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TGFβ1I1 suppressed cell migration and invasion in colorectal cancer by inhibiting the TGF-β pathway and EMT progress

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Abstract. – OBJECTIVE: Colorectal cancer (CRC) is the fourth leading cause of death worldwide and there is a need for more specific therapeutic targets and biomarkers for the disease. Transforming growth factor β1-induced transcript 1 (TGFB1I1) was reported to be downregulated in CRC tissues; however, the precise roles of TGFB1I1 in CRC remain unclear.

PATIENTS AND METHODS: The expression of TGFB1I1 in CRC cell lines and tissues was assessed by quantitative Polymerase Chain Reaction (qPCR). TGFB1I1 was overexpressed in SW620 and RKO cells. Cell viability was analyzed by a CCK-8 assay. The proportion of apoptotic cells was analyzed by flow cytometry. The EdU cell proliferation assay of SW620 and RKO cells after transfection was performed *via* flow cytometry. The migration potency of SW620 and RKO cells was analyzed using a cell migration assay. A wound healing assay was performed to assess the migration potency of SW620 and RKO cells. The invasion potency of SW620 and RKO cells after TGFB1I1 overexpression was analyzed. The protein levels of VEGF, TGF-β, MMP9, p-Smad2/3, N-cadherin, and E-cadherin were analyzed by Western blot.

RESULTS: Decreased expression of TGFB1I1 was found in CRC tissues and cell lines. Overexpression of TGFB1I1 inhibited the proliferation and induced the apoptosis of CRC cells. The overexpression of TGFB1I1 inhibited the migration and invasion of CRC cells. We also found that the overexpression of TGFB1I1 in CRC cells inhibited the TGF-β pathway and epithelial-mesenchymal transition (EMT) progress.

CONCLUSIONS: TGFB1I1 suppressed cell migration and invasion in CRC by inhibiting the TGF-β pathway and EMT progress.

Key Words:
TGFβ1I1, Cell migration, Cell invasion, Colorectal cancer, VEGF, TGF-β, EMT.

Abbreviations

CCK-8: Cell Counting Kit-8; CRC: Colorectal cancer; EGFR: Epidermal growth factor receptor; EdU: thymidine analog 5-ethynyl-2'-deoxyuridine; EMT: epithelial-mesenchymal transition; KLF4: Kruppel-like factor 4; L-FABP: liver fatty acid-binding protein; qRT-PCR: quantitative Real Time-PCR; TGFB1I1: Transforming growth factor beta 1 induced transcript 1; TGF-β: Transforming growth factor-β; VEGF: Vascular endothelial growth factor.

Introduction

Colorectal cancer (CRC) is one of the most frequently diagnosed cancers in the world and kills almost 700,000 people every year[1]. The incidence of CRC is increasing significantly with the shift to Western diets, living standards, and lifestyles. Despite the declining mortality rate of CRC mainly due to advances in screening tests, surgery, radiation therapy, and adjuvant chemotherapy, more than 40% of CRC patients die from tumor recurrence and metastasis[2,3]. Although current methods for the diagnosis and therapy of CRC have achieved remarkable progress, tumor metastasis remains an important factor that affects patient survival[4-7]. A complex structure of oncogene and gene pathways are involved in the occurrence, development, and variation of CRC, and gene therapy is becoming an intense focus of research[8]. Improving therapies and discovering the underlying molecular mechanism of drug resistance in CRC are essential. Therefore, discovering the underlying molecular pathophysiological pathways is necessary for improving the therapeutic strategy.

TGFB1I1, also known as Hic-5, is a focal adhesion scaffold protein primarily expressed in vas-

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cular and visceral smooth muscle cells. It is also induced by transforming growth factor-β1 and hydrogen peroxide[9,10]. As well-known accelerator in fibrosis, TGFB1I1 was found to play an important role in a diverse range of biological processes including senescence[11], myogenesis and pathogenic myofibroblast phenotype[12,13], cancer[14-25], steroid hormone action[26,27], cell growth and survival[28,29]. The expression level of TGFB1I1 in hormone-refractory prostate cancer specimens was significantly lower than that in benign prostatic hypertrophy and untreated prostate cancer samples[15]. TGFB1I1 was overexpressed in hepatocellular carcinoma and correlated well with intra-hepatic and extra-hepatic metastasis. The knockdown of TGFB1I1 inhibited the constitutive migration of hepatocellular carcinoma cells[16]. TGFB1I1 is primarily expressed in cancer-associated fibroblasts. It plays an important role in the deposition and remodeling of the stromal extracellular matrix, and promotes non-cell autonomous breast tumor progression[20]. In CDC, the overexpression of TGFB1I1 induced the cell cycle arrest in LoVo cells and suppressed the growth of subcutaneous tumors[30].

The overexpression of TGFB1I1 in colon cancer cells induced the expression of several gut epithelial differentiation/maturation markers, such as liver fatty acid-binding protein (L-FABP) and Kruppel-like factor 4 (KLF4)[31]. However, the role of TGFB1I1 in the migration and invasion of CRC cells remains unclear.

In our study, decreased expression of TGFB1I1 in colorectal cancer tissues and cell lines was found. Overexpression of TGFB1I1 inhibited the cell proliferation, induced apoptosis, and inhibited the cell migration and invasion of colorectal cancer cells. We also found that the overexpression of TGFB1I1 in colorectal cancer cells inhibited TGF-β pathway and EMT progress.

Patients and Methods

Clinical Samples

Experimental CRC tissues and adjacent tumor tissues were obtained from 20 patients in the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, Zhejiang, China). Inclusion criteria for CRC patients included no history of treatment, no history of other malignant tumors, and CRC confirmed by histopathology. The study was conducted in accordance with the Declaration of Helsinki, and all protocols were approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. All participants provided written informed consent before the study.

Cell Culture

Human CRC cell lines (LoVo, SW620, RKO, HCT-116, SW480 and HT-29) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Procell Life Science & Technology, Wuhan, Hubei, China) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). Human NCM460 colonocytes were purchased from INCELL (San Antonio, TX, USA) and cultured in DMEM.

Cell Viability Assay

3000 cells in each well were seeded and exposed to the indicated treatment. In each well, 90 μL fresh media was used to replace the media and 10 μL of Cell Counting Kit-8 (CCK-8) solution (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) was added. 2.5 h later, absorbance at 450 nm was measured using the Infinite M200 FA plate reader (TECAN, Männedorf, Switzerland).

Apoptosis Assay

The apoptosis of SW620 or RKO cells was analyzed by Annexin V-FITC/PI Detection Kit (Sigma-Aldrich, St. Louis, MO, USA). Briefly, the cells were exposed to different treatments and harvested. Then, the cells were double-stained by Annexin V-FITC and PI in the dark. Afterwards, the cells were analyzed using a BD FACSCalibur Aria system (BD Biosciences, San Jose, CA, USA).

Quantitative Real-Time PCR (qRT-PCR)

The cells were homogenized in 1 ml of TRIzol (Invitrogen, Carlsbad, CA, USA) to extract the total RNA following the protocol. SYBR® Green (BioRad Laboratories Inc., Hercules, CA, USA) was used for qRT-PCR analysis, and qPCR was performed for thymidylate synthase (TS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative expression levels were normalized to that of GAPDH using the comparative cycle threshold method and fold change. The primers used are as follows: TGFB1I1 forward primer, 5'-AAGCCTGTCTGGAAGTTACTTGT-3'; TGFB1I1 reverse primer, 5'-GCAGGGTATCCTTGTGCCA-3'; GAPDH forward primer, 5'-GGAGCGAGATCCCTCCAAAAT-3'; GAPDH reverse primer, 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Western Blot

Cells were homogenized in ice-cold lysis buffer. After centrifuging for 5 min at 10,000 g, equivalent amounts of protein (20 μg) was subjected to 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked for 60 min with phosphate-buffered saline (PBS). Then, the membranes were incubated with respective primary antibodies: anti TGFB1I1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:1000), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:1000), TGF-β (CST, 1:1000), p-Smad23 (Abcam, Cambridge, MA, USA, 1:1000), MMP9 (Abcam, 1:1000), N-cadherin (Sigma-Aldrich, St. Louis, MO, USA, 1:1000), E-cadherin (CST, 1:1000) and anti-rabbit IgG-HRP (CST, 1:1000). Bands were visualized using an enhanced chemiluminescent system (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Transfection

The pcDNA3.0 or pcDNA3.0-TGFB1I1 plasmid were constructed by GenePharma Co., Ltd, Shanghai, China. The SW620 and RKO cells were transfected by pcDNA3.0 or pcDNA3.0-TGFB1I1 plasmid with the Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA).

Transwell Migration and Invasion Assay

For the transwell migration assay, 2×10^5 cells/well were seeded onto the upper well of a Millipore transwell chamber (8 μm) in serum-free medium, and 650 μL media containing 10% FBS in the lower chamber. 24 h later, the non-penetrated cells on the upper surface of the membrane were removed. The cells were fixed with formaldehyde, stained with 0.1% crystal violet, and photographed in different fields using an inverted microscope (CX41, Olympus, Tokyo, Japan). For transwell invasion assay, all the steps were carried out similarly to those in the migration assay except for the Matrigel coating.

EdU Proliferation Assays

The SW620 and RKO cells were transfected with after indicated transfection. For the EdU assay, the cells were sequentially incubated with 10 μM thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) for 3 h and fixed with 3.7% formaldehyde. Then, the stained cells were analyzed by FACS Calibur™ Flow Cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical Analysis

All experiments were performed at least three times. We considered $p<0.05$ to be statistically significant. Unpaired Student's *t*-tests were used to assess differences by Prism statistical software package (Version 8.0, GraphPad Software Inc.).

Results

Decreased Expression of TGFβ1I1 In Colorectal Cancer Tissues and Cell Lines

We first analyzed the expression of TGFB1I1 in the CRC tissues and adjacent tumors. As shown in Figure 1A, the mRNA level of TGFB1I1 was significantly decreased in CRC tissues. Additionally, the expression of TGFB1I1 in the CRC lines and human NCM460 colonocytes was assessed. The

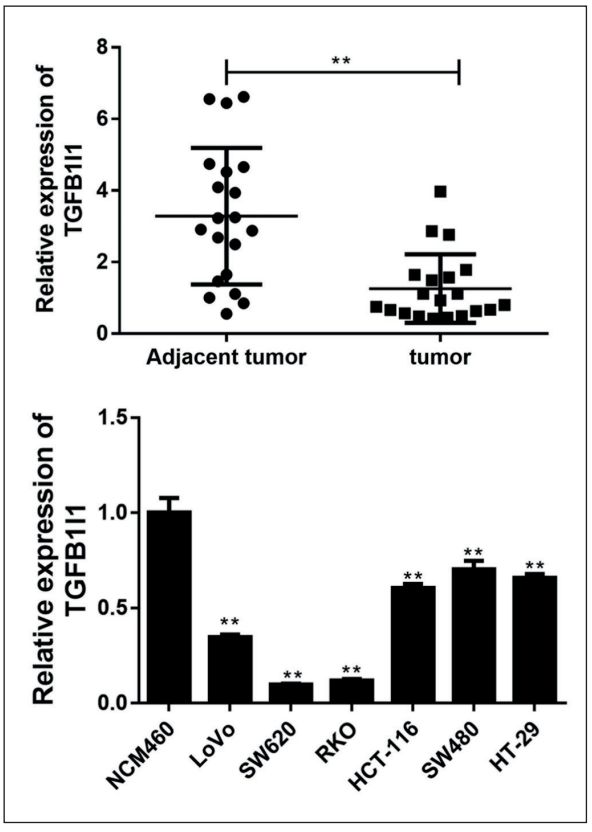

Figure 1. Decreased expression of TGFB1I1 in colorectal cancer tissues and cell lines. **A**, The expression of TGFB1I1 in the colorectal cancer tissues and normal tissues was analyzed by qRT-PCR. **B**, The mRNA level of TGFB1I1 in the colorectal cancer lines (LoVo, SW620, RKO, HCT-116, SW480 and HT-29) and human NCM460 colonocytes was analyzed by Q-PCR. Data represent three independent experiments (average and SEM of triplicate samples). ***$p<0.001$.

mRNA level of TGFB1I1 in the CRC lines (LoVo, SW620, RKO, HCT-116, SW480 and HT-29) was lower than in human NCM460 colonocytes (Figure 1B). Of the CRC lines, the highly metastatic cell lines SW620 and RKO showed the lowest level of TGFB1I1 mRNA (Figure 1B). The SW620 and RKO cell lines were chosen for further experimentation of the role of TGFB1I1 in CRC.

Overexpression of TGFβ1I1 Inhibited Proliferation and Induced Apoptosis of Colorectal Cancer Cells

To ascertain the role of TGFB1I1 in CRC cells, TGFB1I1 was overexpressed in SW620 and RKO cells. Both the mRNA (Figure 2A) and protein (Figures B and C) levels of TGFB1I1 in SW620 and RKO cells were significantly increased. The proliferation of SW620 and RKO was determined by Cell Counting Kit-8 (CCK-8) (Figure 2D) and EdU (Figure 2E) assay. The proliferation was markedly suppressed by TGFB1I1 overexpression. We also assessed the cell apoptosis of SW620 and RKO cells after TGFB1I1 overexpression. As shown in Figure 2F, TGFB1I1 overexpression induced cell apoptosis of SW620 and RKO. Collectively, TGFB1I1 overexpression inhibited the proliferation and induced the apoptosis of CRC cells.

Overexpression of TGFβ1I1 Inhibited the Migration and Invasion of Colorectal Cancer Cells

To ascertain whether TGFB1I1 was involved in the migration and invasion of CRC cells, we overexpressed it in SW620 and RKO cells, and then, preformed cell a migration transwell assay and a wound healing assay. As shown in Figures 3A and B, the migration potency of SW620 and RKO cells was markedly inhibited by TGFB1I1 overexpression. Similar results were found in SW620 and RKO cells after TGFB1I1 overexpression by a wound healing assay (Figures 3C and D). Additionally, we analyzed the invasion potency of SW620 and RKO cells after TGFB1I1 overexpression and found that TGFB1I1 overexpression significantly inhibited the cell invasion (Figures 3E and F).

Overexpression of TGF-β1I1 In Colorectal Cancer Cells Inhibited the TGF-β Pathway and EMT Progress

Given that the overexpression of TGFB1I1 inhibited the cell migration and invasion of CRC cells, we aimed to further ascertain the molecular mechanisms. Collective data showed that matrix metallopeptidase 9 (MMP9) and its associated vascular endothelial growth factor (VEGF) are critical for tumor vascularization and invasion[32]. The protein levels of VEGF and MMP9 were also markedly decreased in TGFB1I1-overexpressed CRC cells (Figure 4). Additionally, the TGF-β and p-Smad23 levels were also decreased by TGFB1I1 overexpression. We also analyzed the epithelial-mesenchymal transition (EMT) markers and found that N-cadherin protein levels were decreased with the increased level of E-cadherin in TGFB1I1 overexpressed CRC cells (Figure 4). Our results indicate that the overexpression of TGFB1I1 in CRC cells inhibits the TGF-β/Smad pathway and EMT progress.

Discussion

MMP9 has been involved in various pathological conditions, degradation of the extracellular matrix, tumor invasion, and metastasis[33]. MMP9 plays a critical role in proteolytic degradation, cell adhesion, cell migration, and EMT. It promotes the EMT of cancer cells by proteolytic activation of latent TGF-β[34]. MMP9 degrades collagen type IV on the basement membrane and the overexpression of MMP9 is found in many types of cancer[35]. Here, the protein levels of MMP9 and VEGF markedly decreased in TGFB1I1-overexpressed CRC cells. Previous studies reported that MMP9 expression was enhanced by epidermal growth factor receptor (EGFR) in several types of cancer *via* PI3K/AKT pathways. As a potent inducer of multi-functional angiogenesis, VEGF plays a critical role in tumor angiogenesis. It could promote the proliferation of vascular endothelial cells and also enhance capillary permeability[36]. VEGF is found to be highly expressed in several types of malignant tumors and plays a critical role in angiogenesis, immune cell infiltration, and metastasis of tumors[37]. In PCa cells, the overexpression of MMP9 enhanced the expression of VEGF, suggesting that MMP9 is involved in EGFR pathways[38]. Additionally, VEGF was reported to be regulated by MMP9 *via* cleaving membrane-bound VEGF[34,39]. Our research shows that TGFB1I1 inhibited VEGF and MMP9.

TGF-β has a broad array of fundamental functions, including specifying tissue pattern formation during embryonic development and maintenance of tissue homeostasis in the adults[40]. It plays critical roles in diverse cellular processes, including cell proliferation, differentiation,

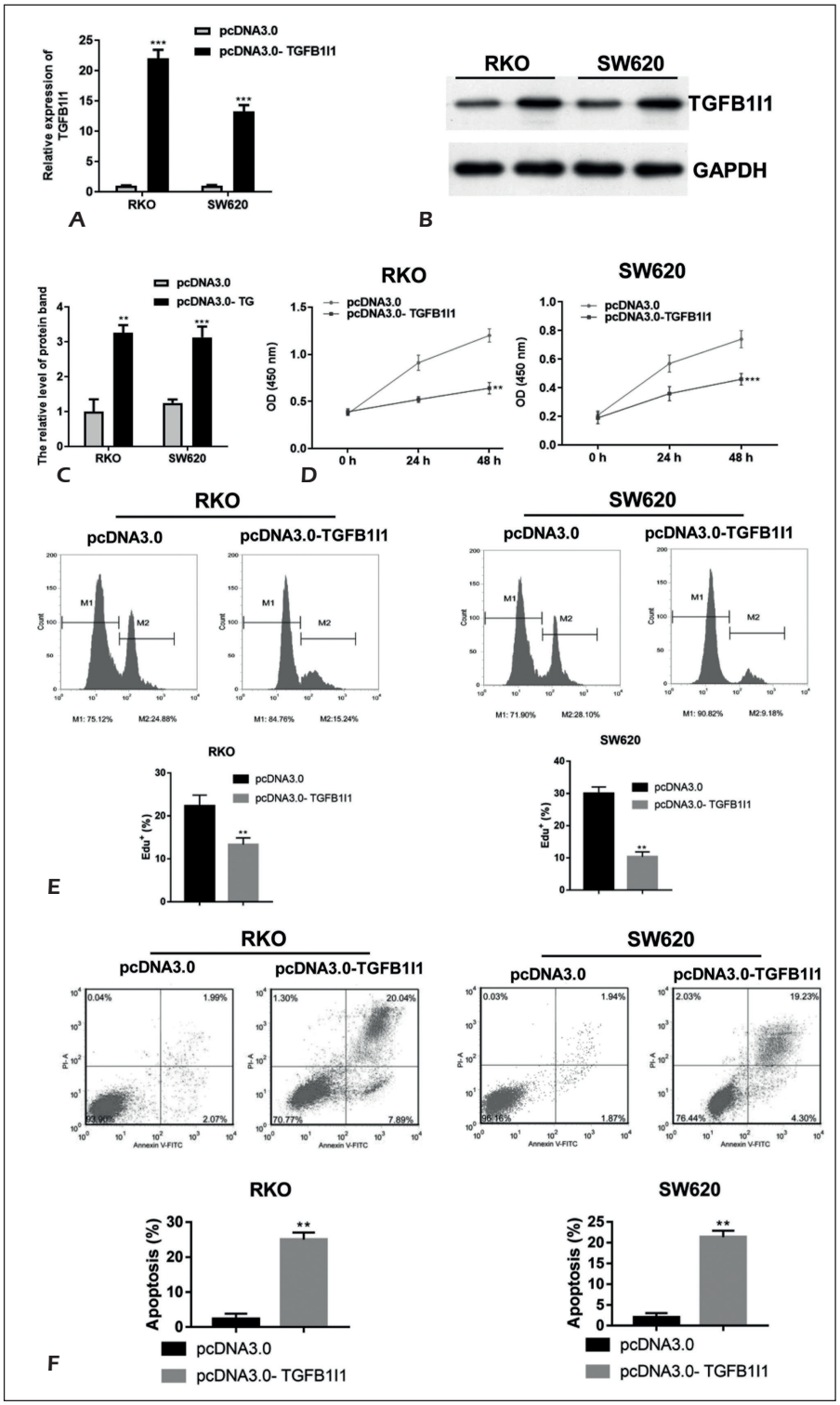

Figure 2. Overexpression of TGFB1I1 inhibited proliferation and induced apoptosis of colorectal cancer cells. The SW620 and RKO cells were transfected by pcDNA3.0 or pcDNA3.0-TGFB1I1 plasmid. **A**, The mRNA level of TGFB1I1 in SW620 and RKO after transfection was analyzed by qPCR. **B**-**C**, Western blot analysis of the protein levels TGFB1I1 in SW620 and RKO cells after transfection. Densitometry plot of results from the blots are shown. **D**, The cell proliferation of SW620 and RKO cells after transfection was analyzed by CCK8. **E**, The EdU proliferation assays of SW620 and RKO cells after transfection was performed via flow cytometry. **F**, Flow cytometry was used to evaluate the cell apoptosis of SW620 and RKO cells after transfection. Data represent three independent experiments (average and SEM of triplicate samples). **p<0.01. ***p<0.001.

migration, and cell metabolism[41]. In the normal colon, TGF-β shows a suppressive function by inhibiting proliferation and induction cell apoptosis[42]. TGF-β functions as tumor suppressor during the early stages of carcinogenesis. In CRC, TGF-β is overexpressed in both cancer and stromal cells and promotes the development of the late stages of carcinogenesis. A dual role of TGF-β was reported in the progression and metastasis of CRC[43]. Here, the TGF-β protein level in CRC cells was

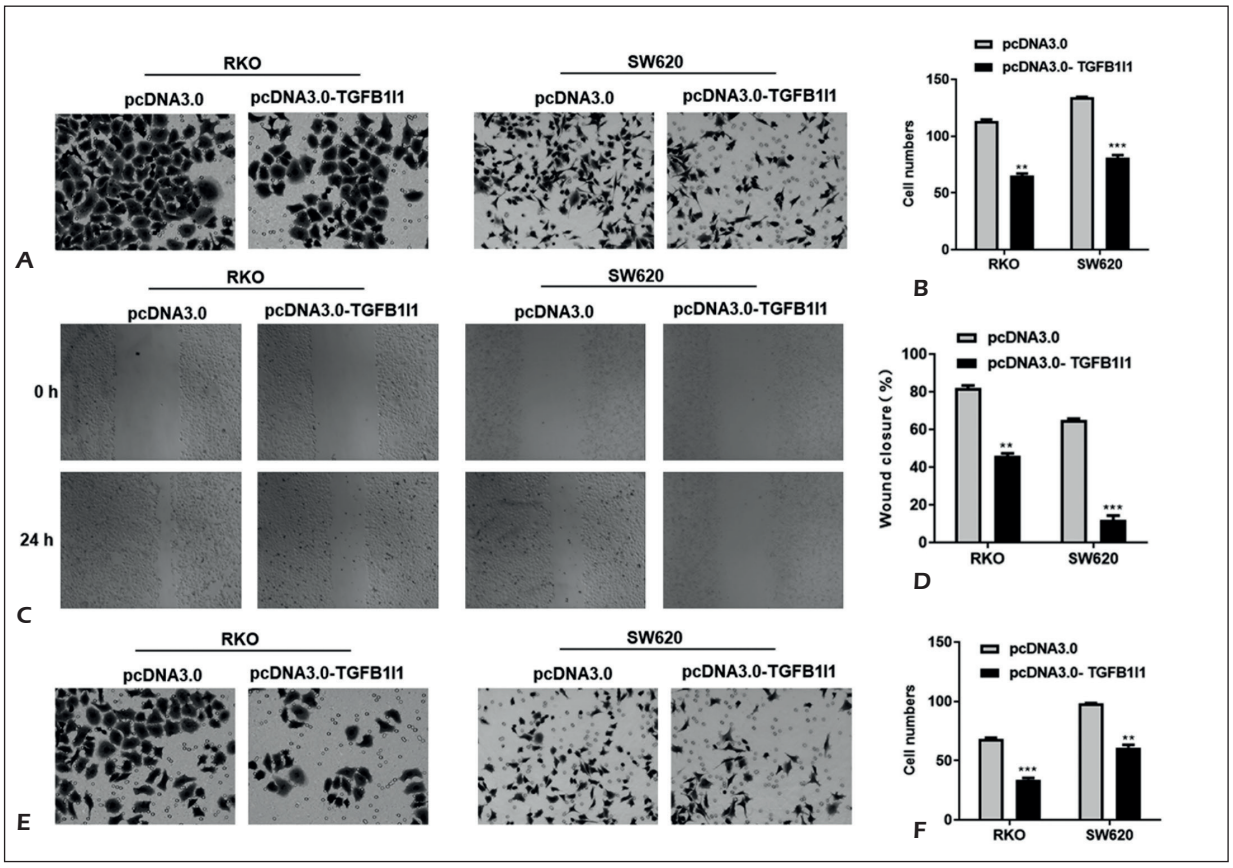

Figure 3. Overexpression of TGFB1I1 inhibited migration and invasion of colorectal cancer cells. The SW620 and RKO cells were transfected by pcDNA3.0 or pcDNA3.0-TGFB1I1 plasmids. **A**-**B**, The migration potency of SW620 and RKO cells was analyzed by cell migration assay. Representative images of the cells are shown (200x). Six fields for each chamber were photographed, the migrated cells were counted, and an average number is shown. **C**-**D**, A wound healing assay was performed to assess the migration potency of SW620 and RKO cells. Representative images of the cells are shown (40x). **E**-**F**, The invasion potency of SW620 and RKO cells after TGFB1I1 overexpression was analyzed. Representative images of the cells are shown (200x). Data represent three independent experiments (average and SEM of triplicate samples). *p<0.05. **p<0.01. ***p<0.001.

decreased by TGFB1I1 overexpression. TGF-β signals lead to the activation of transforming growth factor beta receptor I (TGFβRI), thereby inducing the phosphorylation of SMAD2 and SMAD3[44]. In our TGFB1I1-overexpressed CRC cells, the p-Smad23 level was also decreased. Overexpression of TGFB1I1 in CRC cells inhibited the TGF-β-p-Smad pathway.

It is well known that EMT results in cancer cells losing epithelial characteristics and gaining mesenchymal characteristics[45]. The EMT process enhances the migratory and invasive capacities of the cancer cells[46]. Significantly, the EMT process is derived by TGF-β. TGF-β promotes the cancer stem cell-like traits, migratory and invasion capacities of CRC cells[46,47]. In our study, the N-cadherin protein level decreased with an increased level of E-cadherin in TGFB1I1-overexpressed CRC cells. Overexpression of TGFB1I1 inhibited the migration and invasion of CRC cells. Collectively, our results indicate that the overexpression of TGFB1I1 in CRC cells inhibits cell migration and invasion *via* the TGF-β/Smad pathway and EMT progress.

Conclusions

Our study demonstrates that TGFB1I1 plays a suppressive role and inhibits cell proliferation, migration, and invasion *via* the TGF-β/Smad pathway. Our findings provide new insights into the progression and metastasis of CRC and will be helpful for developing new clinical therapies for the disease.

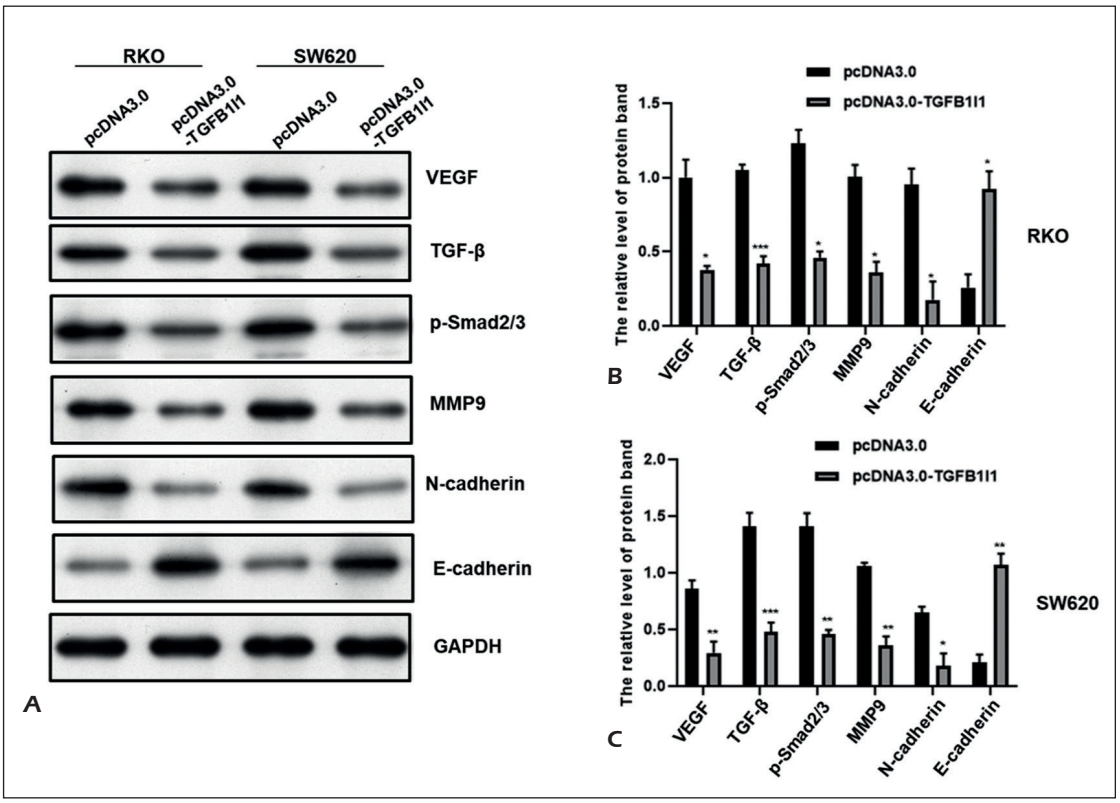

Figure 4. Overexpression of TGFB1I1 in colorectal cancer cells inhibited the TGF-β v pathway and EMT progress. **A**, The protein levels of VEGF, TGF-β, MMP9, p-Smad23, N-cadherin and E-cadherin were analyzed by western blot. **B**-**C**, Densitometry plot of blot results are shown. The relative expression levels were normalized to that of GAPDH. Data represent three independent experiments (average and SEM of triplicate samples). *p<0.05. **p<0.01. ***p<0.001.

Author Contribution

Xiaojiao Ruan, Bailiang Ye and Zhihai Zheng performed the experiments. Xiaojiao Ruan, Shaotang Li analyzed the data. Xiaojiao Ruan, Xiaofeng Zheng and Suzhan Zhang wrote the paper.

Ethics Approval

Ethics approval was obtained from the Ethic Committee of the First Affiliated Hospital of Wenzhou Medical University.

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Conflict of Interests

The authors declare that there are no competing interests associated with the manuscript.

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