TCN1 Deficiency Inhibits The Malignancy of Colorectal Cancer Cells By Regulating The ITGB4 Pathway

Xinqiang Zhu
The Second Affiliated Hospital of Soochow University
Xuetong Jiang
The Affiliated Suqian Hospital of Xuzhou Medical University
Qinglin Zhang
Wuxi People's Hospital Affiliated to Nanjing Medical University
Hailong Huang
The Affiliated Suqian Hospital of Xuzhou Medical University
Xiaohong Shi
The Affiliated Suqian Hospital of Xuzhou Medical University
Daorong Hou
Nanjing Medical University
Chungen Xing (xingcg@suda.edu.cn)
The Second Affiliated Hospital of Soochow University https://orcid.org/0000-0001-7865-1258

Primary research

Keywords: TCN1, proliferation, invasion, colorectal cancer, ITGB4 pathway

Posted Date: November 12th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1038280/v1

Abstract

Background: This study aimed to investigate the biological function and regulatory mechanism of TCN1 in colorectal cancer (CRC).

Methods: We studied the biological functions of TCN1 using gain-of-function and loss-of-function analysis in HCT116 cell lines, and examined the effects of TCN1 on the proliferation, apoptosis, and invasion of CRC cells and determined its potential molecular mechanisms using CRC lines and mouse xenotransplantation models. Tumor xenograft and tumor metastasis studies were performed to detect the tumorigenicity and metastasis of cells in vivo.

Results: TCN1-knockdown attenuated CRC cell proliferation, invasion and promoted cell apoptosis. Overexpression of TCN1 yielded the opposite effects. In addition, TCN1-knockdown HCT116 cells failed to form metastatic foci in the peritoneum after intravenous injection. Molecular mechanism studies showed that TCN1 interacts with integrin subunit β4 (ITGB4) to positively regulate the expression of ITGB4. TCN1-knockdown promoted the degradation of ITGB4 and increased the instability of ITGB4 and filamin A (FLNA). Downregulation of ITGB4 at the protein level resulted in the disassociation of the ITGB4/PLEC complex, leading to cytoskeletal damage.

Conclusion: TCN1 might exert oncogenic role in CRC via regulating the ITGB4 signaling pathway.

Background

Colorectal cancer (CRC) is one of the most common types of cancer in the world [1], and its mortality rate is high [2]. Its incidence rate and mortality rate have increased in China in recent years [3]. Strong local invasion and distant metastasis are the most important factors leading to the death of CRC patients [4]. CRC has been an important public health problem at present [5]. Although the development of surgical resection technology has improved the survival rate of early CRC patients, the long-term prognosis of most CRC patients is still poor, the main reason is recurrence and metastasis [6]. However, the exact mechanism of CRC development is still unclear. Molecular mapping of CRC (including at the DNA and protein levels) has become increasingly important for identifying prognostic biomarkers and developing new therapeutic strategies [7]. Therefore, identifying new key molecules involved in the progression of CRC will help to provide new therapeutic targets.

Transcobalamin 1 (TCN1), also known as vitamin B12 (cobalamin) R binding protein, is one of the three transporters of vitamin B12, which exists in serum and various biological liquids [8]. Vitamin B12 plays an important role in hematopoiesis, cell metabolism, and nervous system function [9, 10]. TCN1 carries vitamin B12 through the stomach and is released by enzymes in the duodenum, where it binds to intrinsic factors [11, 12]. Unexpectedly, the overexpression of TCN1 in tumor tissues is associated with tumorigenesis [13]. TCN1 is overexpressed in malignant tumors, such as hepatocellular carcinoma, leukemia, breast cancer, lung cancer, and gastric cancer [11, 12, 14]. Bioinformatics-based studies have shown that TCN1 is an important oncogene [15], however, its expression and clinical significance in CRC

are still unclear. Next generation sequencing (NGS) confirmed that TCN1 is one of the overexpressed genes in CRC [16]. It is suggested that TCN1 is related to the occurrence and development of colorectal cancer, which is worthy of further study.

Integrin is a transmembrane glycoprotein that forms heterodimers and is part of an important cell adhesion receptor family [17]. The cell adhesion receptor family plays an important role in controlling the interactions between cells, between cells and the matrix, and between the extracellular matrix (ECM) and cytoskeleton. They play an important role in the migration, invasion, and proliferation of cancer cells [18, 19] and regulate cell differentiation, angiogenesis, epithelial-mesenchymal transition (EMT) [20, 21]. These characteristics make integrin an important prognostic indicator and therapeutic target of cancer [22, 23]. Integrin subunit β4 (ITGB4) has a long cytoplasmic domain and unique cytoskeleton and signal function [24]. ITGB4 is related to the actin cytoskeleton, and its basic function in polarized epithelial cells is to form stable cell attachment by forming hemidesmosomes and basement membranes. It has been reported that high expression of ITGB4 promotes the occurrence, metastasis, and poor prognosis of different types of tumors [25, 26]. These results indicated that the expression of ITGB4 may play an important role in the occurrence and development of tumors, and it may become another potential target for tumor therapy. However, the regulatory mechanism of ITGB4 protein expression is still unclear.

In this study, the background expression levels of TCN1 were assessed, and the HCT116 cell line was transfected with TCN1 shRNA for loss-of-function analysis and transfected with TCN1 for overexpression analysis to define the role of TCN1 in CRC cell growth, invasion and survival. We demonstrate that TCN1 deficiency in CRC cells inhibits their growth, adhesion, and invasion. Mechanistically, TCN1 interacts with ITGB4; TCN1 deficiency promotes ITGB4 degradation, facilitates the degradation of ITGB4 and plectin (PLEC), and impairs the stability of FLNA and F-actin networks, and eventually leads to cytoskeleton damage of CRC cells.

Materials And Methods

Cell culture

The human cell lines (including HCT116 and HEK293T) originated from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in Roswell Park Memorial Institute-1640 medium (RPMI 1640) medium with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin in a humidified atmosphere of 5% CO2 at 37 ℃.

Clinical Samples

CRC specimens were collected with informed consent from 80 cases of surgical patients in Suqian Hospital Affiliated to Xuzhou Medical University and Jiangning Hospital Affiliated to Nanjing Medical University between 2011 and 2014 following the protocols approved by the Ethics Committee of the Affiliated Suqian Hospital of Xuzhou Medical University. The samples had paired samples of adjacent

normal CRC tissue. The definitive histological diagnosis of each CRC patient was confirmed after surgery, and no patients received radiotherapy or chemotherapy. All participants signed informed consent forms.

Immunohistochemistry (Ihc) Analysis

Paraffin embedded tumor tissues or peritumor tissues were cut into 4-μm-thick sections. The slide was heated by microwave in 0.01 M citrate buffer (pH = 6.0) for 10 minutes to recover the antigen. Subsequently, the sections were incubated with primary antibodies [anti-TCN1 rabbit antibody (ab202121, Abcam), anti-ITGB4 rabbit antibody (ab182120, Abcam), anti-Ki-67 antibody (ab16667, Abcam), and anti-PCNA antibody (ab92552, Abcam)] in a humidified chamber overnight at 4°C. Horseradish peroxidase (HRP)-labeled secondary antibodies were incubated at room temperature for 1 h. All colorectal cancer sections were examined by two experienced pathologists. TCN1 or ITGB4 staining was independently scored by two pathologists who did not know the clinical data using the H-score system.[27] The intensity of immunostaining was 0–3:0, negative; 1. Weak; 2. Medium; 3. Strong. H score was the product of different staining intensities in 0-3 and the percentage of positive cells.

Q-PCR

Total RNA was extracted from snap-frozen CRC tumor tissues and paired noncancerous tissues using TRIzol Reagent (B5704-1, Takara), followed by treatment with DNase I (2212, Takara). After the RNA concentration was determined by spectrophotometer (NanoDrop 2000c, Thermo Scientific), the cDNA was synthesized using PrimeScriptTM RT reagent Kit (RR037A, Takara). Q-PCR was performed using the Light Cycler PCR QC Kit (6746381001, Roche) and a 7300 Real-Time PCR System (LC96, Roche). Human TCN1- and GAPDH-specific primers (TCN1-F, 5’- AGA TCA AAG CAG ATG AAG GCA GTT T -3’ and TCN1-R, 5’- TCC GTG AGC ACT GTA TTC AGA GTT -3’ for amplifying 208 bp; GAPDH-F, 5’-ACG GAT TTG GTC GTA TTG GG-3’ and GAPDH-R, 5’-CGC TCC TGG AAG ATG GTG AT-3’ for amplifying 214 bp) (Genesprit Biotechnology) were used. The relative expression level of the target genes was normalized to that of the GAPDH and calculated by the ΔCT method [ΔCT = (mean CT−mean CT GAPDH)] [28]. Data analysis was performed using GraphPad Prism 8 software.

Survival Analysis

Survival analysis was performed on 80 patients, and the survival curves were drawn according to the Kaplan-Meier method. Patients were divided into TCN1 and ITGB4 high expression group (ΔCt ≤4.25) and low expression group (ΔCt > 4.25) according to the median levels of TCN1 and ITGB4 in cancer tissues. Overall survival (OS) was the time from surgery to patient’s death. Follow-up was for 60 months, although patients who survived beyond 5 years were still followed up.

Generation Of Stable Cell Lines Using Lentivirus Infection

To generate a lentivirus expressing short hairpin RNA (shRNA) targeting human TCN1 (GenBank Accession No. NM_001062.4), the shTCN1 sequences listed in Supplemental Table 1 were designed and synthesized. HEK293T cells were co-transfected with lentiviral expression constructs (4 μg), viral envelope plasmid (pMD2.G, 4 μg), and viral packaging plasmid (psPAX2, 4 μg) using Lipofectamine 2000 (Invitrogen). The empty vector was used as a shRNA control (TCN1-KDC). The expression constructs (TCN1-KD1, TCN1-KD2, and TCN1-KDC) plasmid maps are listed in Supplemental Figure 1A, B. After 72 h of transfection, the virus supernatant was collected and purified by 0.45 μm filters. The mCherry-expressing lentiviruses, including LV-TCN1-KD1, LV-TCN1-KD2, and LV-TCN1-KDC, were then concentrated by ultracentrifugation (20,000 rpm) for 2 h.

The full-length coding sequence (CDS) of human TCN1 was cloned into pLenti-CMV-PuroR-mCherry lentiviral plasmid (CAVR Gene, Zhenjiang, China) to construct the recombinant TCN1-overexpression lentivirus (TCN1-OE). The empty vector was used as a shRNA control (TCN1-OEC). HCT116 cells were infected with LV-TCN1-KD1, LV-TCN1-KD2, LV-TCN1-KDC, LV-TCN1-OEC and LV-TCN1-OE plus 8 μg/ml polybrene. 24 h after lentiviral infection, the cells were selected using puromycin (2 μg/ml, Sigma) prior to use in experiments. The primer sequences for TCN1 cloning are listed in Supplemental Table 1. The TCN1-OE plasmid maps are listed in Supplemental Figure 1C, D.

Cell Apoptosis Detection

An annexin V-fluorescein isothiocyanate (FITC) detection kit (556547; BD Biosciences) was used to detect the apoptosis rate of HCT116 cells. Briefly, the cells were washed twice with cold phosphate-buffered saline (PBS) and then resuspended in 500 μL of binding buffer containing 5 μL Annexin V-FITC antibody and 5 μL Propidium Iodide and incubated for 15 min at room temperature in the dark. Samples were analyzed using flow cytometry (BD FACSCalibur; BD Biosciences).

Transwell Invasion Assays

For cell migration assays, 1×10^5 cells were seeded in the upper chamber of Transwell apparatus (Corning Costa) in serum-free medium, and 10% FBS medium was added in the lower chamber. After 24 h, the cells on the upper surface that did not pass through the 8-μm pore-size polycarbonate filter were removed using a moistened cotton swab; the cells migrating to the lower membrane surface were fixed in 100% methanol for 10 min, stained with 0.4% crystal violet for 15 min, and counted under a microscope (IX-71, Olympus).

Cell Viability

Cell viability was detected at 0 h, 12 h, 24 h, 36 h, 48 h, and 60 h using Cell Counting Kit-8 (CCK-8) reagent (HY-K0301, MedChemExpress). Briefly, the cells were washed twice with PBS. Then, 100 μL RPMI 1640

medium and 10 μL CCK-8 solution were added to each well. The cells were incubated for 1.5 h at 37°C, and the absorption value was detected at 450 nm. All experiments were repeated three times.

Immunofluorescence Staining

HCT116 cells were fixed with 4% formaldehyde for 15 min at room temperature. After washing the cells 3 times in PBS with 0.1% Triton X-100, cells were blocked with 4% BSA for 30 min. Cells were incubated with primary ITGB4 antibody (ab182858, Abcam), FLNA antibody (4762, Cell Signaling Technology), FITC-phalloidin antibody (P5282, Sigma) and PLEC antibody (ab11220, Abcam) for 1 h at room temperature. Following washes with PBS, 0.1% Triton X-100, cells were incubated with FITC/TRITC-conjugated secondary antibodies for 1 h at room temperature. Following washes with PBS, 0.1% Triton X-100, cells were stained with DAPI (Sigma) and visualized by confocal scanning microscopy (710, Zeiss).

Transmission Electron Microscopy Assays

The cells were fixed in 2.5% glutaraldehyde (pH 7.4) at 4 ℃ for 48 h, and then in 0.5% osmium tetroxide for 24 h. After dehydration, the samples were embedded in epoxy resin to make ultrathin sections (70-nm-thick) and examined by transmission electron microscope (Tecnai G2 Spirit Bio TWIN; FEI).

Rna-sequence Analysis

Total RNA was extracted from TCN1-KD1, TCN1-KD2, TCN1-KDC, TCN1-OE and TCN1-OEC HCT116 cell lines. RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit RNA Assay Kit in Qubit 2.0 Flurometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 2 μg RNA per sample was used as input material for the RNA sample preparations. PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 4000 platform and paired-end 150 bp reads were generated. The genes with a fold change value greater than 2, and a p-value < 0.01 were considered differentially expressed. Relationships of differently expressed genes were determined by GO and GSEA analysis.

Chromatin Immunoprecipitation (Chip) Assays

ChIP assays were performed as described previously [29]. The protein–DNA complexes were immunoprecipitated using anti-TCN1 antibody (ab202121, Abcam), anti-ITGB4 antibody (ab182120, Abcam) or immunoglobulin G (IgG, ab172730, Abcam), which served as a control. ChIP samples were analyzed by quantitative real-time PCR. The primer sequences for Chips are listed in Table S1.

Establishment Of Tumor Xenografts In Nude Mice

All animal experimental procedures were approved by the Animal Care and Use Committee of Nanjing Medical University. Thirty BALB/c nude mice (4 weeks old, male) were randomly divided into five groups (TCN1-KDC, TCN1-KD1, TCN1-KD2, TCN1-OEC and TCN1-OE) and housed at 22°C on a 12/12 h light/dark cycle and freely received standard mouse chow and tap water. A total of 2.5×10^6 cells were inoculated into the mammary fat pads of mice. The growth of the primary tumor was measured with a caliper every week for 6 weeks. The tumor volume was calculated by multiplying the length by the square of the width.

Western Blot Analysis

The cells and cancer tissues were lysed in RIPA lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (Bioteke Company). Protein samples (50 μg) were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. TBST buffer (100 mM NaCl, 10 mm Tris HCl, and 0.1% Tween 20) containing 5% skim milk was used to block the membrane at room temperature for 1 h. Then, the membrane was incubated with primary antibodies against TCN1 (ab202121, Abcam), ITGB4 (ab182120, Abcam), FLNA (4762, Cell Signaling Technology) and GAPDH (sc-166574, Santa Cruz Biotechnology) at 4°C overnight. On the second day, an HRP-conjugated goat anti-rabbit IgG secondary antibody (sc-2004, Santa Cruz Biotechnology, Inc.) or goat anti-mouse IgG HRP binding secondary antibody (sc-2005, Santa Cruz Biotechnology, Inc.) was incubated with the membrane for 1 h at room temperature. Then, the membrane was rinsed three times with TBST. Western blot analyses were performed with Pierce ECL Western Blotting Substrate (32209, Thermo Fisher Scientific) and a ChemiDoc XRS$^+$ molecular imager (Bio-Rad), and quantitative analysis was performed with ImageJ software.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad 8.0 statistical software (GraphPad Software). One way ANOVA was used to compare the differences among the groups. Univariate survival analysis was performed using the Kaplan-Meier method. Survival curves were compared by log-rank test. $P<0.05$ was defined as statistically significant difference.

Results

TCN1 and ITGB4 are overexpressed in CRC and associated with poor prognosis

To investigate the clinical significance of TCN1 and ITGB4 expression in colorectal cancer patients, we detected the protein expression of TCN1 and ITGB4 in colorectal cancer specimens and adjacent tissues. IHC and Western blot analysis showed significant upregulation of TCN1 and ITGB4 expression in human

CRC tissues compared to normal tissues (P<0.01, Fig. 1a, b). Next, we analyzed TCN1 and ITGB4 mRNA expression in CRC specimens. We found that the transcription levels of TCN1 and ITGB4 were significantly higher in CRC tissues than in paired peritumor tissues (Fig. 1c, d). Kaplan Meier survival analysis showed that patients with high expression of TCN1 and ITGB4 had shorter overall survival than other CRC patients (Fig. 1e, f). Notably, the expression of TCN1 and ITGB4 correlated well across all CRC samples analyzed (Fig. 1g). These results indicate that TCN1 and ITGB4 expression levels are upregulated in human CRC tissues and correlate with a poor prognosis in CRC patients, indicating that TCN1 may promote cell proliferation in conjunction with ITGB4 during CRC progression.

Lentivirus-mediated Tcn1 Knockdown And Overexpression In Crc Cells

We analyzed lentivirus mediated TCN1-knockout or overexpression in HCT116 CRC cells using Q-PCR and Western blot (Fig. 2a, b, and Fig. 3c). We found that the expression of TCN1 and ITGB4 in TCN1-knockdown (TCN1-KD) cells was reduced to 20% of the negative control (TCN1-KDC) cells, and TCN1 expression in cells with TCN1 overexpression (TCN1-OE cells) was 10 times higher than that in cells transfected with the scrambled negative control (TCN1-OEC cells) (Fig. 2a, b). The protein levels of TCN1 were confirmed by Western blot analysis (Fig. 3c). Fluorescence microscope analysis showed that almost all HCT116 cells transfected with lentivirus expressing TCN1 shRNA or TCN1 and corresponding control HCT116 cells exhibited mCherry expression (Additional file 1: Fig. 1e). Our results confirmed the successful generation of lentivirus-mediated TCN1-silenced HCT116 cell lines (TCN1-KD1 and TCN1-KD2) and a TCN1-overexpressing HCT116 CRC cell line (TCN1-OE). The effects of TCN1 on apoptosis were assessed by flow cytometric analysis (Fig. 2c, d). Annexin V/PI staining revealed that apoptosis in TCN1-KD1 and TCN1-KD2 cells was significantly increased compared with that in TCN1-KDC cells (P<0.01). Moreover, transwell assays showed the migration ability of HCT116 cells was also significantly decreased in TCN1-KD1 and TCN1-KD2 cells compared with TCN1-KDC cells (P<0.01) and were markedly increased in TCN1-OE cells compared with TCN1-OEC cells (P<0.05, Fig. 2e, f). The effect of TCN1 on human CRC cell proliferation was assessed by CCK-8 assay (Fig. 2g). As shown in Fig. 2g, a significant decrease in cell viability was detected in TCN1-KD1 and TCN1-KD2 cells compared with TCN1-KDC cells at each time point. In contrast, the viability of TCN1-OE cells was markedly increased compared with that of TCN1-OEC cells. These data indicated that knockdown of TCN1 inhibits CRC cell proliferation in vitro.

ITGB4 is a direct downstream transcriptional target of TCN1 in CRC cells

The results above indicated that TCN1 can promote the proliferation of CRC cells. To identify their potential downstream targets and understand their mechanism of action, we performed RNA-sequence assay using mRNA from cells of TCN1-KD1, TCN1-KD2, TCN1-KDC, TCN1-OE and TCN1-OEC (Fig. 3a). Gene set enrichment analysis (GSEA) showed that cell proliferation, cell migration and Wnt signaling gene were enriched in TCN1-KD1 and TCN1-KD2 cells compared to the control (Additional file 1: Fig. S2a-c). Consistently, Gene Ontology (GO) analysis of differentially expressed genes in TCN1 knockdown cells

and control cells showed that several key cellular processes related to cancer progression, such as Wnt signaling, Notch signaling, cell division, cell migration and proliferation, were significantly enriched (Fig. 3b).

From gene profile data, we found a total of 77 downregulated genes in TCN1-KD1 and 156 downregulated genes in TCN1-KD2 versus NC controls with at least a 2-fold change, whereas there were 41 upregulated genes in TCN1-OE versus NC controls with at least a 2-fold change. There were 11 common genes which exhibited at least 2-fold downregulation in all knockdown and 2-fold upregulation in all overexpression profiles (Fig. 3c). Since ITGB4 is a key factor in the Notch signaling pathway, the expression of TCN1 and ITGB4 was consistent in clinical samples and HCT116 cell lines (Fig. 1a, b and Fig. 2a, b), and genes transcriptional analysis showed that ITGB4 was among the 11 overlapping gene list (Fig. 3c), we chose ITGB4 for further investigation as potential downstream target gene of TCN1. We performed ChIP analysis using anti-TCN1 antibodies on the promoter of ITGB4 to determine whether TCN1 directly regulates ITGB4 transcription (Fig. 3d). We analyzed ITGB4-antibody immunoprecipitate from TCN1-knockdown HCT116 cells by immunoblot and found that ITGB4 co-immunoprecipitated with TCN1(Fig. 3d), and knockdown of TCN1 led to a significant reduction in TCN1 enrichment on the promoter of ITGB4 in HCT116 cells (P<0.01, Fig. 3d), suggesting that TCN1 bound to the promoter and directly regulated the transcription of ITGB4. Moreover, knockdown of TCN1 in HCT116 cells led to significantly decreased expression of ITGB4 and FLNA, and overexpression of TCN1 in HCT116 cells results in markedly increased expression of ITGB4 and FLNA at the transcriptional and protein levels (Fig. 2a, b and Fig. 3e).

Tcn1 Deficiency Causes Cytoskeletal Network Damage

The binding of integrin to the cytoskeleton is essential for the stable adhesion of integrin to the ECM [30]. PLEC and ITGB4 are hemidesmosomes, which play an important role in maintaining the integrity of the cytoskeleton [31]. Phalloidin is a cyclic peptide produced by *Amanita phalloides* that can bind to and stabilize F-actin [32]. FITC fluorescent substance-labeled phalloidins can specifically bind to F-actin in eukaryotic cells, thus indicating the distribution of microfilaments in the cytoskeleton of cells [33]. Thus, we analyzed the distribution of F-actin in TCN1 knockdown and TCN1-overexpression cells using FITC-phalloidin staining. In TCN1-KDC cells, the fluorescence signals of ITGB4, PLEC, and phalloidin were clustered and completely colocalized (Fig. 4a and Additional file 1: Fig. S3a, b). In TCN1-KD1 and TCN1-KD2 cells, the fluorescence signal of ITGB4 was weak, while PLEC and phalloidin exhibited a diffuse filamentous distribution. However, the fluorescence signals of ITGB4, PLEC, and phalloidin in TCN1-OE cells were stronger than those in TCN1-OEC cells. These results suggest that the degradation of ITGB4 induced by TCN1 inactivation may directly lead to the structural damage of hemidesmosomes.

Similar to ITGB4, TCN1 knockdown also decreased the level of FLNA in HCT116 cells (Fig. 4b). Immunofluorescence showed that FLNA signal almost disappeared in TCN1 knockdown cells, indicating that TCN1 may regulate the stability of FLNA. However, the fluorescence signal of FLNA in TCN1-OE cells

was stronger than that in TCN1-OEC cells. FLNA can bind F-actin filaments to form a stable cytoskeleton [34]. These results provide evidence that TCN1 knockdown affects cytoskeletal reconstruction.

Transmission electron microscope analysis showed that actin filaments were cross-linked in the cytoplasm, and a dense network structure in the cytoplasm and basal layer of control cells (Fig. 4c). These three-dimensional structures have high strength and can support the extension of pseudopodia. In TCN1-knockdown cells, the three-dimensional network structure of actin filaments is destroyed, resulting in the formation of long and straight filaments. These parallel microfilaments can affect the formation of pseudopodia and lead to the destruction of the cytoskeleton [33]. The results demonstrated that TCN1-knockdown may cause damage to the cytoskeletal network by regulating ITGB4 signaling.

Inactivation of TCN1 inhibits tumorigenesis in a colorectal cancer xenograft model

We established a nude mouse subcutaneous xenograft model using TCN1-KDC, TCN1-OEC, TCN1-KD, TCN1-KD2 and TCN1-OE cells to further evaluate the in vivo tumorigenic effect of TCN1 knockdown and TCN1-overexpressing cells (Fig. 5a). Consistent with the in vitro results, Bioluminescence imaging showed TCN1-knockdown cells produced a significant decrease, and TCN1-overexpression cells produced a marked increase in the tumor size compared with the control group (Fig. 5a-c). Meanwhile, the tumor volume was more markedly decreased in the TCN1-KD and TCN1-KD2 groups than in the TCN1-KDC group (P<0.01, Fig. 5d, e); The tumor volume in the TCN1-OE group was substantially increased compared with the TCN1-OEC group (P<0.01, Fig. 5d, f).

The TCN1 and ITGB4 expression levels in xenograft tumors were evaluated using Q-PCR and Western blotting (Fig. 5g, h). TCN1 and ITGB4 expression was more markedly decreased in the TCN1-KD1 and TCN1-KD2 groups than in the TCN1-KDC group (P<0.01) and was substantially increased in TCN1-OE cells compared with TCN1-OEC cells (P<0.01, Fig. 5g, h). The expression levels of the proliferation-related proteins Ki-67 and PCNA in xenograft tumors were immunohistochemically evaluated (Fig. 5i). Ki-67 and PCNA expression decreased more drastically in the TCN1-KD1 and TCN1-KD2 groups than in the TCN1-KDC group and was increased in the TCN1-OE group compared with the TCN1-OEC group. The results were similar to the in vitro results and further revealed that TCN1 knockdown inhibit tumorigenesis in vivo, indicating TCN1 knockdown synergized with ITGB4-induced inactivation of Ki-67 and PCNA in CRC cells.

TCN1 deficiency inhibits metastatic engraftment in the peritoneum in vivo

Since TCN1 deficiency impairs the adhesion and growth of CRC cells, we assessed the effect of TCN1 knockdown and TCN1 overexpression on the metastasis ability of HCT116 cells in vivo (Fig. 6). Bioluminescence imaging showed that most of the metastatic foci were found in the peritoneal cavity in mice (Fig. 6a). The TCN1-KD1 and TCN1-KD2 groups had few tumor nodules. The area of metastatic foci was decreased more in the TCN1-KD1 and TCN1-KD2 groups than in the TCN1-KDC group and was increased in the TCN1-OE group compared with the TCN1-OEC group (Fig. 6b).

TCN1 and ITGB4 expression was detected in metastatic foci in the peritoneal cavity using IHC (Fig. 6c). TCN1 and ITGB4 expression was decreased more in the TCN1-KD1 and TCN1-KD2 groups than in the TCN1-KDC group, and was increased in the TCN1-OE group compared with the TCN1-OEC group. These results suggest that TCN1 deficiency inhibits the metastasis and implantation of colorectal cancer cells in vivo after intravenous injection.

Discussion

CRC is a pathological tumor in the colon or rectum, which may invade and spread to distant organs [35]. Most CRC patients are elderly or have an unhealthy lifestyle, and only a small number of cases are caused by genetic diseases [36, 37]. Despite significant progress in surgery and treatment, the long-term survival rate is still unsatisfactory, mainly because CRC is often diagnosed at an advanced stage [38, 39]. At present, the diagnosis, recurrence, and metastasis of CRC mainly rely on colonoscopy and other imaging examinations, which are often delayed. Therefore, it is urgent to find new sensitive biomarkers to ensure early diagnosis and timely treatment of colorectal cancer, and even predict the occurrence of CRC.

Increasing evidence has shown that TCN1 is highly expressed in metastatic epithelial tumors such as breast cancer, thyroid cancer, laryngeal cancer, and cervical cancer [9, 11, 12, 14, 40, 41]. Bioinformatics analysis and meta-data analysis based on the COAD database showed that TCN1 was overexpressed as an oncogene in colorectal cancer [15]. The NGS results showed that TCN1 was the second most upregulated mRNA in colorectal cancer [16]. The results provide more evidence for the role of TCN1 in colon carcinogenesis, suggesting that TCN1 may be a potential new gene biomarker. This study found that the high expression of TCN1 and ITGB4 is positively correlated with the poor prognosis of CRC, suggesting that TCN1 may positively regulate the expression of ITGB4 and promote the development of CRC. TCN1 gene knockdown promoted the apoptosis of colorectal cancer cells and inhibited the proliferation and invasion of colorectal cancer cells.

Although it has been reported that TCN1 regulates malignant cell metastasis and glycolysis [11, 12, 14], the mechanism by which TCN1 promotes tumor development is still unclear. To reveal the potential mechanism of TCN1 in colorectal cancer, we selected genes related to TCN1 to determine its mode of action. Because TCN1 has the role of an oncogene [15], we selected genes positively related to TCN1 for GO and GSEA. The results showed that TCN1 was related to the Wnt signaling pathway, Notch signaling pathway, and tumor-related genes involved in cell division, migration, or proliferation. More importantly, our results show that TCN1 interacts directly with the ITGB4 promoter, which complements our understanding of the relationship between TCN1 and integrins. TCN1 deficiency can downregulate ITGB4 signaling and cause damage to the cytoskeletal network. The evidence suggests that TCN1 affects tumorigenesis at least partly by regulating the expression of ITGB4, while overexpression of ITGB4 upregulate the Notch signaling pathway [42]. According to these results, we speculate that integrins, including ITGB4, may be TCN1 receptors on the cell membrane that can promote cell signal transduction, but this needs further study.

ITGB4 is one of the most characteristic integrins and is involved in regulating a variety of cell functions [43]. Integrins affects the migration, invasion, proliferation, and survival of tumor cells and regulates the angiogenesis, connective tissue proliferation, and immune response of tumor host cells, thereby affecting EMT, cancer development, metastasis, and even treatment results [44, 45]. In this study, through the analysis of patient samples and clinical data, we found that the expression level of ITGB4 was upregulated in CRC tissues, which was associated with poor prognosis of colorectal cancer and was positively correlated with TCN1. The overexpression of ITGB4 is also associated with aggressive behavior and poor prognosis in breast cancer, bladder cancer, cervical cancer, head and neck cancer, lung cancer, and pancreatic cancer [46, 47].

Integrins are also the main adhesion molecules connecting the ECM and cytoskeleton [48]. It is reported that ITGB4 interacts with the extracellular matrix and cytoskeleton and plays an important role in many physiological processes such as cell proliferation, carcinogenesis, and immune response [49, 50]. The main structure of hemidesmosomes is composed of PLEC and ITGB4 [51]. Under normal physiological conditions, TCN1 may bind to the ITGB4-PLEC complex to maintain the three-dimensional filamentous structure of hemidesmosomes. Our study showed that TCN1 knockdown decreased the expression of ITGB4 and FLNA. The decrease in ITGB4 protein levels led to the degradation of the ITGB4-PLEC complex. In addition, the decrease in FLNA prevented F-actin from forming vertical branches. These factors lead to the destruction of three-dimensional structure of cytoskeleton microfilaments and cytoskeleton network structure [52].

Metastasis is a complex biological cascade in which tumor cells invade the local environment, migrate to distant tissues, and finally colonize [53, 54]. Upregulation of ITGB4 expression is positively correlated with colorectal cancer progression [55] and promotes EMT in prostate cancer [56]. EMT promotes tumor metastasis by promoting the invasion of epithelial malignant cells [57]. In the process of EMT, cells lose adhesion properties and undergo polarity changes accompanied by the reorganization of the cytoskeleton and the upregulation of extracellular matrix components, which eventually facilitate migration and invasion [58]. Therefore, inhibiting the occurrence of EMT has become a research hotspot in the treatment of colorectal cancer metastasis. GSEA showed that ITGB4 was significantly involved in the focal adhesion signaling pathway [59]. The overexpression of ITGB4 was significantly correlated with the upregulation of focal adhesion-associated genes [42]. Focal adhesion and metastasis are the key to cell migration and invasion [60]. Focal adhesion signaling pathway plays an important role in EMT of prostate cancer [61], and ITGB4 mediates the activation of the focal adhesion signaling pathway in ovarian cancer [62] and hepatocellular carcinoma [63]. In addition, it has been reported that ITGB4 and the focal adhesion signaling pathway are involved in the development of colorectal cancer [64, 65]. Similarly, our study showed that TCN1 inhibited the expression of FLNA, F-actin, and PLEC by inhibiting the ITGB4 pathway.

Only cells with adhesion, migration, invasion, and proliferation abilities can form new tumor lesions [59], and ITGB4 mediates the invasion and migration of gastric cancer cells [66]. The results showed that TCN1 interacts with ITGB4, and the level of ITGB4 decreased significantly in TCN1-deficient cells.

Therefore, this effect will lead to the decrease of adhesion, survival, and proliferation of TCN1 knockdown cells. Naturally, circulating tumor cells in the blood may lose the ability to adhere to the vascular endothelium and thus lose the ability to migrate into new tissues and eventually form metastatic colonies. These results provide a new idea for targeting the TCN1/ITGB4 signaling pathway in the treatment of colorectal cancer metastasis, but further research is still needed.

Conclusion

Our data revealed that TCN1 was significantly overexpressed in CRC tissues and correlated with the pathological features of advanced CRC. TCN1 deficiency causes cytoskeletal network damage, and inhibits cell division, cell migration, and proliferation by regulating ITGB4 signal pathway. Collectively, TCN1 might be a therapeutic target and prognostic marker for the individualized treatment of colorectal cancer.

Abbreviations

CRC : colorectal cancer; TCN1: Transcobalamin 1 (); NGS: Next generation sequencing; ECM: extracellular matrix; EMT: epithelial-mesenchymal transition; ITGB4: Integrin subunit β4; PLEC: plectin; IHC: immunohistochemistry; shRNAs: short hairpin RNAs; SDS-PAGE: sodium dodecyl sulfatepolyacrylamide gel electrophoresis; TBST: tris-buffered saline tween-20; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; CCK-8: Cell Counting Kit-8; OD: optical density; SPF: specific pathogen free; ANOVA: one-way analysis of variance;

Declarations

Authors' Contributions

XQZ, XTJ and QLZ contributed to designing and performing the study; HLH. and XHS contributed to collecting clinical tissues and analyzing the data. DRH contributed to writing the manuscript; CGX contributed to revising the paper and is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. This manuscript was approved by all authors.

Author details

[1] Department of General Surgery, The Second Affiliated Hospital of Soochow University, No.1055 Sanxiang Road, Suzhou 215004, China. [2] Department of General Surgery, The Affiliated Suqian Hospital of Xuzhou Medical University, No.138 Huanghe South Road, Suqian 223800, China. [3] Departments of Gastroenterology, Wuxi People's Hospital Affiliated to Nanjing Medical University, No.299 Qingyang Road, Wuxi 214023, China. [4] Department of Pathology, The Affiliated Suqian Hospital of Xuzhou Medical University,

No.138 Huanghe South Road, Suqian 223800, China. [5] Key Laboratory of Model Animal Research, Nanjing Medical University, No.101 longmian Avenue, Jiangning District, Nanjing 211166, China.

Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Ethics Review Committee of Second Affiliated Hospital of Soochow University (Suzhou, China). The animal study followed the Guidelines for the Animal Care and Use approved by Second Affiliated Hospital of Soochow University.

Funding

This work was supported by Suqian Science and Technology Innovation Special Project (No. S201913).

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, CA, 2018;68(1):7–30.
2. Kawaguchi N, Tashiro K, Taniguchi K, et al. Nogo-B (Reticulon-4B) functions as a negative regulator of the apoptotic pathway through the interaction with c-FLIP in colorectal cancer cells. Biochim Biophys Acta. 2018;864(8):2600–2609.
3. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394–424.
4. Chen WQ, Zheng RS, Baade PD, et al. Cancer statistics in China, 2015. CA Cancer J Clin. 2016;66(2):115–132.
5. Wei W, Zeng H, Zheng R, et al. Cancer registration in China and its role in cancer prevention and control. Lancet Oncol. 2020;21(7):342–349.
6. Lee TY, Liu CL, Chang YC, et al. Increased chemoresistance via Snail-Raf kinase inhibitor protein signaling in colorectal cancer in response to a nicotine derivative. Oncotarget. 2016;7(17):23512–23520.
7. Deng WW, Hu Q, Liu ZR, et al. KDM4B promotes DNA damage response via STAT3 signaling and is a target of CREB in colorectal cancer cells. Mol Cell Biochem. 2018; 449(1–2):81–90.
8. Wang Y, Yue CL, Fang JG, et al. Transcobalamin I: a novel prognostic biomarker of neoadjuvant chemotherapy in locally advanced hypopharyngeal squamous cell cancers. Onco Targets Ther. 2018;11:4253–4261.

9. Chong LY, Cheok PY, Tan WJ, et al. Keratin 15, transcobalamin i and homeobox gene Hox-B13 expression in breast phyllodes tumors: Novel markers in biological classification. Breast Cancer Res Treat. 2012;132(1):143–151.
10. Allen LH, Miller JW, de Groot L, et al. Biomarkers of Nutrition for Development (BOND): Vitamin B-12 Review. J Nutr. 2018;148:1995–2027.
11. Waibel R, Treichler H, Schaefer NG, et al. New derivatives of vitamin b12 show preferential targeting of tumors. Cancer Res. 2008;68(8):2904–2911.
12. Lyon P, Strippoli V, Fang B, et al. B Vitamins and One-Carbon Metabolism: Implications in Human Health and Disease. Nutrients. 2020;12(9):2867.
13. Martinelli M, Luca Scapoli L, Mattei G, et al. A Candidate gene study of one-carbon metabolism pathway genes and colorectal cancer risk. Br J Nutr. 2013;109(6):984–989.
14. Lederer AK, Hannibal L, Hettich M, et al. Vitamin B12 Status Upon Short-Term Intervention with a Vegan Diet-A Randomized Controlled Trial in Healthy Participants. Nutrients. 2019;11(11):2815.
15. Chu CM, Yao CT, Chang YT, et al. Gene expression profiling of colorectal tumors and normal mucosa by microarrays meta-analysis using prediction analysis of microarray, artificial neural network, classification, and regression trees. Dis Markers. 2014;2014:634123.
16. Li M, Zhao LM, Li SL, et al. Differentially expressed lncRNAs and mRNAs identified by NGS analysis in colorectal cancer patients. Cancer Med. 2018; 7:4650–4664.
17. Case LB, Waterman CM. Integration of actin dynamics and cell adhesion by a three-dimensional, mechanosensitive molecular clutch. Nat Cell Biol. 2015;17:955–963.
18. Ishizuka Y, Koshinaga T, Hirano T, et al. NRP1 knockdown promotes the migration and invasion of human neuroblastoma-derived SK–N–AS cells via the activation of β1 integrin expression. Int J Oncol. 2018;53:159–166.
19. Siddharth S, Nayak A, Das S, et al. The soluble nectin-4 ecto-domain promotes breast cancer induced angiogenesis via endothelial Integrin-β4. Int J Biochem Cell Biol. 2018;102:151–160.
20. Longmate W, DiPersio CM. Beyond adhesion: emerging roles for integrins in control of the tumor microenvironment. F1000Res. 2017;6:1612.
21. Hamidi H, Ivaska J. Every step of the way: integrins in cancer progression and metastasis. Nat Rev Cancer. 2018;19(3):553–548.
22. Vicente-Manzanares M, Sánchez-Madrid F. Targeting the integrin interactome in human disease. Curr Opin Cell Biol. 2018;55:17–23.
23. Raab-Westphal S, Marshall JF, Goodman SL. Integrins as therapeutic targets: successes and cancers. Cancers. 2017;9(9):110.
24. Li J, Luo M, Ou H, et al. Integrin β4 promotes invasion and anoikis resistance of papillary thyroid carcinoma and is consistently overexpressed in lymphovascular tumor thrombus. J Cancer. 2019;10(26):6635–6648.

25. Nagata M, Noman AA, Suzuki K, et al. TITGA3 and ITGB4 expression biomarkers estimate the risks of locoregional and hematogenous dissemination of oral squamous cell carcinoma. BMC Cancer. 2013;13:410.
26. Wang H, Rana S, Giese N, et al. Tspan8, CD44v6 and alpha6beta4 are biomarkers of migrating pancreatic cancer-initiating cells. Int J Cancer. 2013;133(2):416–426.
27. Blackhall FH, Peters S, Bubendorf L, et al. Prevalence and clinical outcomes for patients with ALK-positive resected stage I to III adenocarcinoma: results from the European Thoracic Oncology Platform Lungscape Project. J Clin Oncol. 2014;32(25):2780–2787.
28. Colla S, Tagliaferri S, Morandi F, et al. The new tumor-suppressor gene inhibitor of growth family member 4 (ING4) regulates the production of proangiogenic molecules by myeloma cells and suppresses hypoxia-inducible factor-1 alpha (HIF-1alpha) activity: involvement in myeloma-induced angiogenesis. Blood. 2007;110(13):4464–75.
29. Yang N, Gong F, Sun L, et al. Poly (ADP-ribose) polymerase-1 binds to BCL2 major breakpoint region and regulates BCL2 expression. J Cell Biochem. 2010;110(5):1208–18.
30. Wu D, Xu Y, Ding T, et al. Pairing of integrins with ECM proteins determines migrasome formation. Cell Res. 2017;27(11):1397–400.
31. Litjens SH, Koster J, Kuikman I, et al. Specificity of binding of the plectin actin-binding domain to beta4 integrin. Mol Biol Cell. 2003;14(10):4039–4050.
32. Pospich S, Merino F, Raunser S. Structural Effects and Functional Implications of Phalloidin and Jasplakinolide Binding to Actin Filaments. Structure. 2020;28(4):437–49.
33. Hong DB, Zhang X, Li RY, et al. Deletion of TMEM268 inhibits growth of gastric cancer cells by downregulating the ITGB4 signaling pathway. Cell Death & Differentiation. 2019;26(8):1453–166.
34. Wang W, Zuidema A, Te Molder L, et al. Hemidesmosomes modulate force generation via focal adhesions. J Cell Biol. 2020;219(2):e201904137.
35. Weinberg BA, Marshall JL, Salem ME. The growing challenge of young adults with colorectal cancer. Oncology. 2017;31(5):381–389.
36. O'Keefe SJ. Diet, microorganisms and their metabolites, and colorectal cancer. Nat Rev Gastroenterol Hepatol. 2016;13(12):691–706.
37. Durinikova E, Buzo K and Arena S. Preclinical models as patients' avatars for precision medicine in colorectal cancer: past and future challenges. J Exp Clin Cancer Res. 2021;40(1):185.
38. Heinemann V, von Weikersthal LF, Decker T, et al. FOLFIRI plus cetuximab versus folfiri plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial. Lancet Oncol. 2014;15(10):1065–1075.
39. Boyle T, Fritschi, L, Platell, C, et al. Lifestyle factors associated with survival after colorectal cancer diagnosis. J Br J Cancer. 2013;109(3):814–822.
40. Aboulhagag NA, El-Deek HEM, Sherif MF. Expression of galectin-1 and galectin-3 in renal cell carcinoma; immunohistochemical study. Ann Diagn Pathol, vol. 36, pp. 31–37, 2018.

41. Abdulrahman SS, Mohammad DN, Hamied MA, et al. Immunohistochemical evaluation of salivary gland tumors differentiation and proliferation by using calponin and telomerase. Saudi Dent J. 2019;31(1):105–114.
42. Zhuang HK, Zhou ZX, Ma ZY, et al. Characterization of the prognostic and oncologic values of ITGB superfamily members in pancreatic cancer. J Cell Mol Med. 2020;24(22):13481–1393.
43. Giancotti FG. Targeting integrin beta4 for cancer and antiangiogenic therapy. Trends Pharmacol Sci. 2007;28(10):506–511.
44. Wang L, Zhang X, Pang N, et al. Glycation of vitronectin inhibits EGF-induced angiogenesis by uncoupling VEGF receptor-2-alphavbeta3 integrin cross-talk. Cell Death Dis. 2015;6(6):1796.
45. Xiao T, Takagi J, Coller BS, et al. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. Nature. 2004;432(7013):59–67.
46. Zhang WG, Zhang BX, Vu T, et al. Molecular characterization of pro-metastatic functions of beta4-integrin in colorectal cancer. Oncotarget. 2017;8(54):92333–92345.
47. Zhong F, Lu HP, Chen G, et al. The clinical significance and potential molecular mechanism of integrin subunit beta 4 in laryngeal squamous cell carcinoma. Pathol Res Pract. 2020;216(2):152785.
48. Woo JA, Zhao X, Khan H, et al. Slingshot-Cofilin activation mediates mitochondrial and synaptic dysfunction via Abeta ligation to beta1-integrin conformers. Cell Death Differ. 2015;22(6):921–934.
49. Bianconi D, Unseld M, Prager GW. Integrins in the spotlight of cancer. Int J Mol Sci. 2016;17(12):2037.
50. Ginsberg MH. Integrin activation. BMB Rep. 2014;47(12):655–659.
51. Kadeer A, Maruyama T, Kajita M, et al. Plectin is a novel regulator for apical extrusion of RasV12-transformed cells. Sci Rep. 2017;10(7):44328.
52. Bouameur JE, Favre B, Fontao L, et al. Interaction of plectin with keratins 5 and 14: dependence on several plectin domains and keratin quaternary structure. J Invest Dermatol. 2014;134(11):2776–2783.
53. Ganguly KK, Pal S, Moulik S, et al. Integrins and metastasis. Cell Adh Migr. 2013;7(3):251–261.
54. Yoshioka T, Otero J, Chen Y, et al. β4 Integrin signaling induces expansion of prostate tumor progenitors. J Clin Invest. 2013;123(2):682–699.
55. Kröger C, Afeyan A, Mraz J, et al. Acquisition of a hybrid E/M state is essential for tumorigenicity of basal breast cancer cells. Proc Natl Acad Sci USA. 2019;116(15):7353–7362.
56. Masugi Y, Yamazaki K, Emoto K, et al. Upregulation of integrin beta4 promotes epithelial-mesenchymal transition and is a novel prognostic marker in pancreatic ductal adenocarcinoma. Lab Invest. 2015;95(3):308–319.
57. Yao BW, Li YZ, Wang L, et al. MicroRNA-3194-3p inhibits metastasis and epithelial–mesenchymal transition of hepatocellular carcinoma by decreasing Wnt/beta-catenin signaling through targeting BCL9. Artif Cells Nanomed Biotechnol. 2019;47(1):3885–3895.

58. Scheau C, Badarau IA, Costache R, et al. The role of matrix metalloproteinases in the epithelial–mesenchymal transition of hepatocellular carcinoma. Anal Cell Pathol. 2019;2019:9423907.
59. Gan L, Meng J, Xu M, et al. Extracellular matrix protein 1 promotes cell metastasis and glucose metabolism by inducing integrin β4/FAK/SOX2/HIF-1α signaling pathway in gastric cancer. Oncogene. 2018;37(6):744–755.
60. Kurenova E, Liao JQ, He DH, et al. The FAK scaffold inhibitor C4 disrupts FAK-VEGFR-3 signaling and inhibits pancreatic cancer growth. Oncotarget. 2013;4(10):1632–1646.
61. Ning Z, Wang A, Liang J, et al. USP22 promotes epithelial-mesenchymal transition via the FAK pathway in pancreatic cancer cells. Oncol Rep. 2014;32(4):1451–1458.
62. Wu A, Zhang S, Liu J, et al. Integrated Analysis of Prognostic and Immune Associated Integrin Family in Ovarian Cancer. Front Genet. 2020;11:705.
63. Li J, Hao N, Han J, et al. ZKSCAN3 drives tumor metastasis via integrin beta4/FAK/AKT mediated epithelial-mesenchymal transition in hepatocellular carcinoma. Cancer Cell Int. 2020;20:216.
64. Tai YL, Lai I, Peng YJ, et al. Activation of focal adhesion kinase through an interaction with beta4 integrin contributes to tumorigenicity of colorectal cancer. FEBS letters. 2016;590(12):1826–1837.
65. Jang B, Jung H, Choi S, et al. Syndecan-2 cytoplasmic domain up-regulates matrix metalloproteinase-7 expression via the protein kinase Cγ-mediated FAK/ERK signaling pathway in colorectal cancer. J Biol Chem. 2017;292(39):16321–16332.
66. Yang ZY, Jiang H, Qu Y, et al. Metallopanstimulin-1 regulates invasion and migration of gastric cancer cells partially through integrin β4. Carcinogenesis. 2013;34(12):2851–2860.

Figures

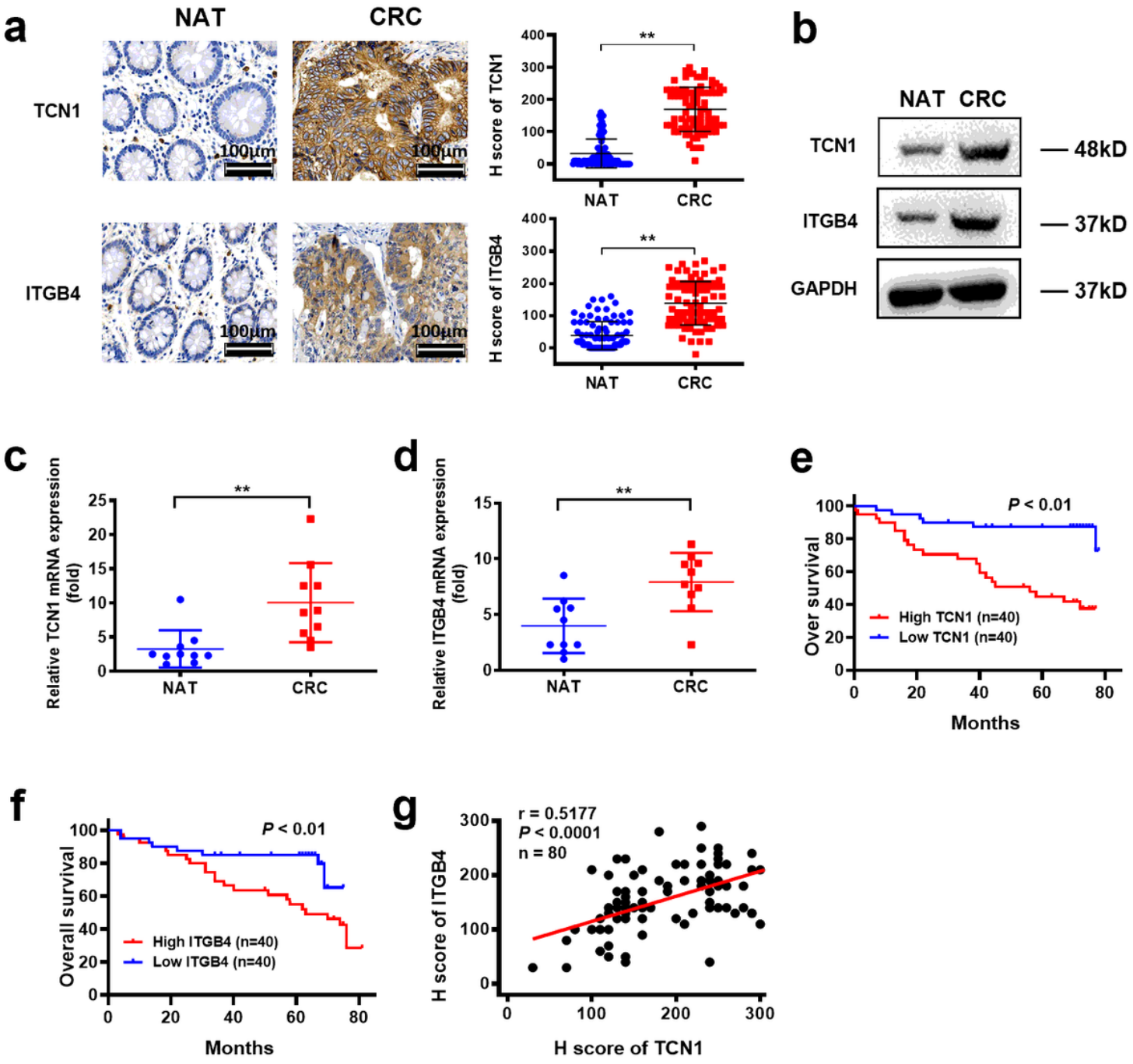

Figure 1

TCN1 and ITGB4 are highly expressed in CRC clinical tissues. a IHC analysis of TCN1 and ITGB4 in CRC tissue. Representative pictures of immunohistochemical staining are shown, n=6. CRC, colorectal cancer; and NAT, adjacent nontumor normal tissue. b Western blot analysis of TCN1 and ITGB4 in CRC tissue specimens, n=6. Representative pictures of Western blot analysis of TCN1 and ITGB4 in 6 pairs of CRC tissues and NATs derived from 6 CRC cases are shown. c-d Q-PCR analysis of TCN1 and ITGB4. RNA samples were extracted from 80 pairs of colorectal cancer tissues and normal tissues for real-time quantitative PCR analysis of TCN1 and ITGB4 expression. The levels of TCN1 and ITGB4 mRNA were expressed as −ΔCT =− (mean CT target−mean CT GAPDH). **, P<0.01 compared with NAT tissue. e

Kaplan-Meier survival curves for CRC patients grouped according to the median expression level of TCN1 indicated that patients with high TCN1 expression displayed a shorter overall survival time after surgery, n=80. f Kaplan-Meier survival curves for CRC patients grouped according to the median expression level of ITGB4 indicated that patients with high ITGB4 expression displayed a shorter overall survival time after surgery, n=80. g Pearson correlation scatter plot of TCN1 and ITGB4 H scores in human CRC, n = 80. All experimental data are presented as the mean ± SD. Statistical significance: ∗ P < 0.05 and ∗∗ P < 0.01.

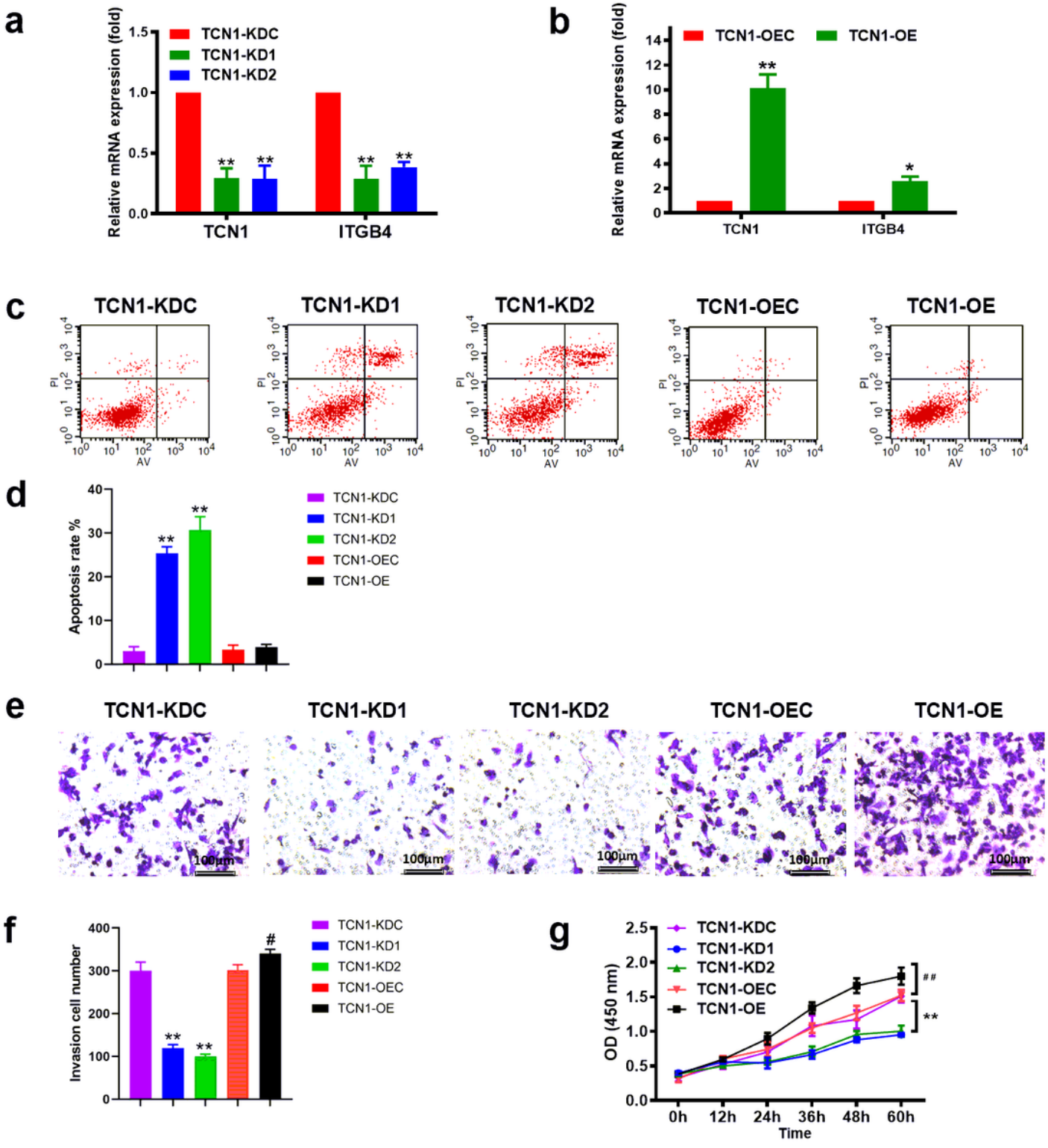

Figure 2

Lentivirus-mediated knockdown and overexpression of TCN1 in HCT116 cells. a, b Expression of TCN1 and ITGB4 in TCN1 knockdown and TCN1-overexpressing HCT116 cells assessed by Q-PCR, n=6. c, d Cell apoptosis was detected in TCN1 knockdown and TCN1-overexpressing HCT116 cells using flow cytometry analysis, as indicated, n=6. e, f Transwell invasion assays were conducted with TCN1 knockdown and TCN1-overexpressing HCT116 cells as indicated, n=6. The numbers of invaded cells are displayed in quantitative bar graphs. g Proliferation of HCT116 cells at 0, 12, 24, 36, 48, and 60 h following TCN1 knockdown and TCN1 expression, n=6. All experimental data are presented as the mean ± SD. Statistical significance: ∗ P < 0.05 and ∗∗ P < 0.01.

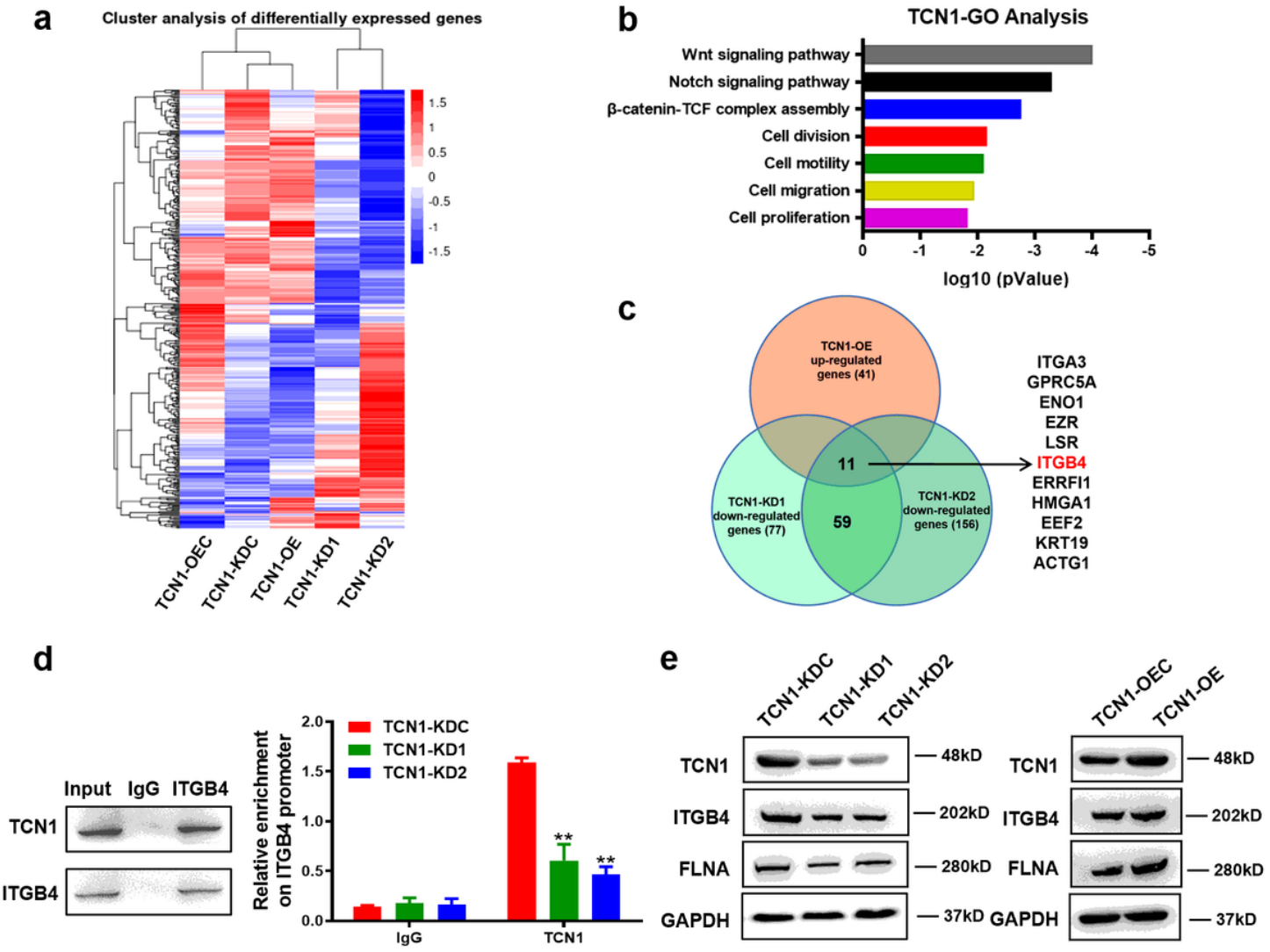

Figure 3

ITGB4 is a direct downstream transcriptional target of TCN1 in CRC cells. a Heat map showing differentially mRNA expression in TCN1 knockdown and TCN1-overexpressing cells (fold change value >1.5 and P < 0.01), as indicated. b GO analysis of the target genes of TCN1 arranged into functional groups. c Venn diagram illustrating the overlap of target genes identified by microarray analysis as being regulated by TCN1 and ITGB4 in HCT116 cells. d ChIP analysis of TCN1 binding to the ITGB4 promoter in TCN1-KDC and TCN1-KD HCT116 cells, n=6. e Expression of TCN1, ITGB4 and FLNA in TCN1 knockdown

and TCN1-overexpressing HCT116 cells assessed by Western blot analysis, n=6. GAPDH served as a loading control. All experimental data are presented as the mean ± SD. Statistical significance: ∗ P < 0.05 and ∗∗ P < 0.01.

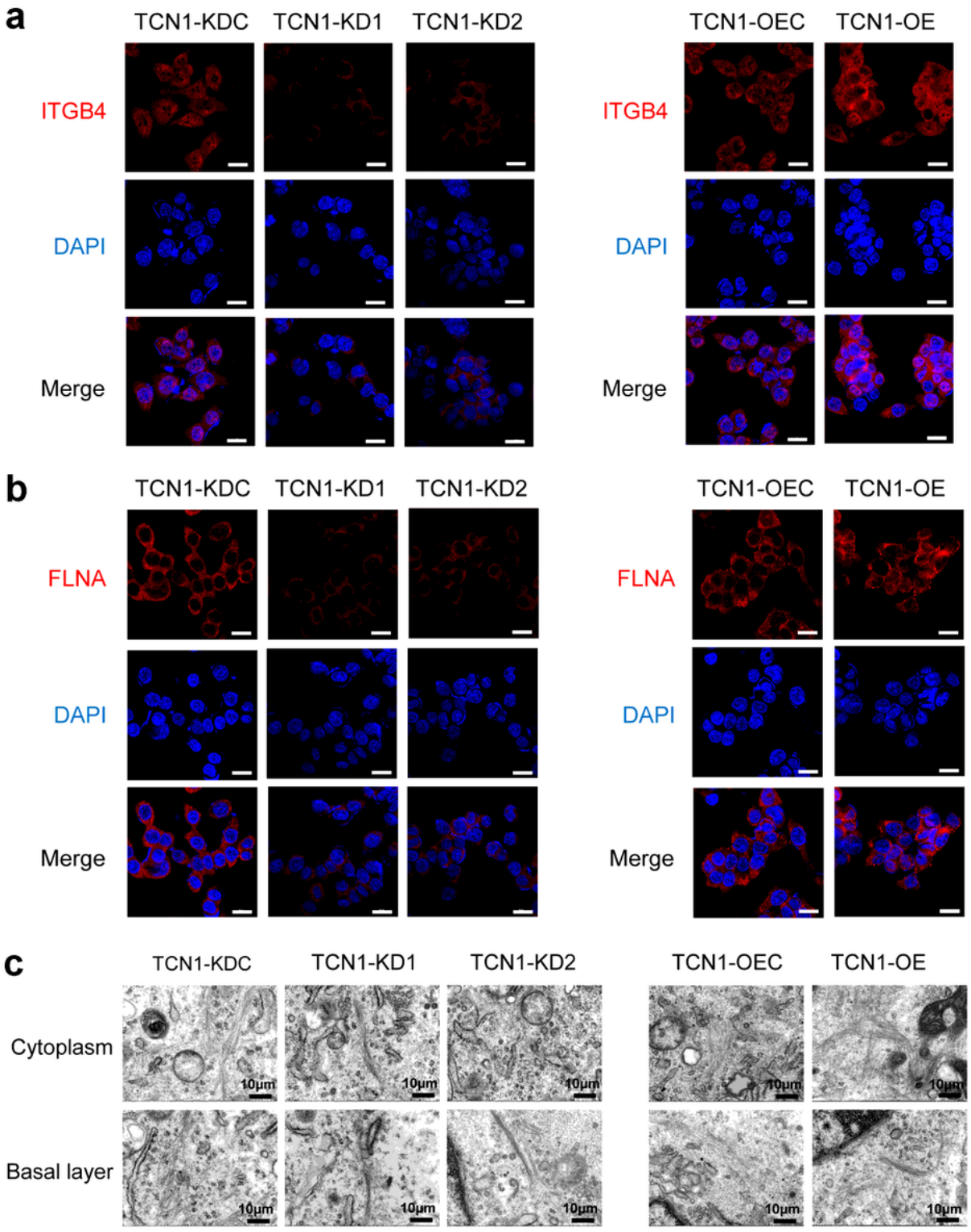

Figure 4

TCN1 deficiency causes cytoskeletal network damage. a, b Representative images of ITGB4 and FLNA in TCN1 knockdown and TCN1-overexpressing HCT116 cells detected by immunofluorescence staining,

n=6. Scale bars=100 μm. The nuclei were stained with DAPI. c Representative images of the microfilament network structure in TCN1 knockdown and TCN1-overexpressing HCT116 cells detected by transmission electron microscopy, n=6. Scale bars=10 μm.

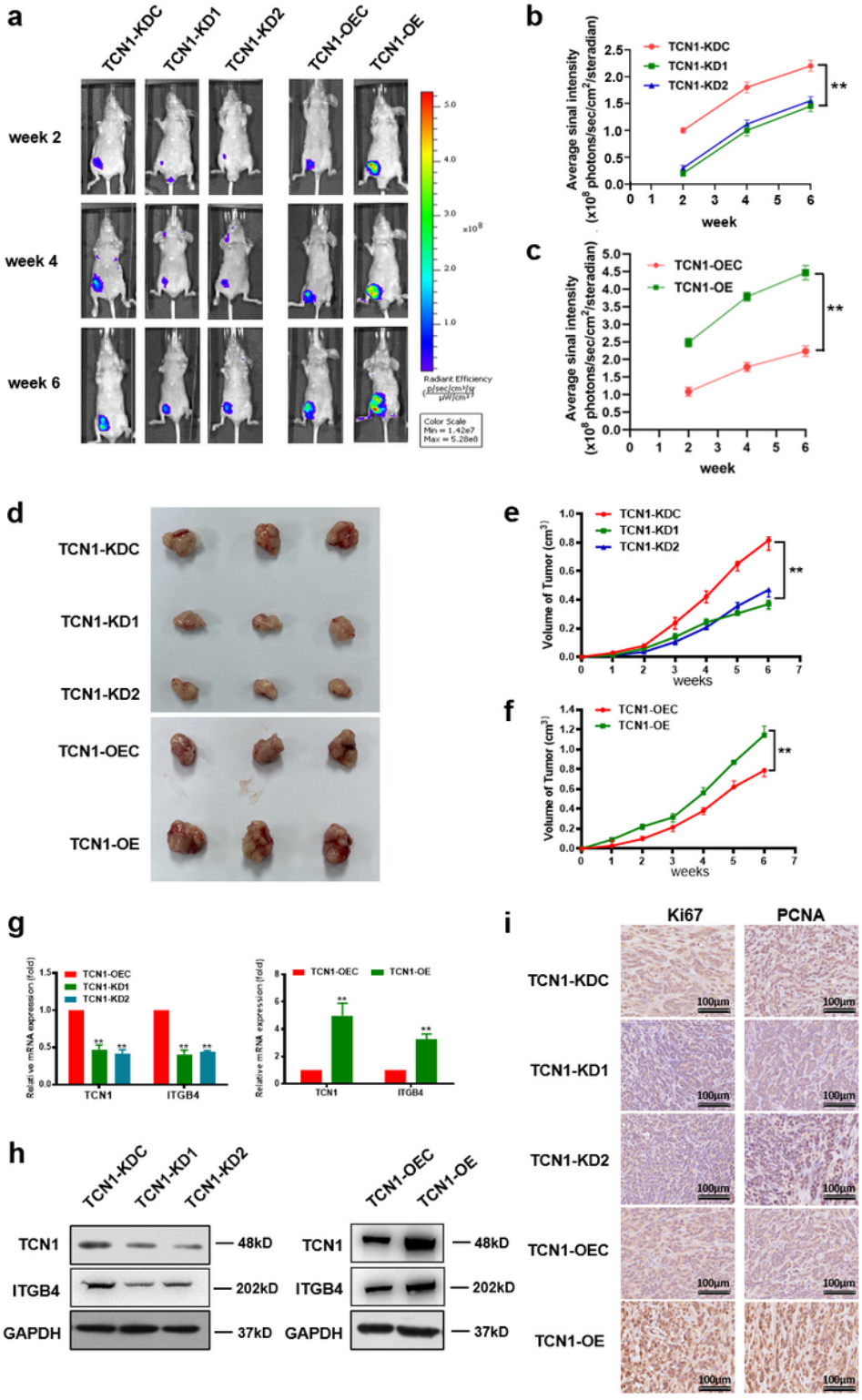

Figure 5

TCN1 deficiency inhibits tumorigenesis in a colorectal cancer xenograft model. a Representative bioluminescent images of tumors in nude mice are shown. b, c The photon flux in bioluminescent images

of tumors was quantified; n=6. d The tumors from each mouse were excised and photographed. e, f The tumor volume was calculated using the formula 0.5 × length × width2; n=6. g The expression of TCN1 and ITGB4 in cancer tissues was assessed by Q-PCR. h The expression of TCN1 and ITGB4 in cancer tissues was assessed by Western blotting. GAPDH served as a loading control. I, The Ki-67 and PCNA expression levels in xenograft tumors were immunohistochemically evaluated. All experimental data are presented as the mean ± SD. Statistical significance: ∗ P < 0.05 and ∗∗ P < 0.01.

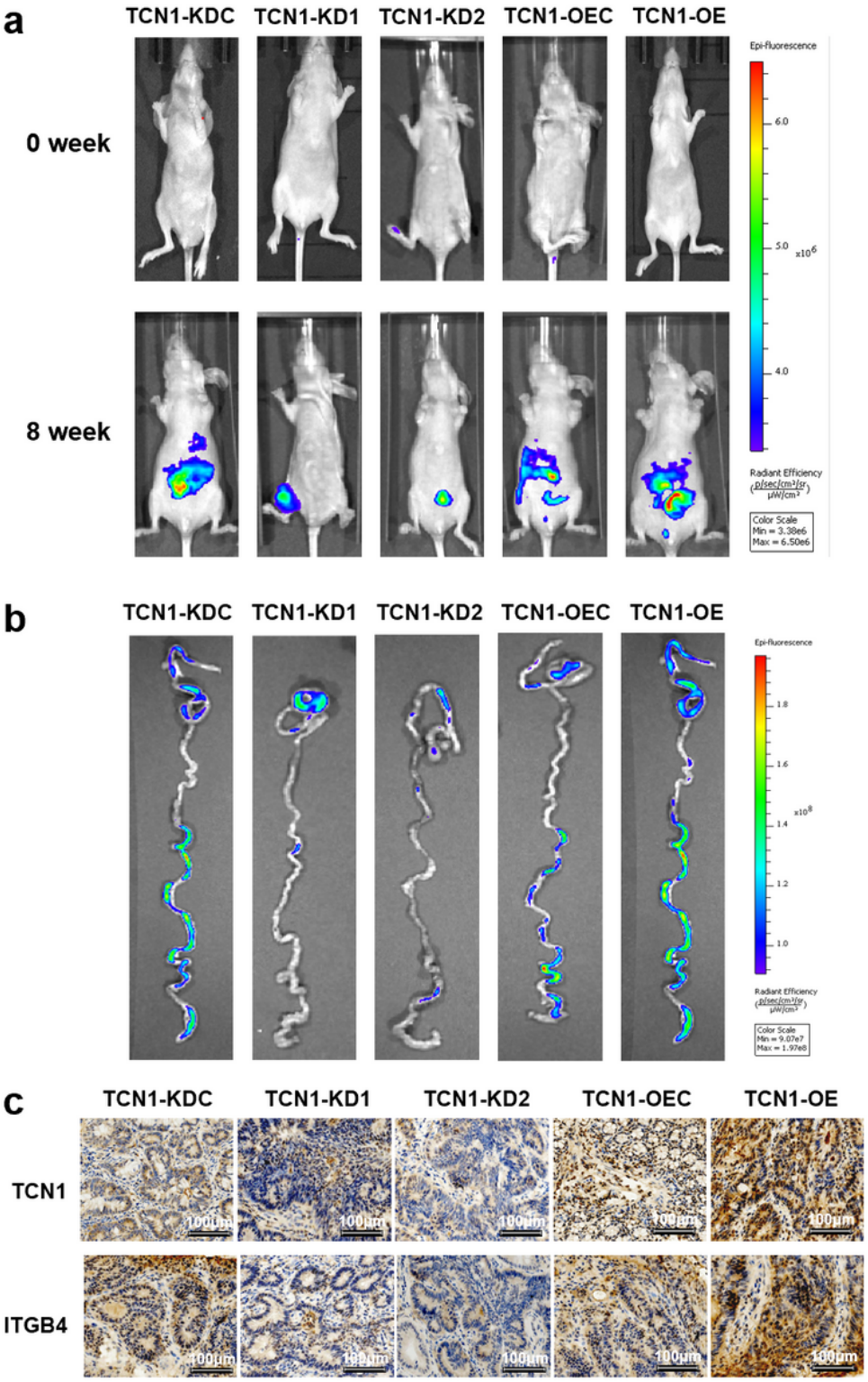

Figure 6

TCN1 deficiency inhibited metastatic engraftment in the peritoneum in vivo. a Representative bioluminescent images of metastatic foci in nude mice after 8 weeks are shown, n=6. b Bioluminescent images of metastatic foci in the intestines of nude mice after 8 weeks are shown, n=6. c Immunohistochemistry analysis of TCN1 and ITGB4 expression in tumor metastatic foci. Representative pictures of immunohistochemical staining are shown, n=6. Scale bars = 100 μm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile.docx