



Up-regulation of FAT4 enhances the chemosensitivity of colorectal cancer cells treated by 5-FU

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Background: Colorectal cancer (CRC) has high mortality, and 5-fluorouracil (5-FU) is a common clinical chemotherapeutic drug. The current study aimed to investigate the role of *FAT4* in chemosensitivity of CRC cells treated by 5-FU.

Methods: The immunohistochemistry and qRT-PCR was conducted to measure the *FAT4* expression in CRC and adjacent tissues. The *FAT4* expression was determined by qRT-PCR and Western blot, comparison of *FAT* expression between normal and several CRC cell lines was then made, so as to identify cell lines with the highest (LS174T) and the lowest (SW-620) expressions of *FAT4*. The effects of 5-FU stimulation at various doses on cell viability were determined by CCK-8, and the level of *FAT4* was also measured. After *FAT4* knockdown in LS174T or *FAT4* overexpression in SW-620 with or without pretreatment of 5-FU (30 µg/mL), cell growth, colony formation, cell migration and invasion, angiogenesis were determined by flow cytometry, wound-healing, transwell assay and tube formation assay, respectively. The expression levels of epithelial-mesenchymal transition (EMT) markers were detected by qRT-PCR and Western blot.

Results: *FAT4* was down-regulated in CRC tissues and cells, cell viability of CRC cells was decreased. The level of *FAT4* was increased with the increase of 5-FU concentrations. Moreover, 5-FU stimulation increased *FAT4* expression, and reduced cell proliferation, migration, invasion, angiogenesis and cell EMT process, furthermore, such effects of 5-FU stimulation could be enhanced by *FAT4* overexpression but reversed by *FAT4* knockdown.

Conclusions: Upregulation of *FAT4* could increase the sensitivity of CRC cells to 5-FU.

Keywords: *FAT4*; proliferation; migration; 5-FU; colorectal cancer (CRC)

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Introduction

Colorectal cancer (CRC) has the fourth highest mortality worldwide, moreover, annually, more than 1.2 million people suffer from CRC (1). 5-fluorouracil (5-FU) is a chemotherapeutic drug widely used as a clinical cancer suppressor in chemotherapy for certain types of cancers,

including CRC (2). 5-FU is considered to be a highly efficient chemotherapeutic agent, as it could successfully increase the 12-month survival for CRC patients (3). However, the toxicity of 5-FU limits its doses in clinical treatments and drug resistance developed by tumor cells reduce its effectiveness as a cancer suppressor (4). Thus, further investigation about the mechanism underlying

the occurrence of CRC and finding mediating genes were urgently required. In the present, some genes have been found to be able to attenuate the process during which 5-FU suppresses CRC, for example, miRNA-522 was reported to enhance 5-FU resistance by moderating *SMAD2* signaling pathway in CRC (5). Through inhibiting the expression of ABC transport protein, lidocaine could increase 5-FU cytotoxicity to choriocarcinoma cells (6).

FAT family, which belongs to the cadherin superfamily, consists of a group of calcium-dependent cell adhesion molecules. Studies showed that FAT family was closely connected to the differentiation of cells and tissues (7), moreover, FAT takes part in the Salvador-Warts-Hippo signaling pathway, which is a widely recognized regulator related to the size of *Drosophila* organ (8). Furthermore, biological properties of cells in epithelial tissues, which are highly associated with the mutation of cell and tumorigenicity, have been reported to be able to moderate the Salvador-Warts-Hippo signaling pathway (9). Qi proposed that *FAT4* could act as a tumor suppressor by observing tumor behavior of breast cancer cells when *FAT4* was low-expressed or over-expressed (10). Studies were less conducted on the relationship between *FAT4* and CRC, but the correlation between *FAT4* and other cancers have been widely investigated. Li reported that *FAT4* could act as a mediator in the down-regulation of lncRNA RNAZFHX4-AS1, so as to inhibit the migration and invasion of breast cancer (11). Han investigated the pathogenesis of prostate cancer, and pointed out that the knockdown of *FAT4* resulted in a more severe condition of prostate cancer (12). Epithelial-mesenchymal transition (EMT) process refers to a physiological process associated with tumor cell migration and invasion that affect greatly tumor metastasis and development (13). In breast cancer, the knockdown of lncRNA PANDAR was found to inhibit EMT process by down-regulating the expression of EMT-related markers, so as to suppress the cell proliferation, invasion of breast cancer cells (14). Moreover, the EMT process is highly associated with the prognosis of lung cancer patients, therefore is seen a predictor of prognosis of lung cancer patients (15). As mentioned above, FAT family, together with some EMT markers such as E-Cadherin, N-Cadherin, all belong to cadherin family, as a result, the possible relationship between them in the process of cancerous progression requires further investigation.

In the present study, we aimed to explore the role of *FAT4* in the cell proliferation, invasion and apoptosis and

EMT process of CRC cells under the treatment of 5-FU.

Methods

Formalin-fixed paraffin-embedded human samples

Human CRC and paired adjacent tissue samples (formalin-fixed, paraffin-embedded, FFPE) were derived from 30 CRC patients (19 males and 11 females) who received tumor resection in the First Affiliated Hospital of Zhejiang Chinese Medical University between 01/11/2016 to 29/01/2018. All patients and their relatives were informed and signed the consent form for allowing their tissues to be used in the research. The study was approved by the First Affiliated Hospital of Zhejiang Chinese Medical University Ethics Committee (2019).

Cell lines

Human normal colon cell line CCD-18Co (CRL-1459TM), human normal colorectal mucosa cell line FHC (CRL-1831), human CRC cell lines LS174T (CL-188), HT29 (HTB-38), HCT116 (CCL-247), HCT8 (CCL-244), HCT-15 (CCL-225), SW-620 (CCL-227) and LOVO (CCL-229) were purchased from American Type Culture Collection (ATCC, USA). All the cells were cultured in a humidified cell incubator with 5% CO₂ at 37 °C. FHC cells were cultured in MEM (32561037, Gibco, ThermoFisher, USA) containing 10% Fetal Bovine Serum (FBS, 16140071, Invitrogen, ThermoFisher, USA), LS174T cells were culture in MEM + 10% FBS + 1% P/S (SU 17920260, HaoranBio, China), while HT29 and HCT116 cells were cultured in McCoy's 5A (12330031, Gibco, ThermoFisher, USA) containing 10% FBS and 1% P/S, and HTC8 and HCT-15 cells were cultured in RPMI-1640 (61870044, Gibco, ThermFisher, USA) containing 10% FBS + 1% P/S. Moreover, SW-620 cells were cultured in DMEM high-glucose (10569010, Gibco, ThermoFisher, USA) containing 10% FBS, 1% P/S, and LOVO cells were cultured in 95% MEM with 5% DMSO (D12345, Gibco, ThermoFisher, USA).

Immunohistochemistry

FFPE samples (CRC and adjacent tissues) were sectioned into 4µm thick, and prepared for immunohistochemistry staining. The sections were incubated with rabbit polyclonal anti-FAT4 antibody (ab243591, 1:200, Abcam,

Table 1 Primers sequence used for qRT-PCR

Gene	Primer	Sequence
<i>FAT4</i>	Forward	5' TTGAAGGAAGGAGAACCCAT3'
	Reverse	5' TCGTCCAATAGTAAAGAGGC3'
<i>VEGF</i>	Forward	5' GAGGAGCAGTTACGGTCTGTG3'
	Reverse	5' TCCTTTCCTTAGCTGACACTTGT3'
<i>E-Cadherin</i>	Forward	5' CGAGAGCTACACGTTACCGG3'
	Reverse	5' GGGTGTGAGGGGAAAAATAGG3'
<i>N-Cadherin</i>	Forward	5' TTTGATGGAGGTCTCCTAACACC3'
	Reverse	5' ACGTTTAAACACGTTGAAATGTG3'
<i>Vimentin</i>	Forward	5' GCCCTAGACGAACTGGGTC 3'
	Reverse	5' GGCTGCAACTGCCTAATGAG 3'
<i>GADPH</i>	Forward	5' CGGAGTCAACGGATTGGTCGTAT3'
	Reverse	5' AGCCTTCTCCATGGTGGTGAAGAC3'

UK) at 4 °C overnight. After washing the sections by PBS, the sections were then incubated with goat anti-rabbit secondary antibody (ab205718, 1:2,000, Abcam, UK) at room temperature for 30 min. The peroxidase reactivity was measured by 3,3'-diaminobenzidine (DAB) (7411-49-6, Suzhou Yacoo Chemical Reagent Co., Ltd, China) and counterstained by haematoxylin. The IHC evaluation was conducted according to the description of Kusinska (16). The score grade of IHC intensity was classified as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). Based on the scores, positive cells were accordingly assigned into four groups, namely, 0 (negative), 1 (less than 10%), 2 (from 10% to 50%), and 3 (more than 50%) groups. The score of IHC intensity multiplied by the percentage of positive cells was expressed as the final score. If the final score was lower than or equal to 1, it was considered to be negative, while the score ranging from 2 to 9 was positive.

Transfections

SiFAT4 (SR312478) and SR312478-FAT4 overexpression plasmid were obtained from Origene, siRNA (A01001) was purchased from Gene pharma. LS174T and SW-620 cell lines were cultured in 6-well plates (5×10^5 cells per well) to reach confluence of 60% to 80%. Lipofectamine TM 2000 transfection reagent (#11668019, ThermoFisher,

USA) was used according to its instruction. A total volume of 75 µg siFAT4 or siRNA was prepared and added to LS174T cells, while *FAT4* and an empty plasmid was respectively added to SW-620 cells, and the cell lines were all cultured in DMEM medium. After culturing the cells in CO₂ for 6 h at 37 °C, DMEM medium was replaced by 10% FBS/DMEM medium to continue the culture for another 24 h.

Quantitative real time PCR

The expression levels of *FAT4* and EMT-related genes were measured by qRT-PCR. Total RNAs were extracted from FFPE tissues or cell samples by PureLink™ RNA Mini Kit (12183018A, ThermoFisher, USA). RNA concentration was determined by NanoDrop 2000c (# ND2000CLAPTOP, Thermo Fisher, USA). Reverse transcription from RNAs to cDNAs was carried out using miScript Reverse Transcription Kit (218160, Qiagen GmbH, German) following the instructions. SYBR-Green PCR kit and the Fast Real-Time PCR system (ABI 7500, Applied Biosystems, Carlsbad, CA) were used for qRT-PCR.

The thermocycling conditions were set as follows: at 95 °C for 20 min, and then 40 cycles at 94 °C for 15 s, at 55 °C for 30 s and at 72 °C for 30 s. 2^{-ΔΔCT} was used to measure the expression levels of *FAT4* and EMT-related biomarkers (17). All primer sequences were listed in *Table 1*.

Group separation

FAT4 expressions in cell lines treated by 5-FU (T0984, Top Science, USA) at different concentrations (0, 1, 5, 10, 30, 60, 120 µg/mL) at 37 °C for 24 and 48 h were determined, so as to identify the cell lines with the highest *FAT4* expression (LS174T) and the lowest (SW-620). The optimal concentration of 5-FU was defined as 30 µg/mL. The cells were divided into four groups, namely, LS174T: siNC, siFAT4, siNC+5-FU (30 µg/mL), siFAT4+5-FU. SW-620: mock, *FAT4*, mock + 5-FU, *FAT4* + 5-FU.

Western blot

The levels of *FAT4*-related proteins and EMT-related proteins were measured by Western blot. RIPA lysis buffer solution was prepared for the lysates of cell lines. After 20-min reaction on ice, the solutions were centrifuged to collect supernatants.

BCA protein assay (Bio-Rad, Hercules, CA, USA) was used to measure the protein concentration. Proteins (30 µL) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Skimmed milk (5%) was used to seal the membranes for 2 h at room temperature, then the membranes were incubated with specific antibody for *FAT4* (anti-rabbit, Abcam, ab130076, 1:500, UK), VEGF (anti-mouse, Abcam, ab1316, 1:100, UK), E-Cadherin (anti-rabbit, Abcam, ab40772, 1:10,000, UK), N-Cadherin (anti-rabbit, Abcam, ab18203, 1:1,000, UK), Vimentin (anti-rabbit, Abcam, ab92547, 1:1,000, UK) at 4 °C overnight. TBST was used to wash the membranes for 3 times, and then the membranes were incubated with the respective secondary antibody (goat anti-rabbit, Abcam, ab150077, 1:1,000, UK, goat anti-mouse, Abcam, ab150113, 1:200, UK) at room temperature for 2 h, and washed by TBST for 3 times and developed by ECL prime Western blot (BRPN2232, Sinopharm Chemical Reagent). The bands were conventionally pressed and photographed. The optical density was analyzed by Image J (Image J 1.8.0, National Institute of Health). GAPDH (anti-mouse, Abcam, ab8245, 1:500, UK) served as the internal references.

Cell counting kit-8 assay (CCK-8)

The cell viabilities of LS174T, SW-620 cell lines and siNC, siFAT4, siNC + 5-FU, siFAT4 + 5-FU, mock, *FAT4*, mock

+ 5-FU, *FAT4* + 5-FU were detected by CCK-8 assay. The cells were seeded in 96-well plates, and stained using CCK-8 solution (HY-K0301, MedChemExpress, USA) at 37 °C for 2 h. Optical density (OD) value was measured at the wavelength of 450 nm.

Flow cytometry assay

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit (70-AP101-100-AVE, MultiSciences, China) was applied to detect cell apoptosis. The cells were separated by 0.25% trypsin (25300054, Gibco, Thermofisher, USA), and cell solution was prepared at the density of 10^6 /mL. After centrifugation, the sediments were collected. A dose of 2 mL PBS was added into 1 mL cells and centrifuged again to collect the sediments. The sediments were then fixed by 70% ethanol at 4 °C for 2 h, then rinsed in PBS twice. After centrifugation, the sediments were resuspended in binding solution. The reaction was conducted using 10 µL Annexin V-FITC and 5 µL PI for 15 min, then in another 300 µL binding buffer in the dark. Then cell apoptosis was measured by flow cytometry (NJ 08855-1327, BD FACSCanto II, BD Biosciences, Franklin Lakes, USA).

Colony formation assay

After cell digestion, the solution was centrifuged, the sediments were collected and cultured in a 6-well plate at the density of 10×10^3 per well for 10 days. Then cells were rinsed in PBS buffer, fixed by 1 mL methanol for 15 min at room temperature and stained by crystal violet solution (0.1%) for 20 min. Finally, the cells were finally photographed, counted and analyzed.

Migration and invasion assays

Cell migration was measured by wound-healing assays. 1.5×10^5 LS174T or SW-620 cells (1.5×10^5 cells/well) were respectively seeded into a 12-well culture plate. After transfection of each cell lines for 24 h, the cells were scratched using a sterilized micropipette. The medium was replaced by serum-free and the wound was photographed at 0 and 24 h using Image J software (NIH Image, Bethesda, MD).

The invasion abilities of LS174T and SW-620 cell lines were detected by transwell assays. A 12-well Transwell cell culture chamber (8 µm, 430172, Corning Costar Corp., USA) was used, and all the procedures were conducted

following the manufacturer's instructions. After the transfection for 24 h, the cells were suspended in the upper chambers at a density of 4×10^4 cells/mL on 50- μ L Matrigel (2.0 mg/mL, 354234, Corning, USA) layer. A total of 500 μ L DMEM medium containing 10% FBS was seen as the lower chemoattractant. The cells were incubated with 5% CO₂ at 37 °C for 24 h. The cells in the upper chambers were stained by crystal violet and cell invasion capacity was measured under a microscope.

Tube formation assay

Ibidi angiogenesis slide (Ibidi, Martinsried, Germany) was used for tube formation measurement. Matrigel was stored at 4 °C overnight in advance, and 10 μ L Matrigel was then added into each well until coagulated. A total of 50 μ L cell suspension (2×10^5 cells/mL) was then respectively added into each well, while cell-free culture medium was added into upper well. The images were regularly observed, and coverage area and cell formation number were collected for statistical analysis.

Statistical analysis

The data were analyzed by SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) and shown as mean \pm standard deviation. Student's *t*-test (two-tailed) and one-way ANOVA followed by Dunnett's post hoc test were carried out for statistical analyses. All the experiment was conducted in triplicate. $P < 0.05$ was considered to be a statistically significant difference.

Results

FAT4 expression in CRC tissues and cells

Immunohistochemistry and qRT-PCR were conducted to detect *FAT4* expressions in CRC and adjacent tissues. Immunohistochemistry results showed a notable *FAT4* staining in adjacent tissues in comparison with a significantly less staining strength in CRC tissues (Figure 1A). The result from qRT-PCR detection showed that the *FAT4* expression was greatly higher in adjacent tissues than in CRC cancer tissues (Figure 1B).

QRT-PCR and Western blot were performed to determine gene and protein expressions of *FAT4* in normal colon cell line, normal colorectal mucosa cell line and CRC cell lines. Compared to FHC cell line, HT29, HCT116,

HCT8, HCT-15, SW-620 and LOVO cell lines had a significantly lower gene and relative protein levels of *FAT4* ($P < 0.001$, Figure 1C,D,E), while CCD-18Co cell line had the highest *FAT4* and relative protein expressions in all the cell lines, showing a significant difference from CCD-18Co and LS174T, HT29, HCT116, HCT8, HCT-15, SW-620 and LOVO cell lines.

The effect of different 5-FU doses on cell viability and FAT4 expression of LS174T and SW-620 cells

According to CCK-8 results, the OD value of LS174T cells was reduced as the concentrations of 5-FU increased, and a significant difference appeared when the 5-FU concentration reached 5 μ g/mL and higher (Figure 2A). The OD value of LS174T cells was higher at 48 h than at 24 h, when the 5-FU concentration was 60 μ g/mL and lower. In contrast, the OD value on 48 h was lower than at 24 h when 5-FU concentration was used at 120 μ g/mL. The similar result was observed on SW-620 cells (Figure 2B), the increases in 5-FU concentrations significantly reduced the OD value of SW-620 cells at 24 and 48 h, moreover, the OD values of SW-620 at 24 h was higher than that at 48 h with any 5-FU concentration.

QRT-PCR and Western blot results showed that 5-FU at 10 μ g/mL or higher could significantly up-regulate the *FAT4* expressions in LS174T and SW-620 cells (Figure 2C,D,E,F,G,H). The *FAT4* expressions kept increasing and reached the highest when the 5-FU concentration was 60 μ g/mL, and then the expression decreased at 120 μ g/mL 5-FU.

FAT4 transfection efficiency in LS174T and SW-620 cells

The transfection efficiency of *FAT4* in LS174T and SW-620 cells were detected by qRT-PCR and Western blot (Figure 3). In LS174T cells, the expression of *FAT4* was silenced, and results showed that both relative mRNA and protein expressions were significantly reduced ($P < 0.001$). In comparison of SW-620 cells transfected with overexpress *FAT4* plasmid, the expression levels of the mRNA and protein were increased ($P < 0.001$).

Effects of FAT4 on cell sensitivity to 5-FU

The cell viabilities of LS174T and S-620 cell lines were detected by CCK-8 assay (Figure 4A,B). In LS17T cell line, silencing *FAT4* expression could significantly increase the

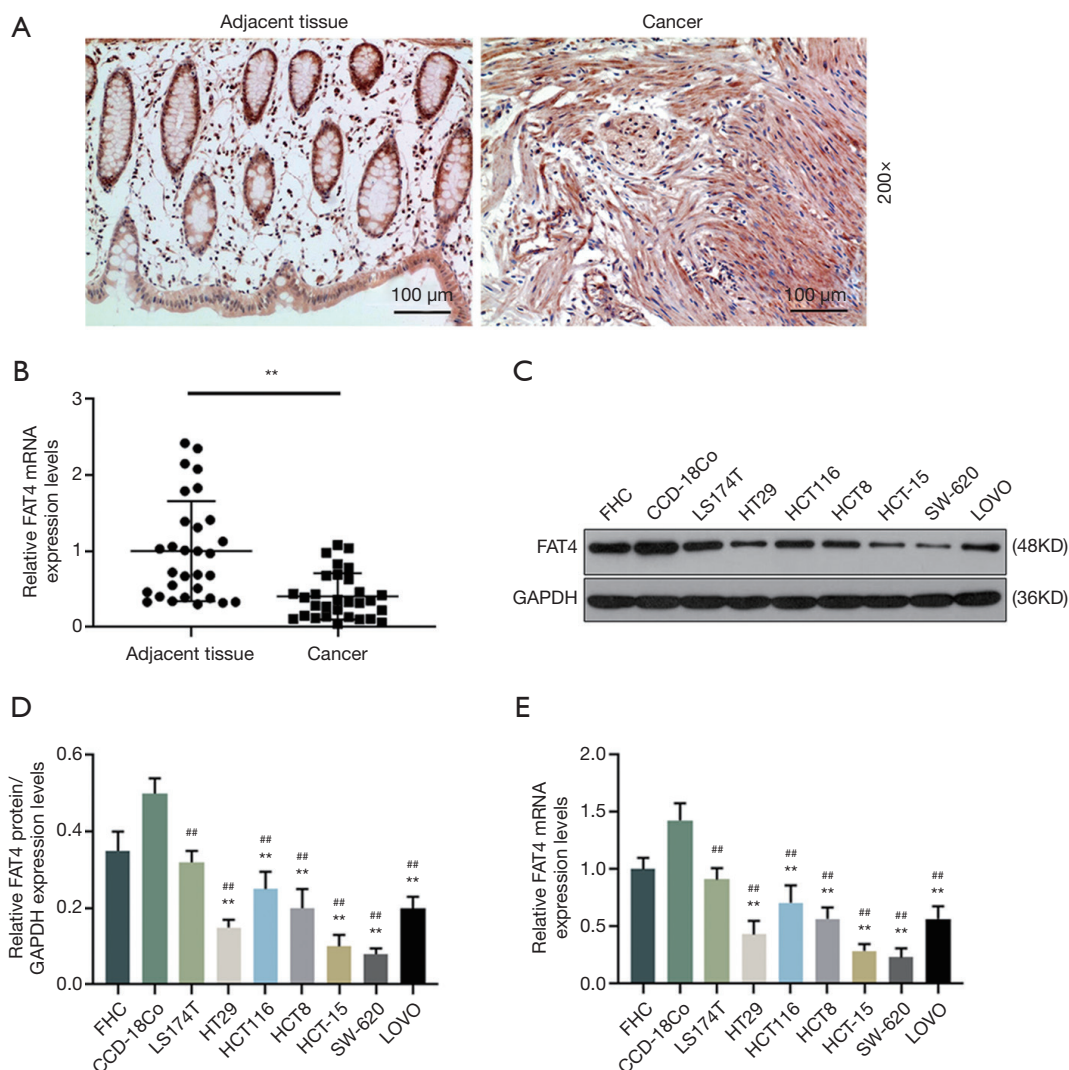


Figure 1 Determination of *FAT4* expression in colorectal cancer (CRC) and adjacent tissues, normal and CRC cell lines. (A,B) *FAT4* expression detected by immunohistochemistry staining and qRT-PCR, **, $P < 0.001$. (C,D,E) *FAT4* expressions comparison between human normal colon cell line CCD-18Co, human normal colorectal mucosa cell lines FHC, human colorectal cancer cell lines LS174T, HT29, HCT116, HCT8, HCT-15, SW-260 and LOVO by qRT-PCR and Western blot. GAPDH served as internal reference. **, $P < 0.001$ vs. FHC; #, $P < 0.001$ vs. CCD-18Co.

cell OD values at 24 h and 48 h, whereas in SW-620 cells, overexpression of *FAT4* had a lower OD value with or without 5-FU addition. Moreover, the OD value of both LS174T and SW-620 cell lines decreased with the addition of 5-FU.

Apoptosis of LS174T and SW-620 was determined (Figure 4C,D), and the results showed that the silence of *FAT4* in LS174T had no significant effect on cell apoptosis rate, however, when *FAT4* was silenced in LS174T pretreated by 5-FU, the apoptosis was significantly reduced compared to the cells only treated by 5-FU. Furthermore,

the result of SW-620 showed that *FAT4* overexpression significantly up-regulated cell apoptosis rate. Moreover, the addition of 5-FU in the two cell lines could significantly increase the cell apoptosis.

The colony formation results showed that silencing *FAT4* significantly up-regulated the cell colony formation rate, which could be greatly reduced by overexpression of *FAT4* and the addition of 5-FU (Figure 4E,F).

The cell migration rate was determined, and results showed that LS174T cell migration rate was significantly

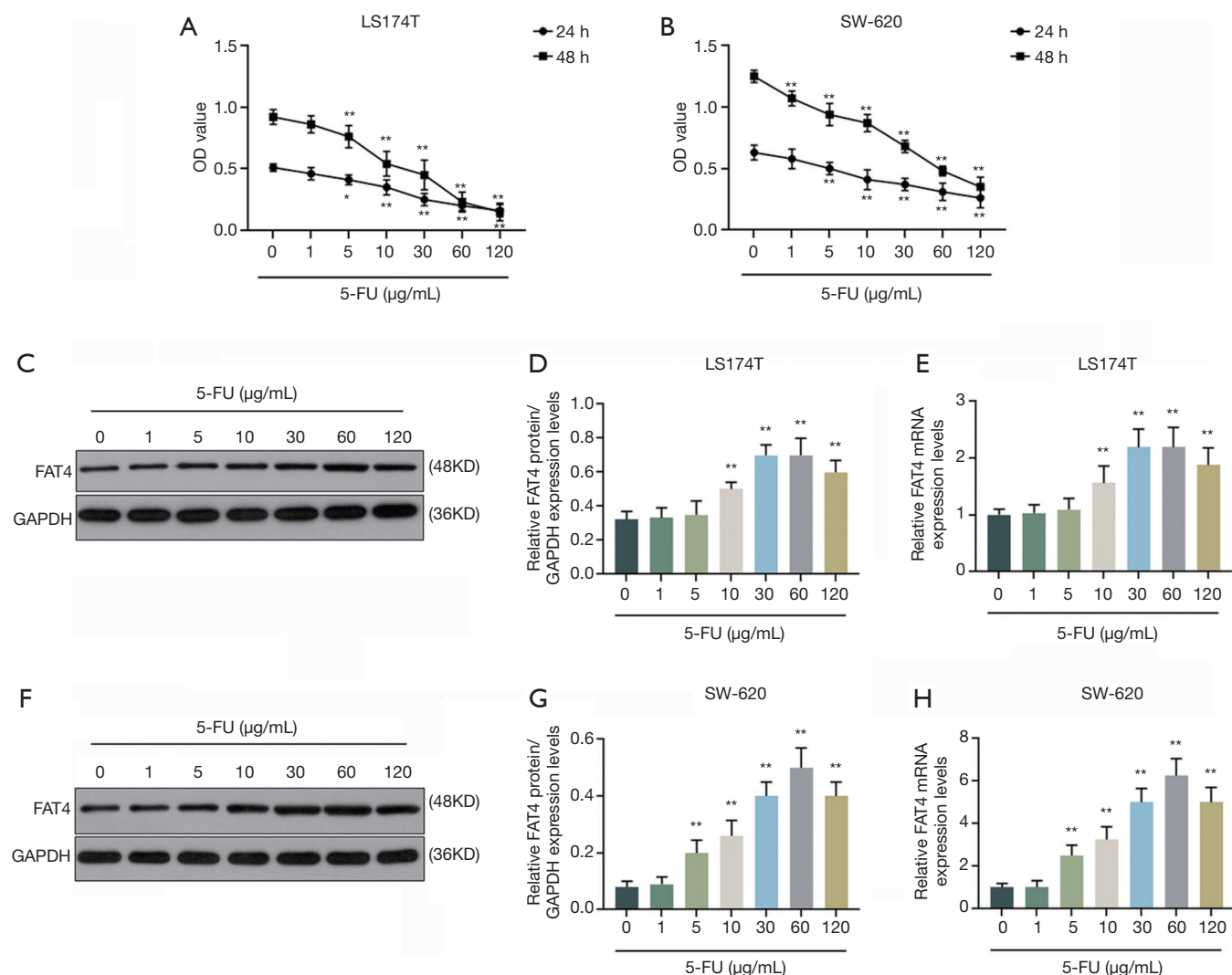


Figure 2 The effect different 5-FU doses on cell viability and *FAT4* expression of LS174T and SW-620 cells. (A,B) Determination of cell viabilities of LS174T and SW-620 cells under the growing concentration of 5-FU at 24 and 48 h; (C,D,E,F,G,H) relative *FAT4* mRNA and protein expressions detected by qRT-PCR and Western blot. GAPDH served as internal reference. *, $P < 0.05$; **, $P < 0.001$ vs. 0 µg/mL 5-FU. 5-FU, 5-fluorouracil.

increased with the silence of *FAT4*, however, the overexpression of *FAT4* greatly reduced the cell migration rate of SW-620. Additionally, the 5-FU treatment noticeably lowered the migration rates of the two cell lines (Figure 5A,B).

Similarly, the cell invasion rates were increased significantly by silencing of *FAT4*, but were decreased significantly when *FAT4* was overexpressed. The pretreatment with 5-FU could significantly reduce the cell invasion rates (Figure 5C,D).

The tube formation was measured, and results showed

that the silence of *FAT4* could significantly increase the tube length, while the *FAT4* overexpression resulted in a significant shorter tube length (Figure 5E,F).

The effect of moderating *FAT4* expression on EMT process in LS174T and SW-620 cells

The protein and mRNA expressions of *FAT4* and EMT were measured by qRT-PCR and Western blot (Figure 6). In LS174T cells, the silencing of *FAT4* decreased the relative protein and mRNA expressions of *FAT4* and

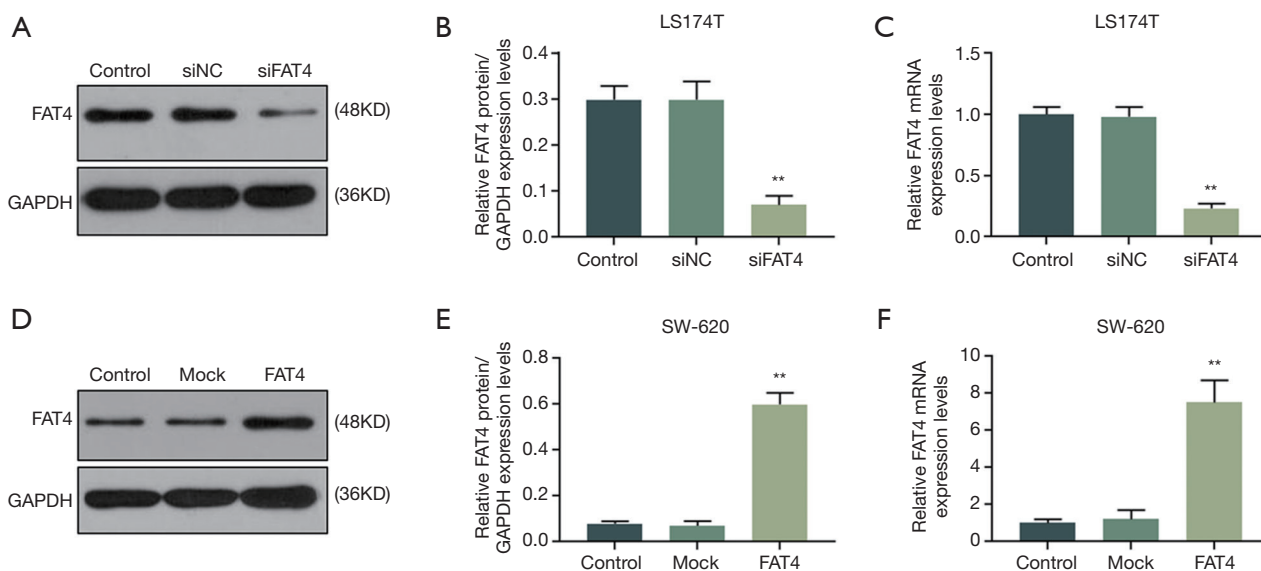


Figure 3 Transfection efficiency of FAT4 in LS174T and SW-620 determined by qRT-PCR and Western blot. (A,B,C) FAT4 gene and protein expression levels in LS174T cells; (D,E,F) FAT4 gene and protein expression levels in SW-620 cells. **, $P < 0.001$ vs. control.

E-Cadherin, and increased the expressions of VEGF, *N-Cadherin*, *Vimentin*. However, increasing *FAT4* expression up-regulated the expressions of *FAT4* and *E-cadherin* and down-regulated the relative protein and mRNA expressions of *VEGF*, *N-cadherin* and *Vimentin*. Moreover, the 5-FU treatment could increase the *FAT4* and *E-cadherin* expressions, and reduce the expressions of *VEGF*, *N-cadherin* and *Vimentin*.

Discussion

FAT gene family is a newly discovered gene member, and has been widely reported to have biological functions (18-20). For the functions of *FAT4* in tumors, Sun investigated the relation between *FAT4* and *SOX11* in gastric cancer, and found that abnormal methylation of both can largely increase the risk developing suffering gastric cancer (21). Moreover, 30% invasive lobular carcinoma showed *FAT4* mutation (22). In the present study, *FAT4* showed a significant lower expression in CRC cancer tissues than adjacent tissues ($P < 0.001$), indicating a potential role of *FAT4* in CRC. Moreover, the prognosis of CRC is likely to be related to FAT expression. Previous study investigated the relation between prognosis of gastric cancer and *FAT4* expressions, and found that high *FAT4* promoter methylation was related to a knockdown of *FAT4* expression, thus resulted in a poor prognosis of gastric cancer (23).

The survival rate of breast cancer was also reported to be associated with *FAT4* hippo signaling pathway, as higher *FAT4* expression greatly increases the potential of a patient having better prognosis (24). In our research, LS174T had the highest *FAT4* expression, while SW-620 had the lowest *FAT4* expression in all the cell lines.

The effects of 5-FU on cell viability were investigated in the present study, and the results showed that the cell viability of SW-620 was reduced more significantly at lower 5-FU dose, indicating that SW-620 cells were more sensitive to 5-FU than LS174T. On the other hand, once the concentration of 5-FU reached higher than $5\mu\text{g/mL}$, the cell viability could be significantly affected, moreover, 5-FU showed great effects on *FAT4* expressions in LS174T and SW-620, as the expression of *FAT4* was greatly increased in the two cells by the pretreatment of 5-FU. However, the *FAT4* expression was significantly inhibited when the concentration of 5-FU reached $120\mu\text{g/mL}$ ($P < 0.001$), indicating the potential negative effect resulted from high 5-FU dose. Kobayashi *et al.* has also found similar relation between high 5-FU concentration and prognosis of pancreatic cancer patients, as they observed that a high 5-FU concentration caused poor prognosis in cancer pancreatic patients (25).

The expression of *FAT4* in LS174T and SW-620 cells was determined when *FAT4* was silenced or overexpressed. In the cells with high *FAT4* expression, silencing of *FAT4*

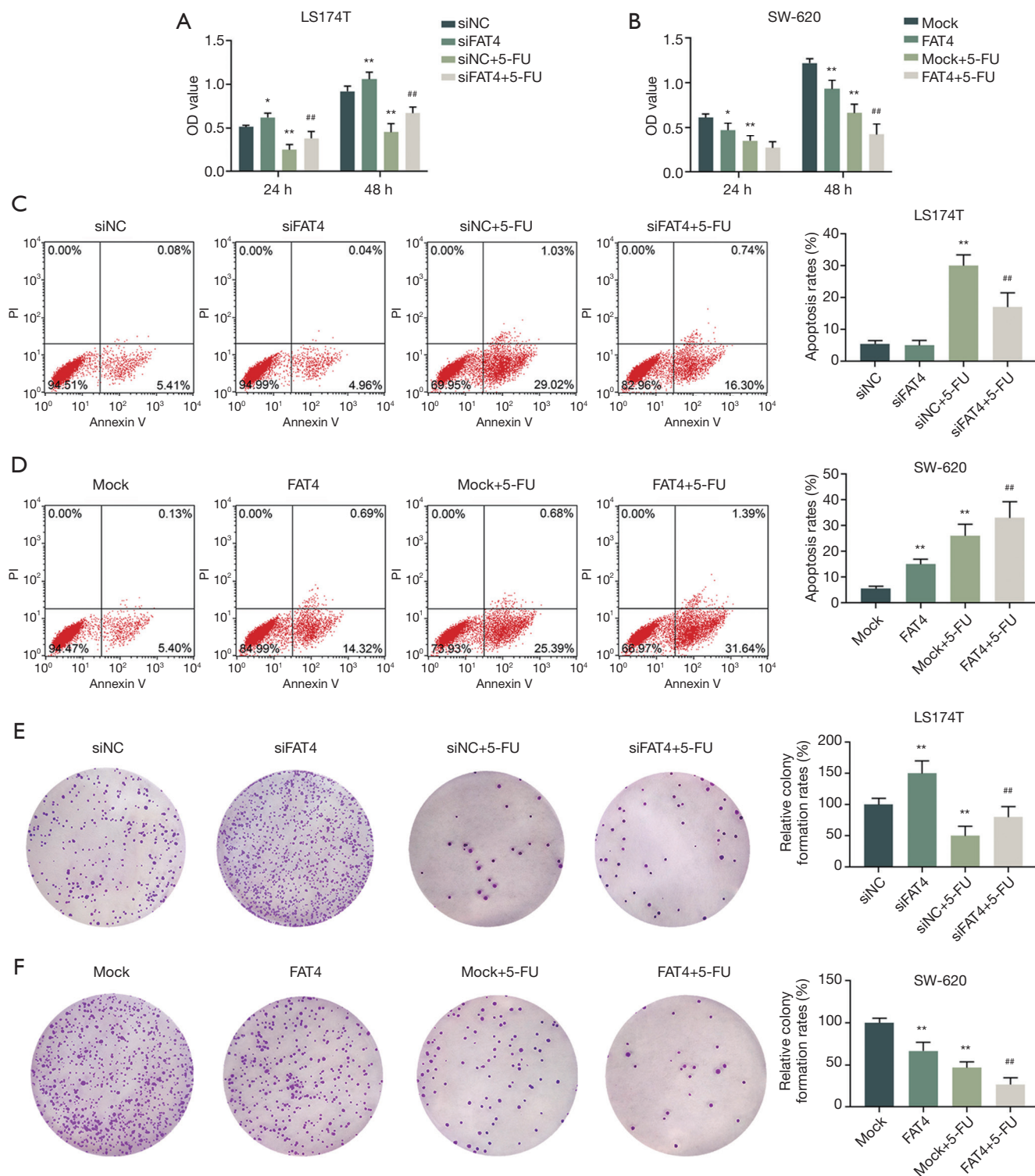


Figure 4 Determination of viability, apoptosis and colony formation rate in LS174T and SW-620 cell lines. (A,B) Cell viabilities of LS174T and SW-620 cell lines; (C,D) cell apoptosis rate of LS174T and SW-620 cell lines; (E,F) colony formation rate of LS174T and SW-620 cell lines. Cells were stained by crystal violet solution (0.1%). $\times 1$ magnification. LS174T cells were divided into siNC, siFAT4, siNC + 5-FU, siFAT4 + 5-FU. SW-620 cells were divided into mock, *FAT4*, mock+5-FU, *FAT4* + 5-FU. *, $P < 0.05$, **, $P < 0.001$, vs. siNC (LS174T) or mock (SW-620). ##, $P < 0.001$ vs. siNC + 5-FU (LS174T) or mock + 5-FU (SW-620). 5-FU, 5-fluorouracil.

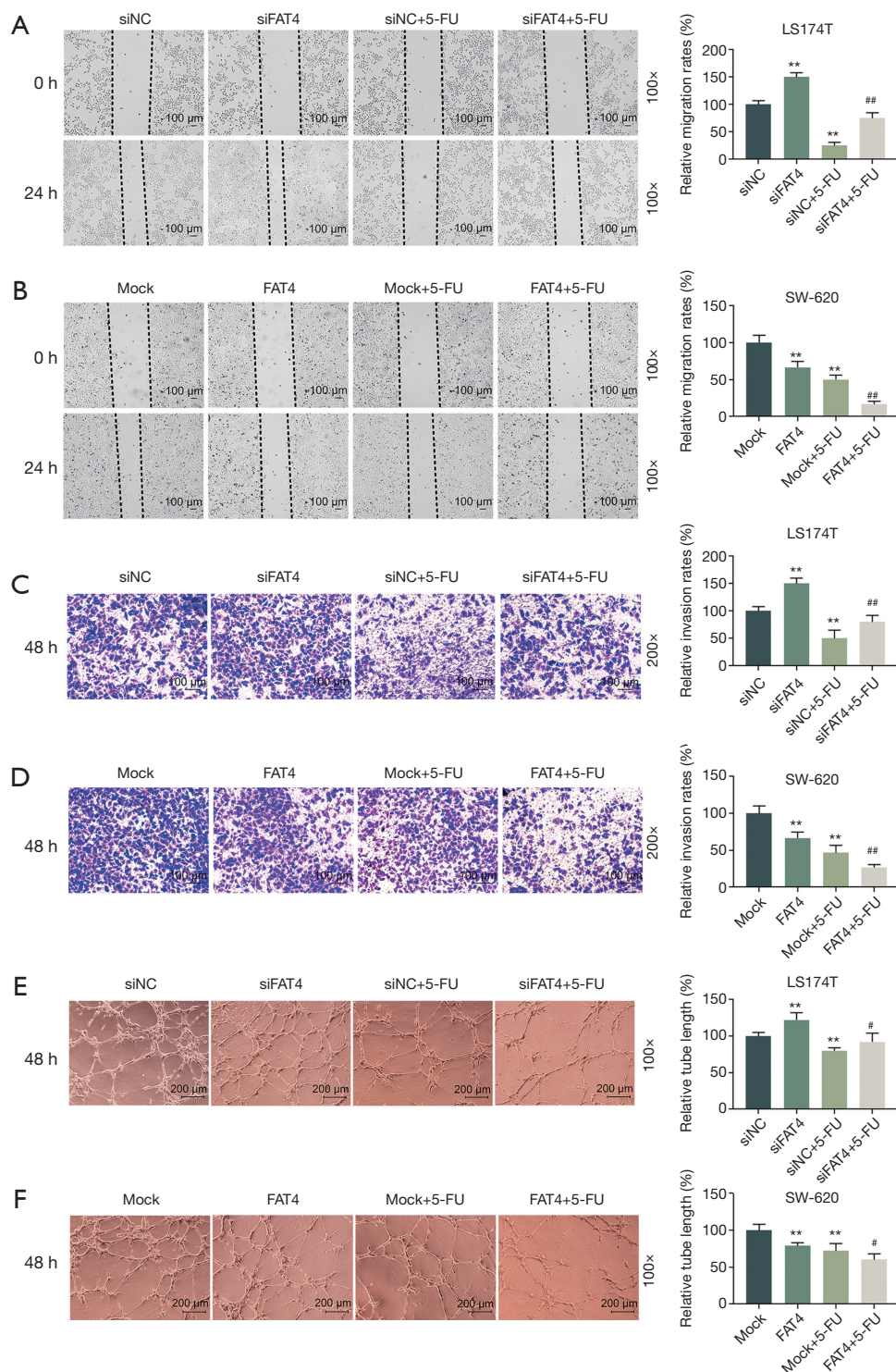


Figure 5 Determination of migration and invasion rates and tube formation length of LS174T and SW-620 cell lines. (A,B) Cell migration rates of LS174T and SW-620 cell line; (C,D) cell invasion rate of LS174T and SW-620 cell lines; (E,F) tube formation length of LS174T and SW-620 cell lines. The cells in the upper chambers were stained by crystal violet. LS174T cells were divided into siNC, siFAT4, siNC + 5-FU, siFAT4 + 5-FU. SW-620 cells were divided into mock, FAT4, mock + 5-FU, FAT4 + 5-FU. **, $P < 0.001$, vs. siNC (LS174T) or mock (SW-620). #, $P < 0.05$, ##, $P < 0.001$ vs. siNC + 5-FU (LS174T) or mock + 5-FU (SW-620). 5-FU, 5-fluorouracil.

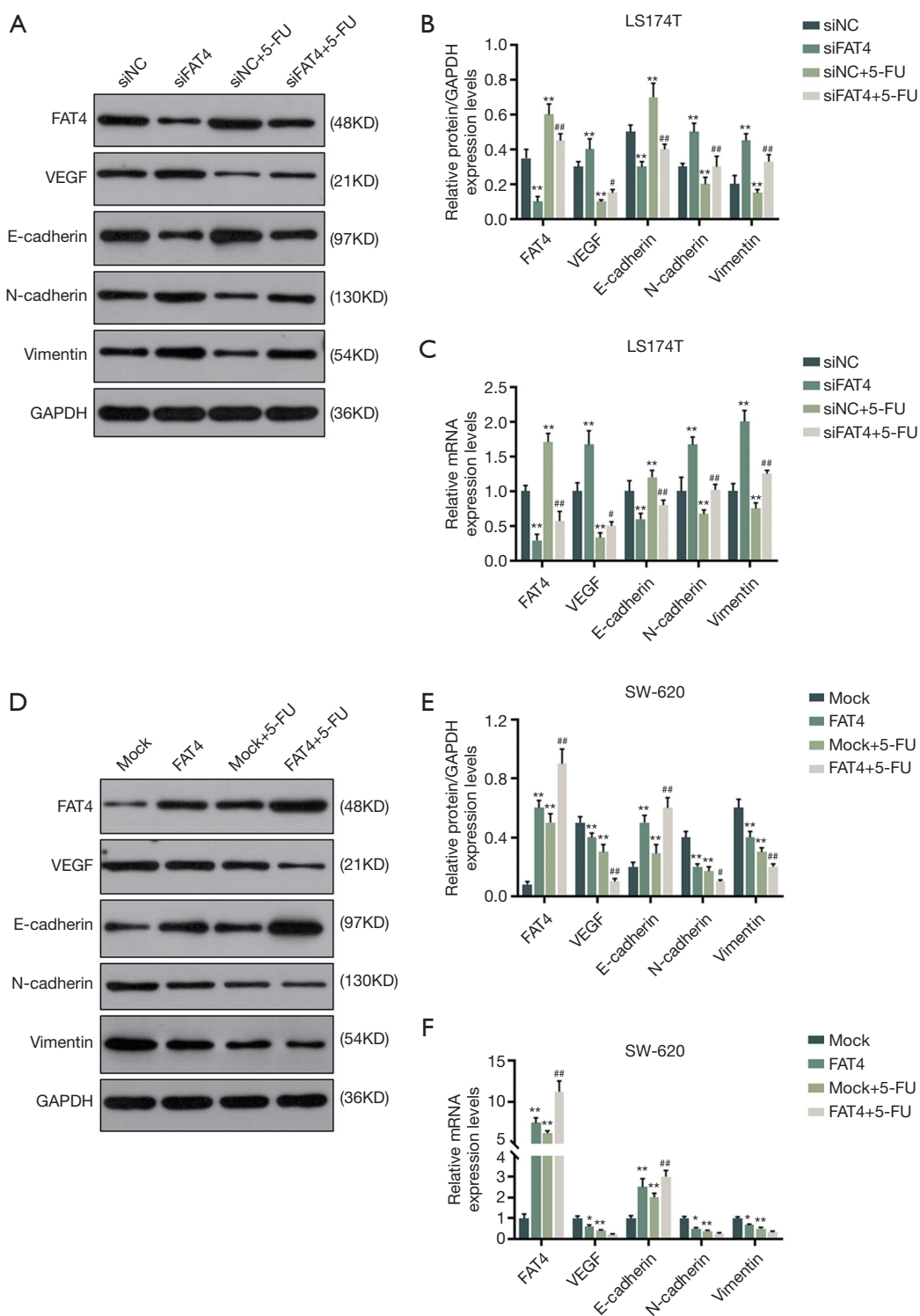


Figure 6 Expression levels of *FAT4*, *VEGF*, *E-Cadherin*, *N-Cadherin*, *Vimentin* in LS174T and SW-620 cell lines detected by qRT-PCR and Western blot. (A,B,C) Relative mRNA and protein expressions of LS174T cell line in the group of siNC, siFAT4, siNC + 5-FU, siFAT4 + 5-FU; (D,E,F) relative mRNA and protein expressions of SW-620 cell line in the group of mock, *FAT4*, mock + 5-FU, *FAT4* + 5-FU. *, P<0.05, **, P<0.001, vs. siNC (LS174T) or mock (SW-620). #, P<0.05, ##, P<0.001 vs. siNC + 5-FU (LS174T) or mock + 5-FU (SW-620). 5-FU, 5-fluorouracil.

expression could significantly lower *FAT4* expression ($P < 0.001$). However, SW-620 first showed a rather low *FAT4* expression, but after transfection with *FAT4* overexpression plasmid, the up-regulated *FAT4* was observed in SW-620 cells ($P < 0.001$). *FAT4* has been reported to control organ size by regulating cell cycles (26,27). Research showed that the block of *FAT4* expression could increase tumor size, and results in more aggressive invasion and migration of tumor cells (28). Moreover, silencing *FAT4* expression significantly activated cell viability, reduced cell apoptosis rate and increased colony formation, cell migration and invasion rates ($P < 0.001$), suggesting that *FAT4* could strongly inhibit CRC cell activity. Moreover, tube formation plays a critical role in the process of tumor development and metastasis, in the present study, tube formation was longer after the knockdown of *FAT4* expression, indicating that *FAT4* inhibition was possibly related to tumor metastasis. However, the *FAT4* overexpression results indicated that the presence of *FAT4* could suppress tumor metastasis. The addition of 5-FU also showed a similar results. Thus, the data suggested that *FAT4* could promote the effects of 5-FU on suppressing cancer cell activity on cell proliferation, migration, and promoting CRC cell apoptosis. It is reported that down-regulation of Hippo pathway was able to attenuate the resistance of CRC cells to 5-FU (29). As explained above, *FAT4* acts as a mediator in Salvador-Warts-Hippo signaling pathway, thus, *FAT4* might play a role in the correspondence between 5-FU and *FAT4*.

E-Cadherin and *N-Cadherin* are EMT markers, apart from the two, *VEGF* and *Vimentin* are also commonly considered to be EMT markers (30,31). Gravidal reported that high level of *N-Cadherin* and low level of *E-Cadherin* was strongly associated with death caused by prostate cancer (32). Cai *et al.* reported that *FAT4* played a tumor suppressor role in gastric cancer by inhibiting EMT (33). In the present research, the expression levels of *VEGF*, *E-Cadherin*, *N-Cadherin* and *Vimentin* and the *FAT4* expression were determined, the results showed that *E-cadherin* was low-expressed, but *VEGF*, *N-Cadherin*, and *Vimentin* were high-expressed in case that *FAT4* was silenced. However, the high *E-Cadherin* and low *VEGF*, *N-Cadherin*, *Vimentin* expression levels were observed after overexpressing *FAT4*. Such results suggested a possible connection between *FAT4* and EMT process, specifically, *FAT4* was likely to increase the expression levels of *E-Cadherin* but suppress the expressions of *VEGF*, *N-Cadherin*, *Vimentin*, thus inhibiting the EMT process.

Conclusions

In conclusion, the present study finds the relationship between *FAT4* and CRC, specifically, *FAT4* functions as a tumor suppressor and has the ability to enhance the 5-FU effect on suppressing the growth, migration, invasion, and tube formation of CRC cells, in other words, CRC cells are more sensitive to 5-FU with overexpressed *FAT4*. Furthermore, *FAT4* is likely to inhibit EMT of CRC cells by up-regulating the expression of *E-Cadherin* and down-regulating the expressions of *VEGF*, *N-Cadherin* and *Vimentin*.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.12.74>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All patients and their relatives were informed and signed the consent form for allowing their tissues to be used in the research. The study was approved by the First Affiliated Hospital of Zhejiang Chinese Medical University Ethics Committee (2019).

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