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TRAF6 promotes the progression and growth of colorectal cancer through nuclear shuttle regulation NF-kB/c-jun signaling pathway

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ABSTRACT

Aims: TRAF6 is an intracellular signal adapter molecule plays a significant role in tumor development. However, the specific mechanism causes and promotes of colorectal cancer keep largely unknown. Therefore, we sought to investigate the roles and the molecular mechanisms of TRAF6 in regulation colorectal cancer.

Material and methods: The immunohistochemistry analyzed the expression of TRAF6 in colorectal cancer samples and analyzed the effects of expression of TRAF6 on the prognosis in colorectal cancer. The roles of TRAF6 in regulating colorectal cancer cell proliferation, colony formation, cell migration, cell wound healing and cell invasion were evaluated in vitro. Animal studies were performed to investigate the effects of TRAF6 on tumor growth. mRNA abundance of key genes was analyzed via qPCR. Protein level of TRAF6 and NF-κB/AP-1 signaling pathways was examined by Western blot. Luciferase reporter and Immunofluorescence assays were used to identify the activities NF-κB/AP-1 signaling pathways.

Key findings: TRAF6 high expression in colorectal cancer tissues. And colorectal cancer patients with high expression of TRAF6 had a poor survival rate. TRAF6 knockdown can inhibit proliferation, migration, and invasion of colorectal cancer cells in vitro and in vivo experiments. TRAF6 activates the TRAF6-NF-κB/AP-1 signaling pathway by entering the nucleus, causing biobehavioral changes in colorectal cancer cells.

Significance: TRAF6 plays a vital role in the progression of colorectal cancer. What's more, research elucidating the biological mechanisms of TRAF6 can treated as potential therapeutic target for colorectal cancer.

1. Introduction

Colorectal cancer (CRC) is the world's fourth mostly deadly cancer [1]. CRC has a poor prognosis and several etiological risk factors were associated the progression including sedentary lifestyle, smoking, alcohol consumption, low levels of physical activity, red meat intake [2,3]. However, the specific mechanism causes and promotes of colorectal cancer keep largely unknown.

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is an intracellular signal adapter molecule that mediates the tumor necrosis factor receptor superfamily and the interleukin-1 receptor/Toll-like receptor family [4]. TRAF6 is essential for many biological processes, including the innate immune system and the formation of bone, lymphoproliferative disease, and tumor angiogenesis [5–8]. TRAF6 has been also found to be involved in promoting tumor progression in cancers [9,10]. TRAF6 can promote the metastasis of esophageal squamous cell carcinoma and tumorigenicity of pancreatic cancer cells [11,12]. TRAF6 is highly expressed in lung cancer tissues and regulated the proliferation of osteosarcoma cells [13,14].

However, the role and mechanism of TRAF6 in colorectal cancer remains poorly understood. Therefore, in this study, we investigated the expression, translocation and function of TRAF6 in colorectal cancer and studied its effect on proliferation, migration and invasion.

2. Materials and methods

2.1. Patient samples and data collection

The study of human colorectal cancer tissue was approved by the Ethics Committee of the First Hospital Affiliated of Fujian Medical University. 100 formalin-fixed and paraffin-embedded colorectal cancer samples and 100 samples of normal colorectal tissues that were located

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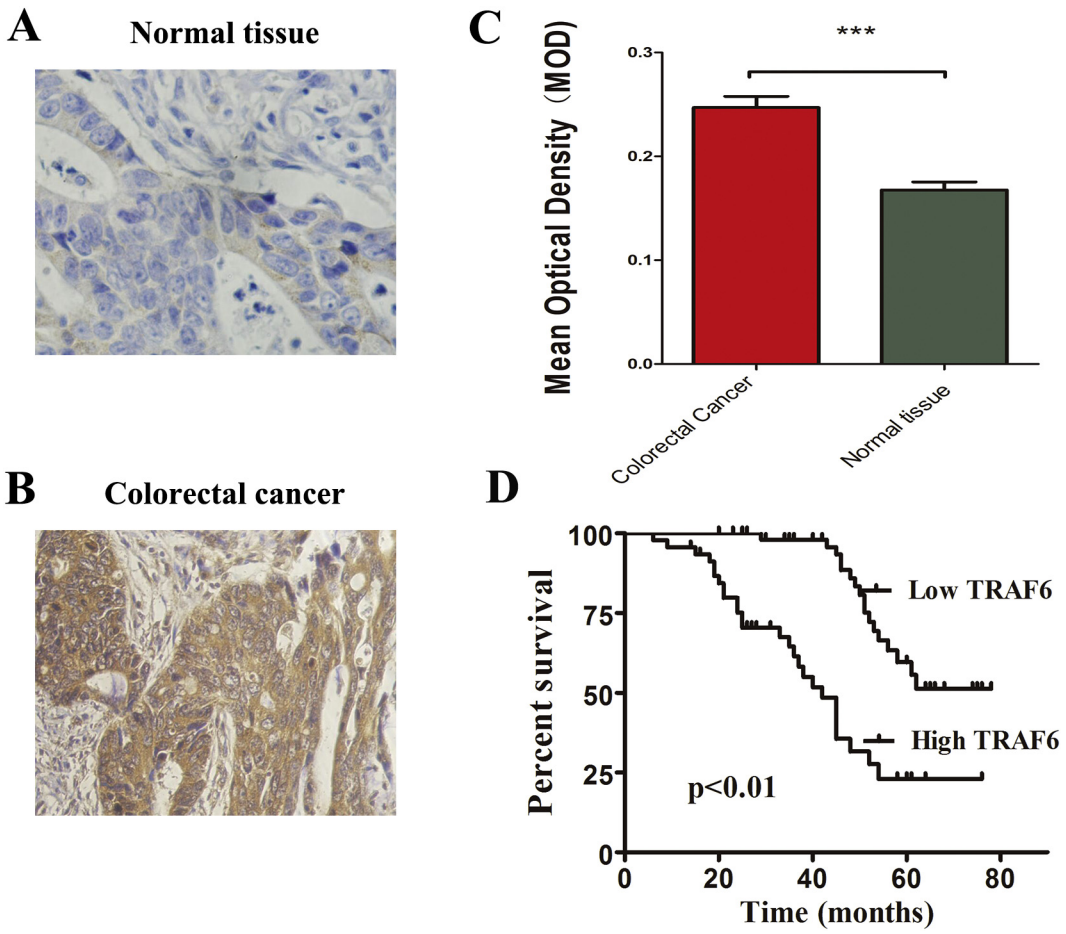

Fig. 1. Expression of TRAF6 in colorectal cancer clinical samples. (A) The staining intensity of TRAF6 was lower in normal colorectal tissues than in colorectal cancer tissues (B) at 200× magnification. (C) The MOD of TRAF6 was higher for tumor tissues than normal tissues. (D) The survival and prognosis of the low expression TRAF6 group was significantly longer than the high expression of TRAF6 group. Data are shown as mean ± SD. (***$p < 0.001$).

away from the cancer. The patient group samples were conserved during the period from 2010 to 2012, and the patient group was composed 51 men and 49 women. All patients with colorectal cancer were diagnosed by pathologist. The colorectal cancer patients we collected were sporadic, with no familial and genetic predispositions including Lynch syndrome, hereditary familial polyposis and other genetic colorectal cancers. And in order to exclude the influence of microsatellite instability (MSI), we did not select MSI patients. All colorectal cancer patients included in present study had not been given any preoperative chemotherapy or other therapy such as radiotherapy. The patients were followed-up by telephone or outpatient service.

2.2. Immunohistochemistry and evaluation

The immunohistochemistry and evaluation were carried out as previously described [15]. The following primary antibodies were used: mouse monoclonal anti-human TRAF6 antibody (1:500, Affinity). The expression of TRAF6 was evaluated using the Image-Pro Plus 6.0 software (Media Cybernetics, Inc. USA). Semiquantitatively analyzed the expression of TRAF6 in each group using Mean Optical Density (MOD) = the Integral Optical Density (IOD) / the positive area.

2.3. Cell culture

SW480 and HCT116 cell lines were obtained from cell bank of the Chinese Academy of Science (Shanghai, China). Both cell lines were culture in RPMI1640 with L-glutamine supplemented with 10 fetal bovine serum in 5% CO_2.

2.4. TRAF6 siRNA transfection and construction of lentiviral vectors and infection

According to the siRNA design principle, two suitable siRNA target sequences (si1329 and si1108), from human TRAF6 gene GenBank, were synthesized as follows respectively: si1329: F:5′-GCAGTGCAATG GAATTTATTT-3′; R:ATAAATTCCATTGCACTGCTT si1108: F:5′-TTCTC CGAACGTGTCACGTTT-3′ R:5′-ACGTGACACGTTCGGAGAATT-3′. In order to create better stable transfection for later experiments, we construction lentiviral-mediated siRNA targeting TRAF6. Si1329 and si1108 sequences were inserted in the psuper plasmids. And co-transfected with lentivirus plasmids pMDL, p-VSV-G and p-REV in 293T cells. The specific experimental steps were carried out as previously described [16].

2.5. RNA extraction and real-time quantitative PCR

RNA extraction was performed according to the kit instructions (Qiagen RNeasy kit). The extracted RNA was reversely transcribed into cDNA, the reagents were obtained from the RT Reagent Kit (TaKaRa, Dalian, China), and the experimental procedures refer to the operation manual of the kit. All the primers (TRAF6:GAPDH) were purchased from BioSune Biotechnology Co., Ltd. Fuzhou, Fujian province as follows: TRAF6 F:5′-ATGCGGCCATAGGTTCTGC-3′ R:5′-TCCTCAAGATGT CTCAGTTCCAT-3′; GAPDH F:5′-GAAGGTGAAGGTCGGAGTC-3′ R: 5′-GAAGATGGTGATGGGATTTC-3′. The specific experimental steps were carried out as previously described [16].

Table 1
Relationship between TRAF6 expression and colorectal cancer clinicopathologic parameters.

Factors	N	TRAF6 expression (MOD ± SD)[a]	*p*
Age (year)			
< 60	53	0.2609 ± 0.0149	0.1071
≥60	47	0.2269 ± 0.0144	
Gender			
Male	51	0.2352 ± 0.0135	0.3916
Female	49	0.2535 ± 0.0166	
Tumor size (cm)			
≤5	56	0.2349 ± 0.0136	0.2054
> 5	44	0.2625 ± 0.0172	
Gross pathology classification			
Infiltrating type	55	0.1734 ± 0.097	0.3678
Ulcerating type	45	0.2587 ± 0.1241	
Clinical stage[b]			
I–II	29	0.1944 ± 0.0154	0.0017*
III–IV	71	0.2680 ± 0.0130	
T stage			
II	29	0.1944 ± 0.0832	0.0018*
III	48	0.2823 ± 0.1149	
IV	23	0.2388 ± 0.09925	
Lymph node metastasis			
Positive	72	0.2787 ± 0.0126	< 0.0001*
Negative	28	0.1674 ± 0.0112	
Histological grade			
Poorly	30	0.2523 ± 0.1012	0.0181*
Moderately	48	0.2689 ± 0.1176	
Well	22	0.1911 ± 0.07965	

[a] MOD ± SD: Mean Optical Density ± Standard Deviation.
[b] The 7th edition of TNM staging criteria of colorectal cancer the American Joint Committee on Cancer (AJCC) and Union for International Cancer.
* $p < 0.05$.

2.6. Western blot analysis

The experiments and results analysis were carried out as described previously [15,16]. The following primary antibodies were used from the different companies under the listing, respectively: Flag (1:1000, Abcam), TRAF6 (1:1000, Affinity), GAPDH (1:1000, Abcam), NF-κB p65 (1:1000, Affinity), p-NF-κB p65 (1:1000, Abcam), c-jun (1:1000, Bioss), p-c-jun (1:1000, Bioss), and LaminB (1:1000, Abcam).

2.7. Cell proliferation, colony formation and invasion assays

Colorectal cancer cells are planted in 96-well plates at a density of 1000 per well, 3 replicate holes in each experimental group. The cells were cultured in a 10% fetal bovine serum (FBS) medium in a 5% CO_2, 37 °C incubator for 1 h, 24 h, 48 h, and 72 h, and then discarded the culture solution, add 10% CCK-8 (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) serum-free medium, counted by microplate reader (Bio-Tek, Winooski, VT, USA) after continuing to culture for 1 h.

For colony formation assay, 500 colorectal cancer cells were transplanted on a 6 cm plate, HCT116 was cultured for 10 days, and SW480 was cultured for 14 days in a culture medium containing 10% fetal bovine serum (FBS). Then discard the culture solution and infiltrate the methanol. After 10 min, the cells were stained with crystal violet. Washed several times with water, dried, photographed and counted.

For cell invasion assay, about 5×10^4 cells with serum-free medium were seeded into the upper chamber of 8 μm pore transwells (Corning, NY 14831, USA) coated with Matrigel (BD Bioscience, USA). Medium containing 10% FBS was added to the lower chamber. After 48 h incubation, cells attaching to lower surface of the chamber were fixed with methanol and then stained with 0.1% crystal violet. Experiments were independently repeated in triplicate.

2.8. Wound healing and cell migration assays

For wound healing assay, colorectal cancer cells were implanted in 6-well plates, when the cells were covered with the 6-well plate, scratch the cell layer with a 20 μl pipette tips, then replaced the cell culture supernatant with serum-free medium and continue to culture cells in 5% CO_2, 37 °C. The cells were photographed at 0, 24, 48 and 72 h.

For migration assay, the Transwell migration chamber experiment was used and the remaining steps were the same as the invasion experiments.

2.9. Dual luciferase reporter assay

Seeding 10×10^4 colorectal cancer cells in a 24-well plate, after the cells are attached to the culture dish, the cells are transfected with pNF-KB (Promega, USA), pAP-1 (Promega, USA) and pRL-TK report vector (Addgene) by transfection reagent Lip3000 (Invitrogen, USA). After 48 h, the relative activity of luciferase was determined by the dual luciferase reporter kit (Promega, USA). The pRL-TK vector was used as the internal reference plasmid, pNF-KB and pAP-1 as the plasmid of interest.

2.10. Cellular immunofluorescence staining assay

Planting colorectal cancer cells in 8-μm-thick chip, after the cells were attached, pcDNA3.1 and pcDNA3.1-Flag-TRAF6 plasmids were transfected as control group and experimental group. After 48 h of transfection, through pre-cooled PBS washed, 4% paraformaldehyde fixed, 0.5% Triton-100 permeabilized, 10% BSA blocked, 1:50 diluted TRAF6, p-p65, p-c-jun antibody incubation, 1:200 fluorescence and DAPI staining, then visualized the cells under a laser scanning confocal microscope (Leica, Germany).

2.11. Animal studies

Animal experiments were conducted at the Animal Experimental Center of Fujian Medical University and approved by the Animal Protection and Use Committee of Fujian Medical University. 4×10^6 HCT116-pSuper and HCT116-pSupe-sh2 cells in serum-free DMEM medium were injected into the left and right thighs of the nude mice of about 5 weeks old. A few days later, when the tumor on the surface of the nude mouse can be touched, the tumor volume is tested every 4 days by the formula: length × width 2/2.32 days after the tumor could be touched, the nude mice were sacrificed by drugs and removed the tumor, then weighed the tumor and photographed. Soak the tumor in formalin for further experiments.

2.12. Statistical analysis

The experimental data obtained were analyzed using GraphPad Prism 5 software, and statistical analysis was performed using one-way ANOVA or *t*-test. All data were presented as mean ± SD, the *p*-value < 0.05 has statistically significant.

3. Results

TRAF6 was high expressed in human colorectal cancer and was associated with tumor progression and poorly prognosis. Analyzed the expression of TRAF6 in 100clinical samples from colorectal cancer patients and 100 normal colorectal tissues located away from the cancer site using immunohistochemistry technology. In the normal tissues, low levels of TRAF6 expression were found in the colorectal tissues (Fig. 1A); however, the colorectal cancer tissues had high levels of expression of TRAF6 (Fig. 1B). The MOD of TRAF6 expression in normal colorectal tissues (0.1678 ± 0.0077) was markedly lower than colorectal cancer tissues (0.2471 ± 0.0108, $p < 0.001$) (Fig. 1C).

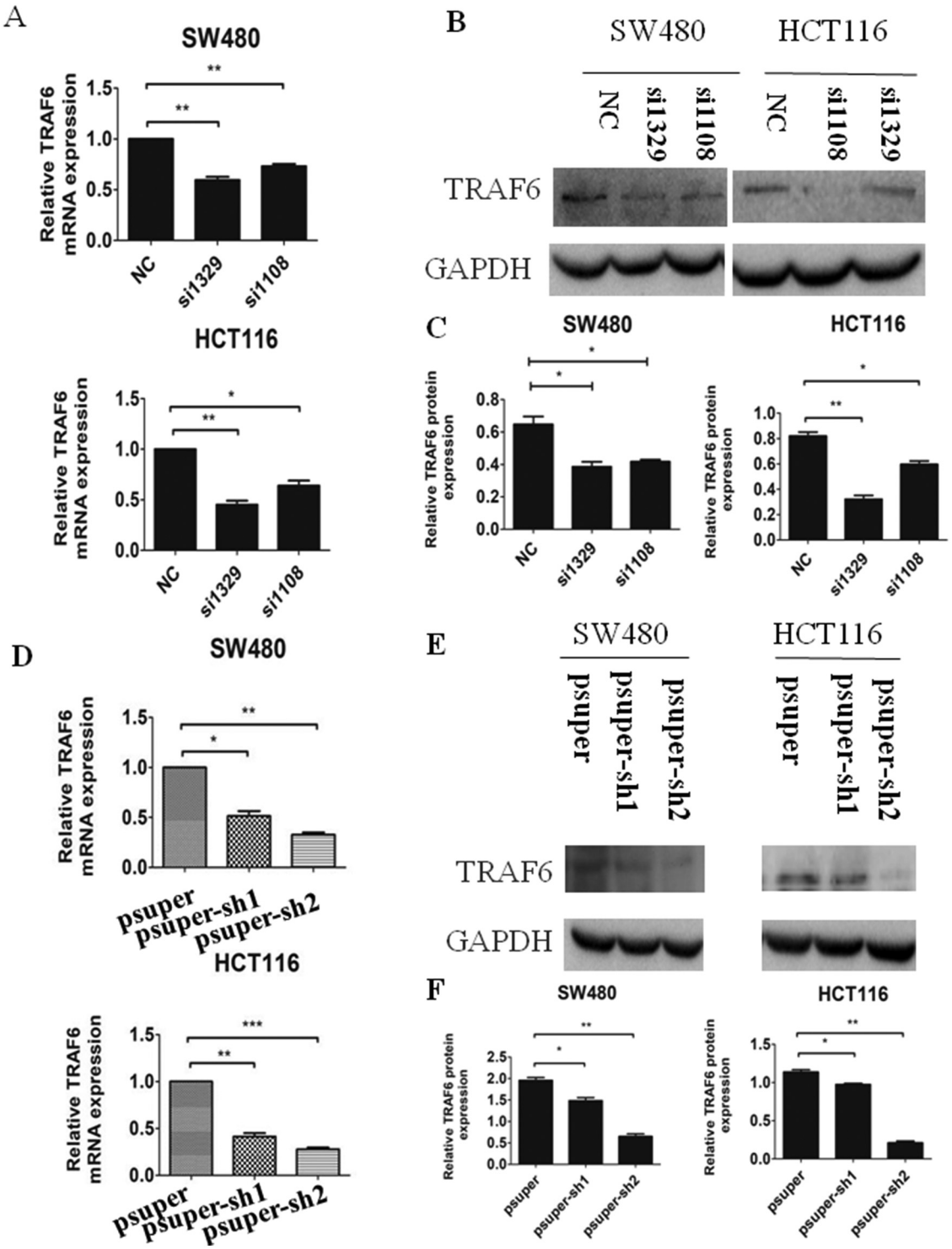

Fig. 2. Construction of stable TRAF6 knockdown colorectal cancer cell lines. (A) qPCR confirmed that si1329 and si1108 can knockdown the TRAF6 mRNA in the SW480 and HCT116 cells. (B and C) Western blot experiment showed that that si1329 and si1108 inhibited the expression of TRAF6 protein in the SW480 and HCT116 cells. (D) qPCR confirmed that psuper-sh1 and psuper-sh2 can knockdown the TRAF6 mRNA in the stable transfection siRNA sequences SW480 and HCT116 cells. (E and F) The efficiency of knockdown TRAF6 protein in the SW480 and HCT116 cells was confirmed by western blot. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Thus, TRAF6 high expression may promote progression of colorectal cancer.

Table 1 reveals the relationship between TRAF6 expression and clinicopathologic parameters. TRAF6 expression did not very markedly with age, gender, tumor size and gross pathology classification. But there was a significantly relationship between TRAF6 high expression and clinical stage ($p = 0.0026 < 0.05$), T stage ($p = 0.0018 < 0.05$), lymph node metastasis ($p = 0.038 < 0.05$), histological grade ($p = 0.0181 < 0.05$). The colorectal cancer samples were split into two groups according the MOD expression levels: a low TRAF6 expression levels group (< 0.2498, which is the average MOD values of 100 colorectal cancer patients of tissues) and a high TRAF6 expression

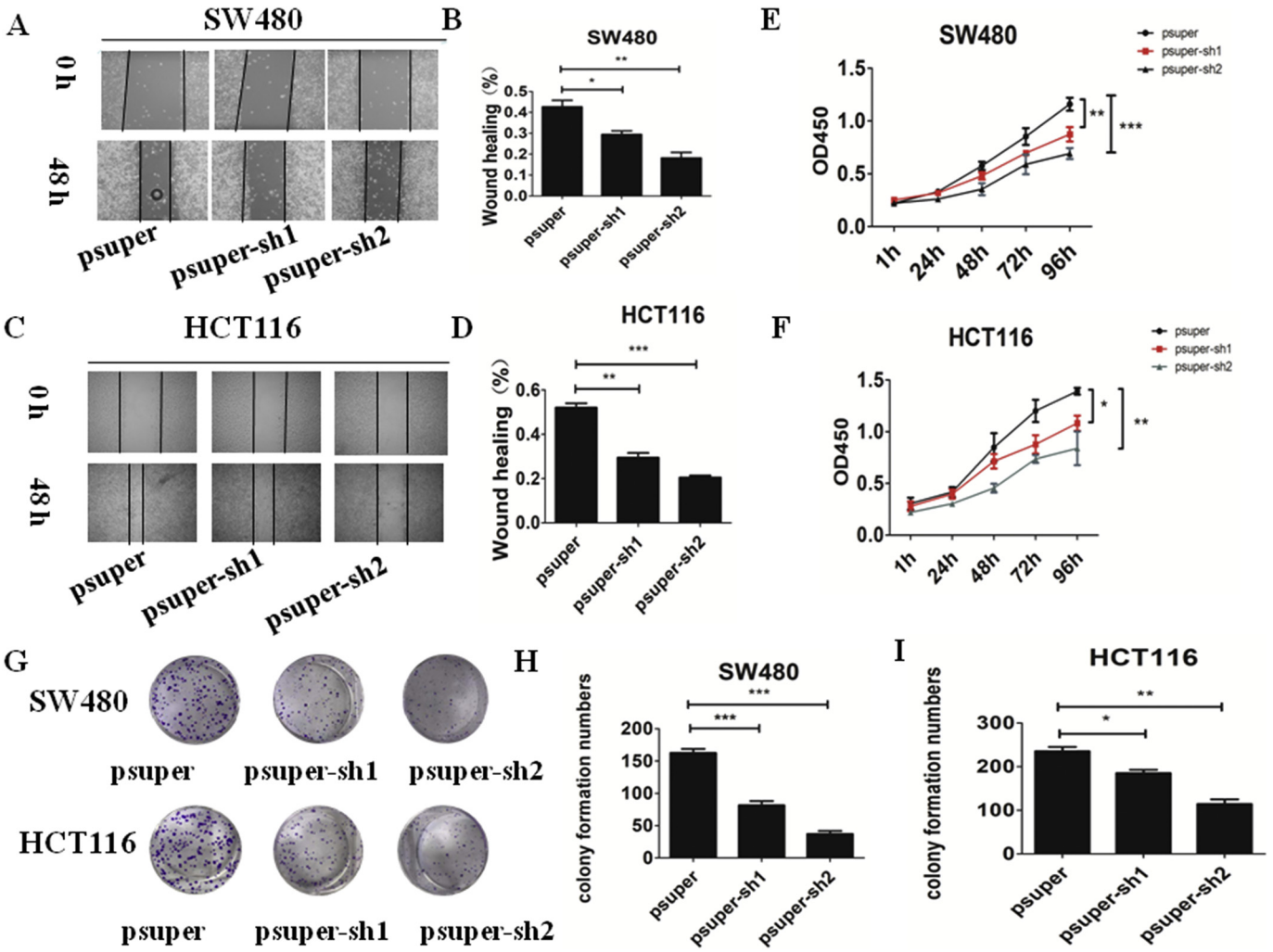

Fig. 3. Knockdown TRAF6 inhibits colorectal cancer cells proliferation, colony formation and wound healing in vitro. (A, B, C and D) Wound healing assays were used to estimate the effects of knockdown TRAF6 on the SW480 and HCT116 cells wound healing abilities. (E and F) CCK-8 assays revealed the effect of down-regulation of TRAF6 on SW480 and HCT116 cells proliferation. (G, H and I) Cells colony formation assays were used to evaluate the effects of knocking down TRAF6 on SW480 and HCT116 cells proliferation. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

levels group (≥0.2498). Additionally, the patients with high expression of TRAF6 had very discouraging prognosis; however, the patients with low expression of TRAF6 had a better prognosis. The survival and prognosis of the low expression of TRAF6 group was significantly longer than the high expression of TRAF6 group ($p < 0.01$) (Fig. 1D).

3.1. Si1329 and si1108 target TRAF6 knockdown the mRNA and protein of TRAF6 in temporary transfection and stable infection SW480 and HCT116 cells

To investigate the effect of TRAF6 in colorectal cancer cells, we used a method of silencing TRAF6. Si1329 and si1108 were transiently transferred into SW480 and HCT116 cells. qPCR revealed a decreased in TRAF6 mRNA in the si1329 and si1108-transfected cells. The TRAF6 mRNA was higher suppressed in si1329 and si1108-transfected cells than negative control (NC) cells in the SW480 and HCT116 cells (Fig. 2A). The same as TRAF6 mRNA expression, the protein expression levels of TRAF6 was also markedly inhibited by the si1329 and si1108 in the SW480 and HCT116 cells (Fig. 2B and C). Because the semi-quantitative analysis by qPCR and western blot showed that there was significantly inhibition between NC and si1329, si1108 groups in the SW480 and HCT116 cells. So, selected si1329 and si1108 sequences to construct lentivirus- mediated vectors to knock down the TRAF6 in further studies named as psuper, psuper-sh1 and psuper-sh2. The viral packing system was used to obtain the viral supernatants including the psuper, psuper-sh1 and psuper-sh2. The supernatants were using to infect SW480 and HCT116 cells. After 2 weeks of screening with puromycin got the stable cell lines that stably expression the relevant sequences of psuper, psuper-sh1 and psuper-sh2. By qPCR and western blot experiments verified the successful construction of knockdown of TRAF6 stable strains, and the mRNA and protein expression of TRAF6 in the psuper-sh1 and psuper-sh2 groups markedly decreased compared with the psuper group (Fig. 2D–F).

3.2. Knockdown the TRAF6 inhibits colorectal cancer cell proliferation, migration and invasion in vitro

To verify the role of TRAF6 in colorectal cancer cells, a series of in vitro cell biology assays were performed. Wound healing and cell migration assays showed that knockdown the TRAF6 significantly inhibited SW480 and HCT116 cells migration, respectively, compared with their negative controls (Fig. 3A–D, and Fig. 4A and B). The role of TRAF6 in suppressing proliferation of colorectal cancer cells was confirmed by CCK-8 and colony formation assays (Fig. 3E–I). Subsequently, transwell assays showed that knockdown of TRAF6 could significantly weaken, respectively, the invasive abilities of SW480 and HCT116 cells

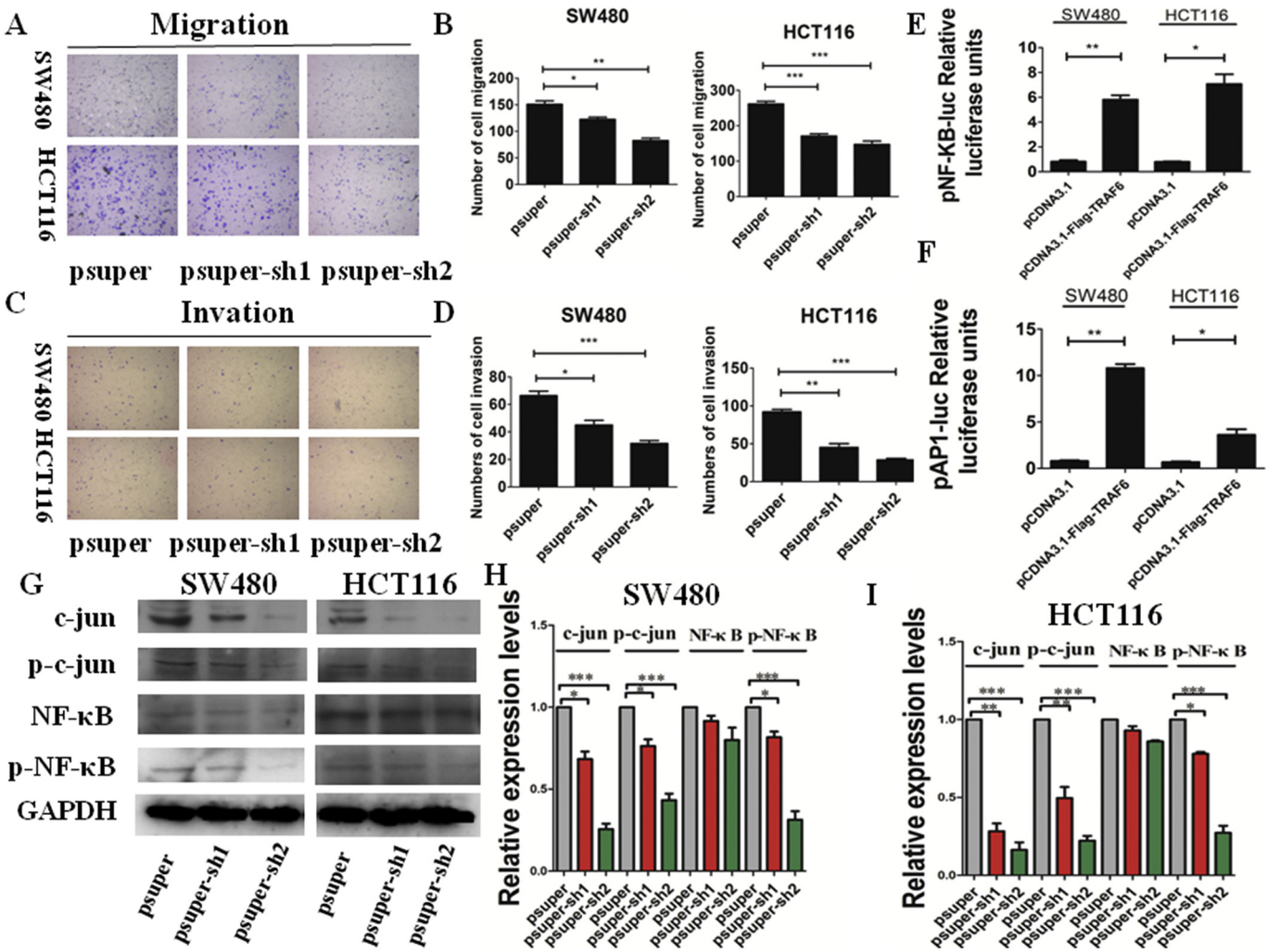

Fig. 4. Knockdown TRAF6 inhibits colorectal cancer cells migration, invasion and decreases the activity of NF-κB and AP-1 signaling pathways. (A, B and C, D) Transwell assays were performed to determine the migratory and invasive abilities of psuper, psuper-sh1 and psuper-sh2 infected SW480 and HCT116 cells. (E and F) The over-expression of TRAF6 significantly increased the luciferase activities of pNF-κB and pAP-1 in the SW480 and HCT116 cells. (G, H and I) TRAF6 knockdown expression down-regulated the protein expression of NF-κB and AP-1 signaling pathways in the SW480 and HCT116 cells. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

(Fig. 4C and D). These data indicate that TRAF6 could affect colorectal cancer progression in vitro.

3.3. Knockdown of TRAF6 inhibits NF-κB and AP-1 signal pathways

In vitro experiments, knockdown TRAF6 affects the biobehavioral changes of colorectal cancer cells. The underlying mechanisms of TRAF6 cause changes in the SW480 and HCT116 cells remain largely unknown. Some research have shown that TRAF6 can affect the NF-κB and AP-1 signal pathways. So, the dual luciferase reporter experiments of NF-κB and AP-1 were performed to confirm these views, and the results showed that when transfected pcDNA3.1-Flag-TRAF6 plasmids into colorectal cancer cells, pNF-κB luciferase activity was markedly increased than that of transfected pcDNA3.1ss (Fig. 4E). The same as pNF-κB, pAP-1 luciferase activity was also increased several fold after transfection of pcDNA3.1-Flag-TRAF6 plasmids into SW480 and HCT116 cells (Fig. 4F). Next, western blot assays to verify the effect on NF-κB and AP-1 protein levels, results found that the protein expression levels of pNF-κB, AP-1 and pAP-1 were decreased in different degrees in SW480 and HCT116 stably transfected with TRAF6 knockdown (Fig. 4G–I). These revealed that knockdown of TRAF6 suppressed the activity of NF-κB and AP-1 signal pathways.

3.4. TRAF6 nuclear shuttle affects pNF-κB and p-c-jun into the nucleus

After 48 h transfection of pcDNA3.1-Flag-TRAF6 plasmids into SW480 and HCT116 cells, separated nucleus and cytoplasm proteins of colorectal cancer cells. Then western blot analyzed the Flag, p-p65 and p-c-jun proteins expression in nucleus and cytoplasm proteins. When transfected pcDNA3.1-Flag-TRAF6 plasmids into SW480 and HCT116 cells, the Flag protein can be found in the nucleus and cytoplasm protein (Fig. 5A). However, the transfected pcDNA3.1 plasmids into SW480 and HCT116 cells, the Flag protein cannot be found in the nucleus and cytoplasm protein (Fig. 5A). And the more p-p65 and p-c-jun proteins can be detected in nucleus, when transfected pcDNA3.1-Flag-TRAF6 plasmids into SW480 and HCT116 cells, than pcDNA3.1 plasmids transfection colorectal cancer cells (Fig. 5A). The same as western blot assays, the cellular immunofluorescence staining assay showed that TRAF6, p-p65 and p-c-jun co-localization in the nucleus of SW480 and HCT116 cells after transfection of pcDNA3.1-Flag-TRAF6 was significantly more than that in transfected pcDNA3.1 (Fig. 5B). These results suggested that TRAF6 nuclear shuttle affects p-p65 and p-c-jun into the nucleus, which in turn affects the biological behavior of SW480 and HCT116 cells.

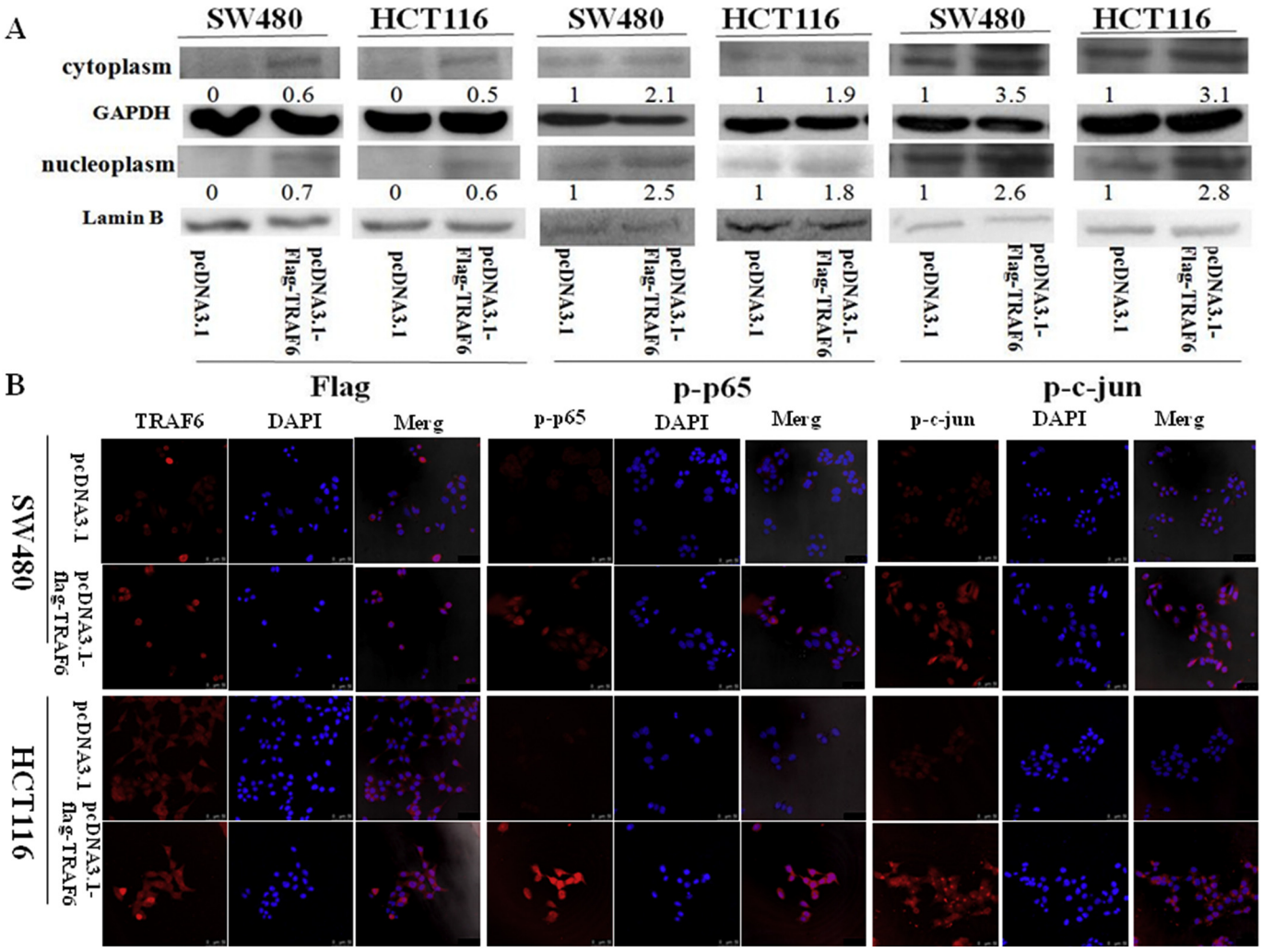

Fig. 5. The nuclear shuttle of TRAF6 increases p-p65 and p-c-jun into the nucleus. (A) Transfection of pcDNA3.1-Flag-TRAF6 into SW480 and HCT116 cells detected Flag protein in the cytoplasm and nucleus. Similarly, transfection of pcDNA3.1-Flag-TRAF6 into SW480 and HCT116 cells increased the amount of p-p65 and p-c-jun in the nucleus. (B) The over-expression of TRAF6 could increase p-p65 and p-c-jun into nucleus using Immunofluorescence method.

3.5. TRAF6 knockdown inhibits the growth of subcutaneous tumors in vivo

The study has verified that TRAF6 knockdown can affect the biological behavior of colorectal cancer cells. Next, further we explored whether TRAF6 knockdown colorectal cancer cells have the same effect in animals. In this experiment, 2×10^6 HCT116-psuper and HCT116-psuper-sh2 cells were injected into the bilateral thigh regions of nude mice. We made a tumor volume growth curve, and found the growth rate of subcutaneous tumors in psuper-sh2 is significantly slower than that on the psuper groups (Fig. 6E). We did not find metastasis of the tumor in the liver, lung, etc. after dissecting the mouse, at the same time, we made a model of tumor contrast, the result revealed that the size of psuper-sh2 is significantly smaller than that of the psuper group (Fig. 6A). Similarly, the weight of the tumor in the psuper group was significantly heavier than that in the psuper group (Fig. 6D). We further performed immunohistochemistry of tumors and found that the intensity of TRAF6 staining was significantly weaker in the psuper-sh2 group (Fig. 6B and C).

4. Discussion

TRAF6 is a key signal transduction molecular of the toll-like receptor (TLR) pathway [17], which plays an important role in innate and adaptive immunity [18]. TRAF6 plays an important role in NF-κB and mitogen-activated protein kinase (MAPK/AP-1) signaling pathways [19]. Recent studies reported that TRAF6 promotes tumorigenesis by inhibiting apoptosis, promoting tumor proliferation and invasion [20]. In addition, TRAF6 has also been reported to be highly expression in various cancers [21]. Therefore, TRAF6 may be as a potential therapeutic target for tumors and it is of significance to study the role of TRAF6 in colorectal cancer and to explore its potential therapeutic target for colorectal cancer.

In this study, we found that TRAF6 high expression in colorectal cancer tissues. And there was a significantly relationship between TRAF6 high expression and clinical stage, lymph node metastasis, T stage and histological grade. Consistent with Zhang et al. [22] research, we also found that the survival and prognosis of the low expression of TRAF6 group was significantly longer than the high expression of TRAF6 group. We constructed vectors for TRAF6 interference and verified its knockdown at RNA and protein levels by qRT-PCR and western-blot techniques. Next, we verified whether knocking down TRAF6 can affect the biological behavior of colorectal cancer cells. And through relevant cell scratch assays, cell proliferation assays, plate colony formation assays, cell migration and invasion assays, we found that knockdown of TRAF6 can attenuate the proliferation, migration and invasion of colorectal cancer cells SW480 and HCT116 cells. Since TRAF6 can affect the biological behavior of colorectal cancer cells in in vitro cell experiments, does it have the same effect in the in vivo? We

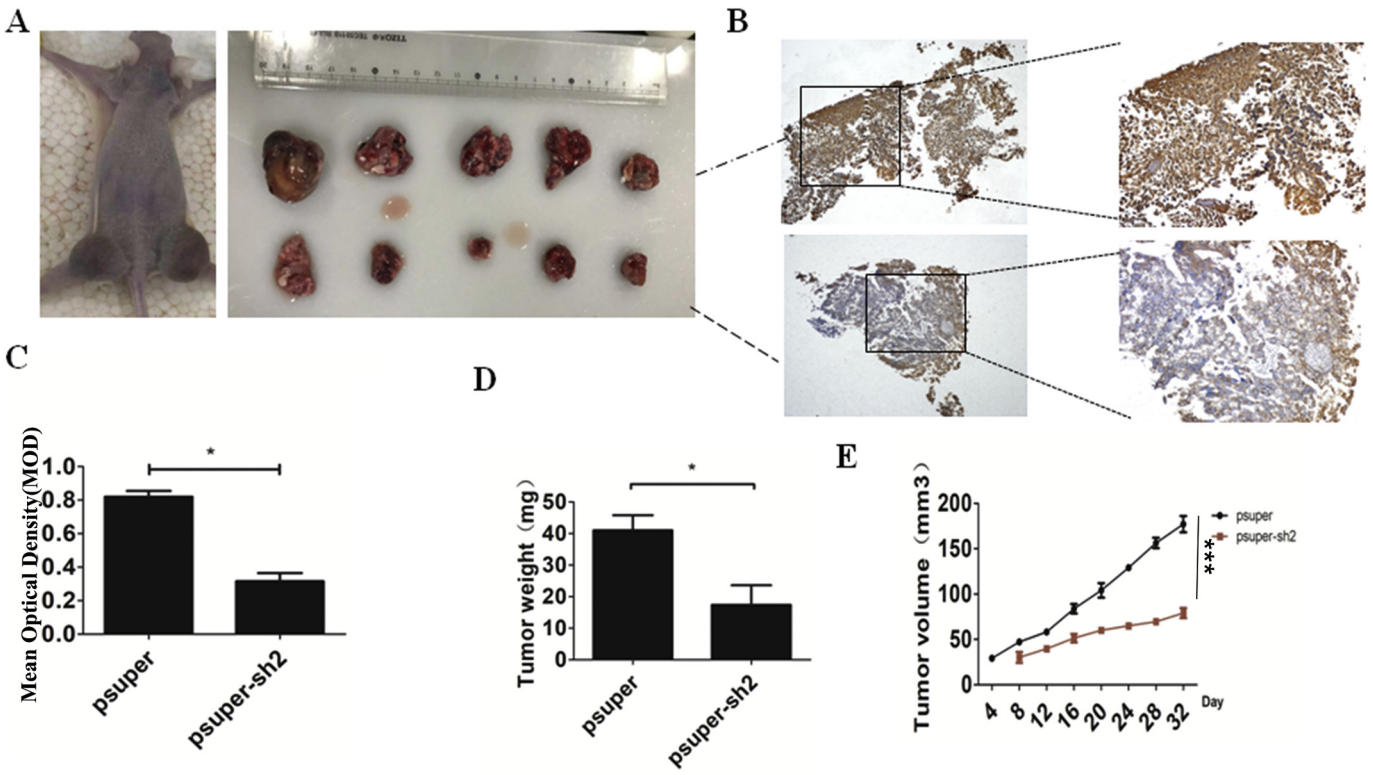

Fig. 6. Knockdown of TRAF6 in HCT116 cells inhibits subcutaneous tumor growth. (A and E) Tumors in nude mice were harvested, and volumes were measured 5 weeks after initiation. (B) Immunohistochemistry analysis of the expression of TRAF6 in HCT116 cells subcutaneous xenograft tumors showed the expression of TRAF6 (40×; 200×) in psuper groups and psuper-sh2. (C) The results of MOD showed that the expression of TRAF6 in psuper-sh2 group was decreased compared with the psuper group. Bars represent the Mean Optical Density (MOD) ± Standard Deviation (SD). (D) The weights of tumors were weighed. The weights of psuper-sh2 group were decreased compared with the psuper group. (*$p < 0.05$; ***$p < 0.001$).

then conducted animal experiment to inject colorectal cancer cells into the subcutaneous of nude mice. Similarly, TRAF6 knockdown can also attenuate tumor proliferation in nude mice.

How TRAF6 activates NF-κB and c-jun has not been fully elucidated so far. TRAF6 was initially identified as a signal transducer [23]. Song et al. [24] reported that overexpression of TRAF6 activates NF-κB and c-jun; the mechanisms could be the NF-κB-inducing kinase (NIK) which can activate the NF-κB and c-jun binds with TRAF6. And studies indicate that TRAF6 can bind directly to the receptor intra cellular domain, such as CD40 receptor activated NF-κB [25,26]. And TRAF6 can be recruited into the signaling pathways (IL-1, IL-8, IL-6 and LPS signaling pathway) as very crucial adapter proteins to activate the NF-κB and AP-1 [27–29]. The ubiquitination of TRAF6 may be essential for NF-κB and MAPK signaling pathway activation [30,31].

Next, we are interested in why TRAF6 knockdown can cause biohavioral changes in colorectal cancer cells. Some studies have shown that TRAF6 can induce toll-like receptors (TLRs)/NF-κB and MAPK/AP-1 signaling pathways [32–34]. These signaling pathways cause the release of inflammatory cytokines and affect cell apoptosis and proliferation [35,36]. We then examined the luciferase activity of NF-κB and AP-1 after overexpression of TRAF6 to confirm that TRAF6 promotes the expression of luciferase activity of NF-κB and AP-1. In addition, we also found that TRAF6 knockdown inhibits the expression of p-p65, c-jun and p-c-jun at protein levels in colorectal cancer cells SW480 and HCT116. These results confirm that TRAF6 knockdown attenuates the activation of downstream pathway signals NF-κB and AP-1. But, it is still unclear how TRAF6 worked on colorectal cancer cells.

TRAF6 is distributed in the cytoplasm and nucleus and mainly distributed in the cytoplasm [37]. NF-κB is distributed in the cytoplasm and translocates into the nucleus when stimulated, and MAPK is involved in the activation of NF-κB [35]. We hypothesized that activation of TRAF6 and its downstream NF-κB and MAPK signaling pathways occurred in the nucleus, through the cytoplasmic-nuclear approach. We confirmed that TRAF6, p-p65, and p-c-jun had more nuclear colocalization in the overexpressed TRAF6 group than in the control group. In addition, we also verified the expression of TRAF6-flag in the nucleus, the expression of p65 and c-jun in the cytoplasm and nucleus by separating the cytoplasm and nucleus of the cells. The results implied that activation of the TRAF6-NF-κB/AP-1 signaling pathway is carried out in the nucleus of colorectal cancer cells.

5. Conclusion

In summary, we detected that TRAF6 high expression in colorectal cancer tissues. And there was a significantly relationship between TRAF6 high expression and clinical stage, lymph node metastasis. We demonstrated that TRAF6 knockdown can inhibit proliferation, migration, and invasion of colorectal cancer cells in vitro and in vivo experiments. We also verified that TRAF6 activates the TRAF6-NF-κB/AP-1 signaling pathway by entering the nucleus, causing biobehavioral changes in colorectal cancer cells. These studies showed TRAF6 plays a vital role in the progression of colorectal cancer and TRAF6 can treated as a potential therapeutic target for colorectal cancer.

Declaration of competing interest

The authors declare that they have no competing interests and the manuscript is approved by all authors for publication.

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