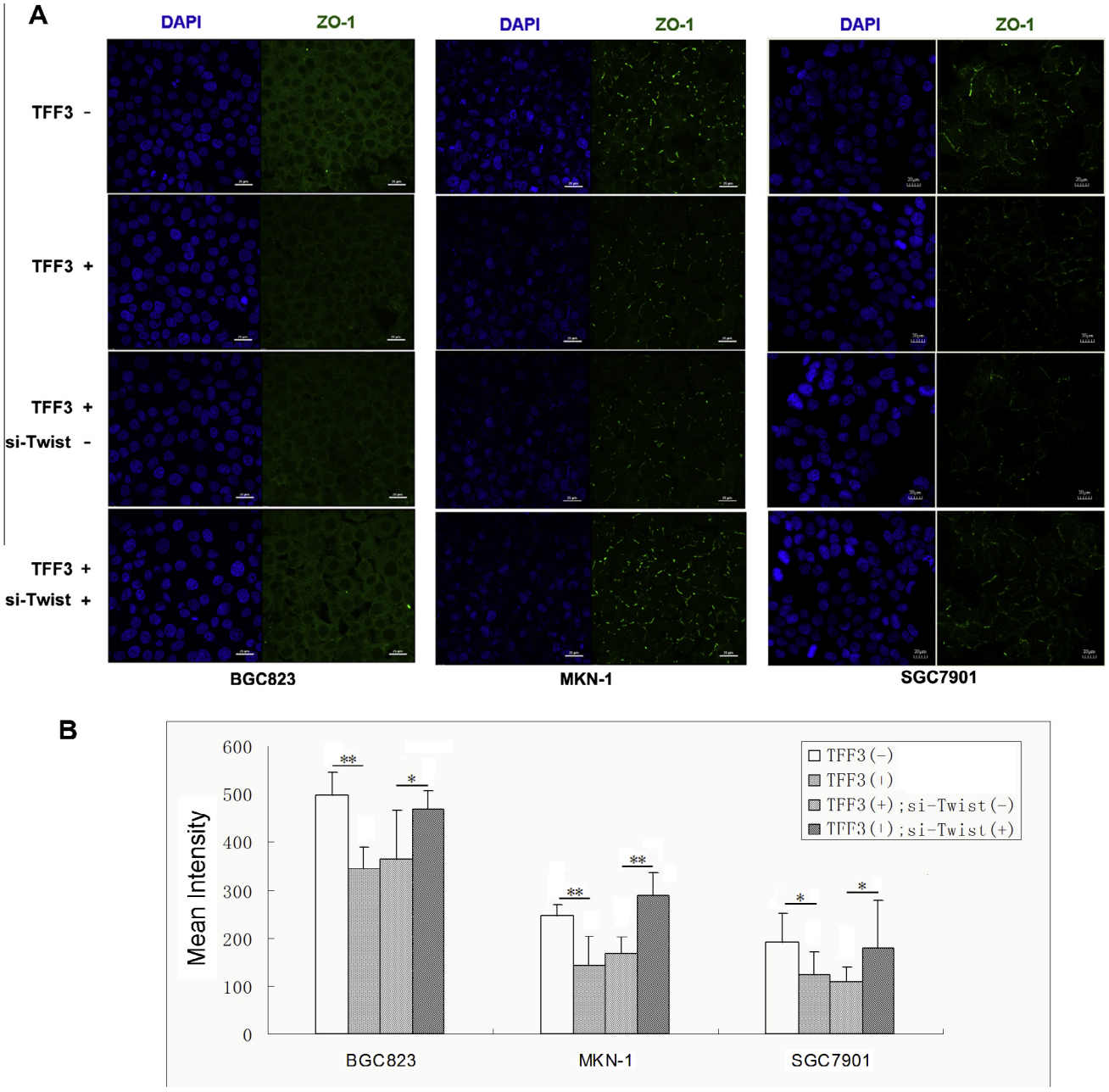

Fig. 4. TFF3-regulated ZO-1 expression was mediated by Twist in gastric cancer cell lines. (A) Immunofluorescence changes of ZO-1 regulated by TFF3 with/without Twist silencing in BGC823, MKN-1 and SGC7901cells. (B) Quantification of immunofluorescence changes of ZO-1 in three gastric cancer cell lines. $^{**}p < 0.01$, $^{*}p < 0.05$.

of immunofluorescence microscopy confirmed that ZO-1 was up-regulated by Twist knockdown in TFF3-expressing cells (Fig. 4A, Row 3 and 4, $p < 0.05$, $p < 0.01$ and $p < 0.05$). The results showed that Twist, at least partially, mediated ZO-1 expression regulated by TFF3.

4. Discussion

The biological role of TFF3 in cancer cell migration remains poorly defined, even though it induced mucosal healing [10,11] and cell migration addressed in bronchial [4], breast [32], biliary [6] and normal intestinal [16] epithelial cells. Our study demonstrated that TFF3 induced SGC7901 cell migration and we, for the first time, found that up-regulation of Twist played an essential role in cell motility in TFF3-expression gastric cancer cells.

Twist, a class II member of the highly conserved family of basic helix-loop-helix (bHLH) transcription factors, is believed to be a master regulator of EMT for the metastasis of tumor cells [33]. Studies reported that Twist was closely related to the adherent proteins and the MMPs, which promoted cells migration and cancer metastasis [30,34,35]. In this study, the results indicated that SGC7901 cell procured the motility ability when Twist was activated by TFF3. In contrast, siRNA-mediated knockdown of Twist abolished TFF3-induced cell migration (Fig. 1 and 2). It provided the evidence that the TFF3-induced cell migration was mediated by Twist.

It has been acknowledged that migration is modulated through the deregulations of cell–cell adhesion complex and interruption of intercellular matrix. In this study, we found that CK-8 (a potential migration character marker) was down-regulated by TFF3, which was an important indication in SGC7901 cell scattering. Furthermore, the expression of cell–matrix molecule such as MMP-9 was enhanced by TFF3, which facilitated cell migration. These results were in line with the studies by other groups, i.e., in rat fibroblast cells [8], glioblastoma multiforme (GBM) cells [31] and prostate cancer cells [30]. Previous study reported that there was no effect of TFF3 on ZO-1expression in HT29/B6 and MDCK cells [7]. However, we found that ZO-1 was down-regulated induced by TFF3 in SGC7901 cell. To demonstrate the phenomenon furtherly, another two different gastric cancer cell lines BGC823 and MKN-1 were detected (Fig. 4). This result confirmed that the negative regulation of TFF3 might be due to cell type-specificity. The decreased CK-8 and ZO-1 manifested the loss of epithelial characteristics and cell–cell adhesion character, which will facilitate the cell detachment from original site to migration, while the up-regulated expression of MMP-9 implied the degradation of intercellular matrix when TFF3 was enhanced in SGC7901. Interestingly, knockdown of Twist could reverse the expressions of these migration-related markers.

The results in this study suggested that Twist is a critical downstream effector of TFF3 in the gastric cancer cell migration. It is supported by the following observations: (1) Twist expression was up-regulated by TFF3 in the SGC7901 cell line. (2) the TFF3-induced cell migration was abolished when Twist was knockdown. (3) the TFF3-induced cell migration was dependent on Twist modulation. (4) Twist mediated downstream migration-related effectors such as CK-8, ZO-1 and MMP-9, which played the key roles for cell migration.

In summary, this study demonstrated that TFF3-induced migration was Twist-dependent in SGC7901 cells. As a downstream mediator of TFF3, Twist contributed to the modulation of ZO-1, CK-8 and MMP-9 expressions induced by TFF3. Our study show that the activation of Twist plays an essential role in promoting migration induced by TFF3 and can be a therapeutic target against the intestinal-type gastric cancer.

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