Tristetraprolin activation by resveratrol inhibits the proliferation and metastasis of colorectal cancer cells

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Abstract. Resveratrol (RSV) is a polyphenolic compound that naturally occurs in grapes, peanuts and berries. Considerable research has been conducted to determine the benefits of RSV against various human cancer types. Tristetraprolin (TTP) is an AU-rich element-binding protein that regulates mRNA stability and has decreased expression in human cancer. The present study investigated the biological effect of RSV on TTP gene regulation in colon cancer cells. RSV inhibited the proliferation and invasion/metastasis of HCT116 and SNU81 colon cancer cells. Furthermore, RSV induced a dose-dependent increase in TTP expression in HCT116 and SNU81 cells. The microarray experiment revealed that RSV significantly increased TTP expression by downregulating E2F transcription factor 1 (E2F1), a downstream target gene of TTP and regulated genes associated with inflammation, cell proliferation, cell death, angiogenesis and metastasis. Although TTP silencing inhibited TTP mRNA expression, the expression was subsequently restored by RSV. Small interfering RNA-induced TTP inhibition attenuated the effects of RSV on cell growth. In addition, RSV induced the mRNA-decaying activity of TTP and inhibited the relative luciferase activity of baculoviral IAP repeat containing 3 (cIAP2), large tumor suppressor kinase 2 (LATS2), E2F1, and lin-28 homolog A (Lin28) in HCT116 and SNU81 cells. Therefore, RSV enhanced the inhibitory activity of TTP in HCT116 and SNU81 cells by negatively regulating cIAP2, E2F1, LATS2, and Lin28 expression. In conclusion, RSV suppressed the proliferation and invasion/metastasis of colon cancer cells by activating TTP.

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Introduction

Colorectal cancer is one of the most common cancer types worldwide and the second most lethal cancer in the USA (1); its incidence has been increasing in Asian countries, including South Korea, and it has become a severe public health problem worldwide (2-4). Although surgery remains the most effective treatment for patients with colorectal cancer, the majority of patients experience relapse within 5 years following complete surgical resection (5-7). Given the heterogeneous properties of colorectal cancer tumors, patients with colorectal cancer have a poor overall survival in response to treatment (8,9). In addition, similar histopathological tumors may elicit considerably different clinical courses (10,11). Therefore, for improved treatment and management of patients with colorectal cancer, knowledge of each cancer property that is associated with the differential responses to drug treatment is imperative for predicting patient outcomes and developing novel therapeutic targets may be beneficial.

Resveratrol (3,5,4'-trihydroxystilbene; RSV) is a polyphenolic compound that naturally occurs in grapes, peanuts and berries (12). Considerable research has been conducted to determine the benefits of RSV against various human cancer types (12-16). RSV has anti-proliferative properties against numerous cancer types, including those of the liver, breast, prostate, lung and colorectum (13,17-22). RSV induces apoptosis and inhibits cell growth, cell cycle progression, migration and invasion (19,22). RSV has antitumor effects in colorectal cancer by inhibiting various carcinogenic processes (21,23-26). Gong *et al* (27) demonstrated that RSV inhibited colorectal cancer proliferation by suppressing cyclooxygenase-2 (COX-2) expression. Karimi Dermani *et al* (28) reported that RSV inhibited proliferation, invasion and epithelial-mesenchymal transition by increasing miR-200c expression in HCT-116 colorectal cancer cells. Furthermore, sirtuin 1 is required for RSV-mediated chemopreventive effects in colorectal cancer cells (29). Hence, RSV serves an important role in colorectal

cancer. However, the precise molecular mechanism underlying the effect of RSV on colorectal cancer has not been clearly understood.

Tristetraprolin (TTP) is an AU-rich element (Res)-binding protein that regulates mRNA stability (30) and is a key protein involved in regulating cytokine expression (31). Decreased TTP expression is observed in patients with colorectal, lung, breast and pancreatic cancer, and TTP dysfunction serves as an important indicator of cancer development (32-37). TTP suppresses the growth of human colon cancer cells by regulating vascular endothelial growth factor expression (38). Recent studies have addressed the association between RSV and TTP in cancer. Ryu *et al* (39) reported that RSV induced apoptosis by activating TTP in glioma cells, whereas RSV inhibited MCF-7 cell proliferation by upregulating TTP (31). However, the precise association between TTP and RSV in colorectal cancer has not been completely understood. The present study reports for the first time, to the best of our knowledge, that RSV induced apoptosis in colorectal cancer cells by activating TTP. RSV-induced TTP expression inhibited baculoviral IAP repeat containing 3 (cIAP2), large tumor suppressor kinase 2 (LATS2), E2F transcription factor 1 (E2F1), and lin-28 homolog A (Lin28) and MDM2 proto-oncogene (MDM2) mRNAs by binding to their 3' untranslated regions (3'UTRs). Furthermore, RSV-induced TTP upregulation inhibited the cell proliferation and metastasis of human colorectal cells. The results of the present study may increase understanding of the antitumor effects of RSV and suggest future applications for RSV as an anticancer agent in clinical cancer therapy.

Materials and methods

Cell lines and reagents. Colorectal cancer cells (HCT116 and SNU81) were purchased from the Korean Cell Line Bank (Seoul, Korea) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (all Capricorn Scientific GmbH, Ebsdorfergrund, Germany) at 37°C in a humidified 5% CO_2 atmosphere. RSV was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany; cat. no. 274666) and suspended in dimethyl sulfoxide (DMSO; cat. no. D2650; Sigma-Aldrich; Merck KGaA). Colorectal cancer cells were treated with RSV in medium containing 2.5% FBS for 24 h at 37°C.

Plasmid construction and luciferase assay. The pGL3/TTPp-1411 promoter construct (40) and variable target gene of TTP in the psiCHECK2 luciferase reporter constructs [psiCHECK2/cIAP2 (41), E2F1 (42), LATS2 (43), Lin28 (44) and MDM2 3'UTR constructs] were as previously described. Cells were transfected with various types of plasmid constructs using iN-fect™ *in vitro* transfection reagent (Intron Biotechnology, Inc., Seongnam, Korea). For the luciferase assays, HCT116 and SNU81 cells were seeded $1x10^5$ cells/well in a 12-well plate and transfected with the constructs (500 ng) for 24 h, and treated with RSV (5, 10 and 20 μM) for 24 h. Transfected cells were lysed with a lysis buffer (Promega Corporation, Madison, WI, USA) and mixed with the luciferase assay reagent (Promega Corporation). The chemilu-

minescent signal was measured using the Wallac Victor 1420 multilabel counter (PerkinElmer Inc., Waltham, MA, USA). In each sample, the firefly luciferase activity was normalized to the *Renilla* luciferase activity. All luciferase assays reported herein represent at least three independent experiments, each comprising three wells per transfection.

SDS-PAGE and immunoblotting. The total protein was extracted from HCT116 and SNU81 cells using ice-cold radio-immunoprecipitation assay buffer [50 mM Tris HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% (v/v) Triton X-100; 0.1% (w/v) SDS] and a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentrations were determined using the bicinchoninic acid assay, according to the manufacturer's protocol (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The protein samples (10 μg) were loaded onto a 10% SDS-PAGE gel, and transferred onto Hybond-P membranes (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Membranes were blocked using 5% skim milk for 1 h at room temperature and probed using appropriate dilutions of rabbit anti-human TTP (cat. no. T5327; Sigma-Aldrich; Merck KGaA; 1:3,000) and anti-β-actin (cat. no. A2228, Sigma-Aldrich; Merck KGaA; 1:3,000) antibodies overnight at 4°C. Secondary antibody rabbit-IgG (cat. no. ADI-SAB-300; Enzo Life Sciences, Inc., Farmingdale, NY, USA; 1:5,000) was probed for 90 min at room temperature. Immunoreactivity was determined using an enhanced chemiluminescence detection system (GE Healthcare Bio-Sciences). Films were exposed at multiple time points to ensure that the images were not saturated. ImageJ (v.1.51j8; National Institutes of Health, Bethesda, MD, USA) was used to analyze the blot images.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA isolation was performed using TRIzol reagent (Thermo Fisher Scientific, Inc.) and was synthesized to cDNA using Moloney murine leukemia virus reverse transcriptase kit (cat. no. 3201; Beams Biotechnology, Seongnam, Korea) and Oligo-dT primer (cat. no. 79237, Qiagen, Hilden, Germany), according to manufacturer's protocol for 60 min at 37°C. For the PCR (5 μl), the cDNA product, PCR master mix (cat. no. RT500; Enzynomics, Inc., Daejeon, Korea), and a Bio-Rad system (CFX96 Optics Module; Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used while monitoring, in real-time, the increase in the fluorescence of the SYBR Green dye (cat. RT500, Enzynomics Co., Ltd., Daejeon, Korea). The specificity of each primer pair was confirmed by melting curve analysis (45,46). The PCR primer pairs used were as follows: TTP, CGCTACAAGACTGAGCTAT and GAGGTAGAACTT GTGACAGA; β-actin, CCCTGGAGAAGAGCTACGAG and AGGTAGTTTCGTGGATGCCA. PCR cycling conditions were 94°C for 10 min to activate the DNA polymerase, followed by 40 cycles of 94°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec.

MTT assay. For the MTT assay, $1x10^4$ cells/well were seeded in 96-well culture plates with complete RPMI-1640 medium. The cells were subsequently incubated with different RSV concentrations (10, 20 or 50 μM) for up to 72 h. The culture medium was removed, and the cells were incubated with MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA) for 90 min at

37°C. Following incubation for 90 min, the MTT solution was removed, and the formazan product was extracted and diluted with DMSO (cat. no. D2650; Sigma-Aldrich; Merck KGaA) with gentle agitation for 15 min. The absorbance was measured using the VICTOR3 Multilabel Reader 1420 (PerkinElmer) at 490 nm. Three independent experiments were performed in four duplicated wells.

Migration and invasion assay. The effect of RSV on the invasive properties of HCT116 and SNU81 cells was assessed using Boyden chambers (Neuro Probe, Inc., Gaithersburg, MD, USA) that were precoated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and incubated for 1 h at room temperature. The bottom wells were filled with 28 μl medium with 2% serum. In total, $1x10^5$ cells/56 μl were seeded into the upper compartment and incubated for 24 h at 37°C and 5% CO_2. Following incubation for 24 h, the cells attached to the upper surface of the filter were removed using a cotton swab, and those attached to the lower surface were stained using Diff-Quik reagents (Sysmex Corporation, Kobe, Japan) for 3 min at room temperature and counted (five fields/well). The invasion percentage was expressed as the percentage of invading cells through the Matrigel. A representative graph of six independent experiments is presented. Images from each well were immediately captured using the Axiovert 40 CFL inverted fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) in five random fields at x40 magnification. For the migration assay, the membrane was pre-coated with collagen (cat. no. C7661; Sigma-Aldrich; Merck KGaA) and 10% acetic acid for 1 h at room temperature.

Clonogenic assay. HCT116 and SNU81 cells were seeded into 12-well plates at $1x10^4$ cells/well and incubated for 24 h at 37°C and 5% CO_2. The cells were subsequently treated with RSV in a dose-dependent manner and incubated for 10 days at 37°C and 5% CO_2. Fresh medium containing RSV (5, 10 and 20 μM) was added on the 3rd day. On the 10th day, the medium was removed from the plates, and the cells were washed once with PBS. The colonies were fixed and stained with methanol (25% v/v) that contained crystal violet (0.05% w/v) for 30 min at room temperature. Thereafter, the residual staining solution was removed, and the plates were washed with water. When the plates had been rinsed three times with PBS and air-dried, the colonies were counted using ImageJ.

siRNA transfection and treatments. TTP small interfering RNA (siRNA; cat. no. sc-36760) or control siRNA (cat. no. sc-37007; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was transfected (100 nM) into HCT116 and SNU81 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. After 24 h, cells were treated with RSV (20 μM) for 24 h.

Microarray. The mirVana™ miRNA isolation labeling kit (Ambion; Thermo Fisher Scientific, Inc.) was used for isolating total RNA from the HCT116 cells at 90% confluence, according to the manufacturer's protocol. The Illumina Total Prep RNA amplification kit (Illumina, Inc., San Diego, CA, USA) was used for hybridization with biotin-labeled cRNA. Illumina Human-12 BeadChip V.4 microarray (Illumina, Inc.)

was used for hybridizing samples, and data were extracted using the Genome Studio (Illumina, Inc.). R software (R-3.50; http://crans.us.r-project.org) was used for data analysis, and the Cluster 3.0 (Eisen Lab; University of California Berkeley, Berkeley, CA, USA) and Treeview (Eisen Lab; University of California Berkeley) software packages were used for generating the gene expression heat map.

Statistics. GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analyses. Data are presented as the mean ± standard deviation. Comparisons among the groups were performed by paired Student's t-test and a two-way analysis of variance with Duncan's multiple range test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

RSV suppresses colon cancer cell proliferation. To investigate the effect of RSV on colorectal cancer progression, MTT and clonogenic assays were performed. The MTT assay demonstrated that treatment with RSV had a dose-dependent inhibitory effect on HCT116 and SNU81 cell viability (Fig. 1A). The clonogenic assay additionally demonstrated that treatment with RSV significantly inhibited HCT116 and SNU81 cell proliferation in a dose-dependent manner (Fig. 1B). Therefore, RSV inhibited the progression and proliferation of colorectal cancer cells.

RSV suppresses the invasive and migratory effects of colon cancer cells. The effect of RSV on the migration and invasion of colorectal cancer cells was also investigated. RSV-treated HCT116 and SNU81 cells were assessed using Matrigel invasion and collagen migration assays. RSV significantly inhibited the invasive ability of colorectal cancer cells in a dose-dependent manner (Fig. 2A). The migration assay also confirmed that RSV significantly inhibited the migratory ability of colorectal cancer cells in dose-dependent manner (Fig. 2B). Therefore, these results suggested that RSV inhibited the migration and invasion of colorectal cancer cells.

Microarray data reveal an RSV-induced increase in TTP gene expression. To investigate the effect of RSV on gene expression levels in colorectal cancer cells, the microarray experiment was performed. RSV administration significantly regulated several genes associated with inflammation, proliferation, cell death, angiogenesis and metastasis (Fig. 3). Increased ZFP36 (TTP) gene expression was observed in RSV-treated HCT116 cells. In addition, RSV decreased the expression of the oncogenes Myc proto-oncogene (Myc), KRAS proto-oncogene GTPase (KRAS) and Fos proto-oncogene AP-1 transcription factor subunit (FOS), and the downstream target genes of TTP, including E2F1. Therefore, RSV-induced TTP upregulation may inhibit the growth and metastasis of colorectal cancer cells.

RSV induces the mRNA and protein expression of TTP. The present study further determined the endogenous mRNA and protein expression levels of TTP in HCT116 and SNU81 cells using RT-qPCR and western blot analysis (Fig. 4A).
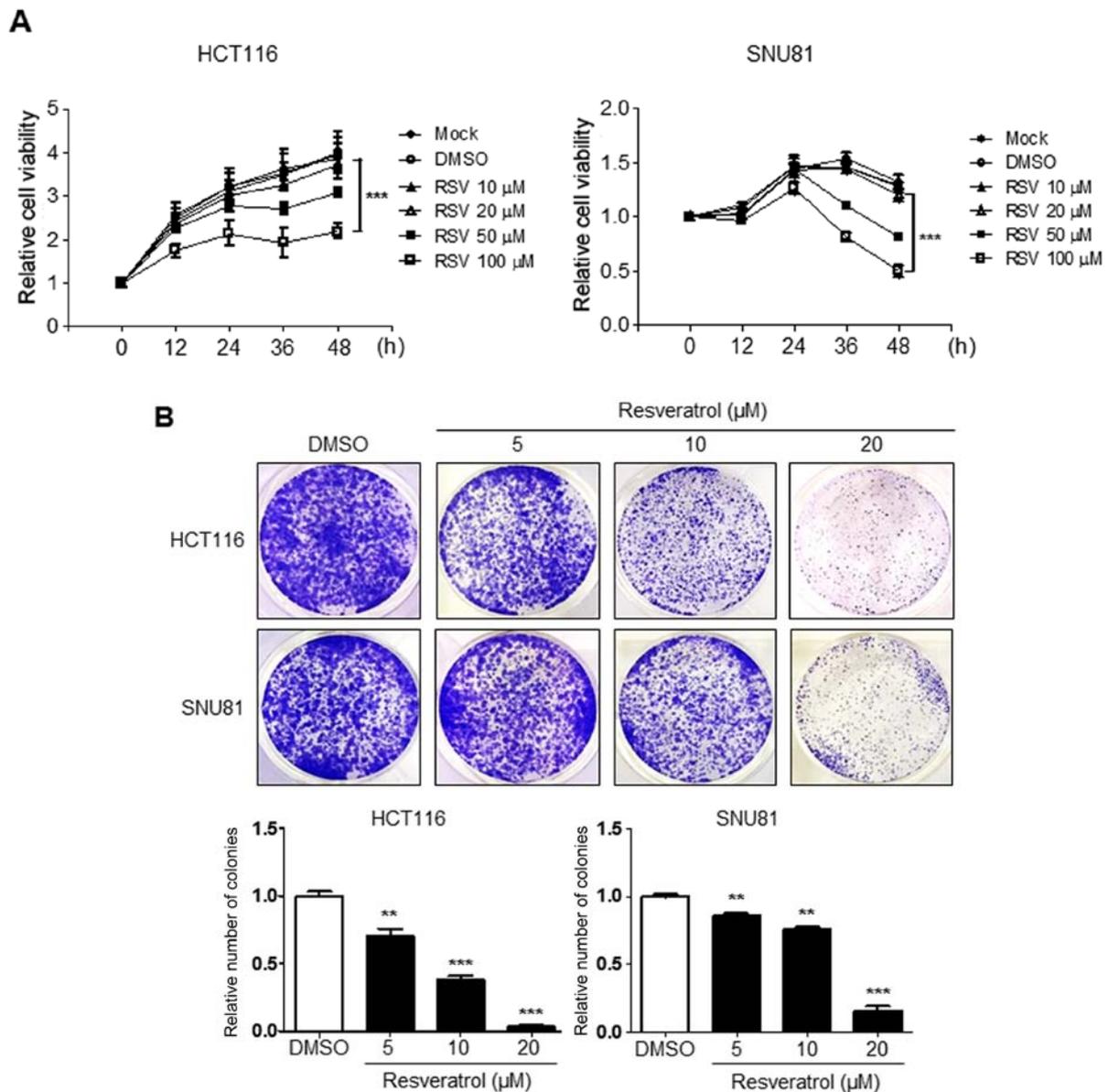
Figure 1. RSV suppresses the proliferation of colorectal cancer cells. (A) Regulation of TTP expression by RSV and suppression of tumor progression in cancer cells. MTT assays demonstrated a decline in cancer cell viability in HCT116 and SNU81 cells treated with RSV, in a dose-dependent manner. Data are presented as the mean ± SD of three different experiments. ***P<0.001. (B) A clonogenic assay was performed on HCT116 and SNU81 cells treated with RSV at increasing doses in order to assess proliferation. Colonies were counted under a light microscope and were photographed. Data are presented as the mean ± SD of three different experiments. **P<0.01; ***P<0.001 vs. DMSO. RSV, resveratrol; TTP, tristetraprolin; DMSO, dimethyl sulfoxide; SD, standard deviation.

Endogenous mRNA and protein expression levels of TTP were increased in HCT116 and SNU81 cells (Fig. 4A). In addition, siTTP decreased the mRNA and protein levels of TTP in HCT116 and SNU81 cells (Fig. 4A). To determine whether RSV regulated TTP expression in colorectal cancer cells, HCT116 and SNU81 cells were treated with 0, 10, 20 and 50 μM RSV. RSV significantly increased the mRNA and protein expression levels of TTP in HCT116 and SNU81 cells in a dose-dependent manner (Fig. 4B). In particular, >20 μM RSV significantly increased TTP expression in colorectal cancer cells (Fig. 4B). To further test whether RSV regulates TTP expression, HCT116 and SNU81 cells were transfected with scramble RNA or siTTP (100 nM). It was observed that RSV restored TTP mRNA expression in HCT116 and SNU81 cells whose TTP expression was reduced by siTTP (Fig. 4C).

Treatment with RSV reversed the inhibition of TTP expression. In addition, siRNA-induced TTP inhibition attenuated the effects of RSV on the cell growth of HCT116 and SNU81 cells (Fig. 4D). Therefore, these data indicate that TTP mediated the apoptotic effects of RSV in colorectal cancer cells and that RSV induced TTP expression in colorectal cancer cells.

RSV controls the downstream target gene of TTP. The present study further assessed whether RSV increased TTP promoter activity in a reporter assay. HCT116 and SNU81 cells were transiently transfected with a pGL3/hTTPp-1411 construct, followed by treatment with RSV. Following treatment with RSV for 24 h, the luciferase activity was measured, revealing significantly increased TTP promoter activity in HCT116 and
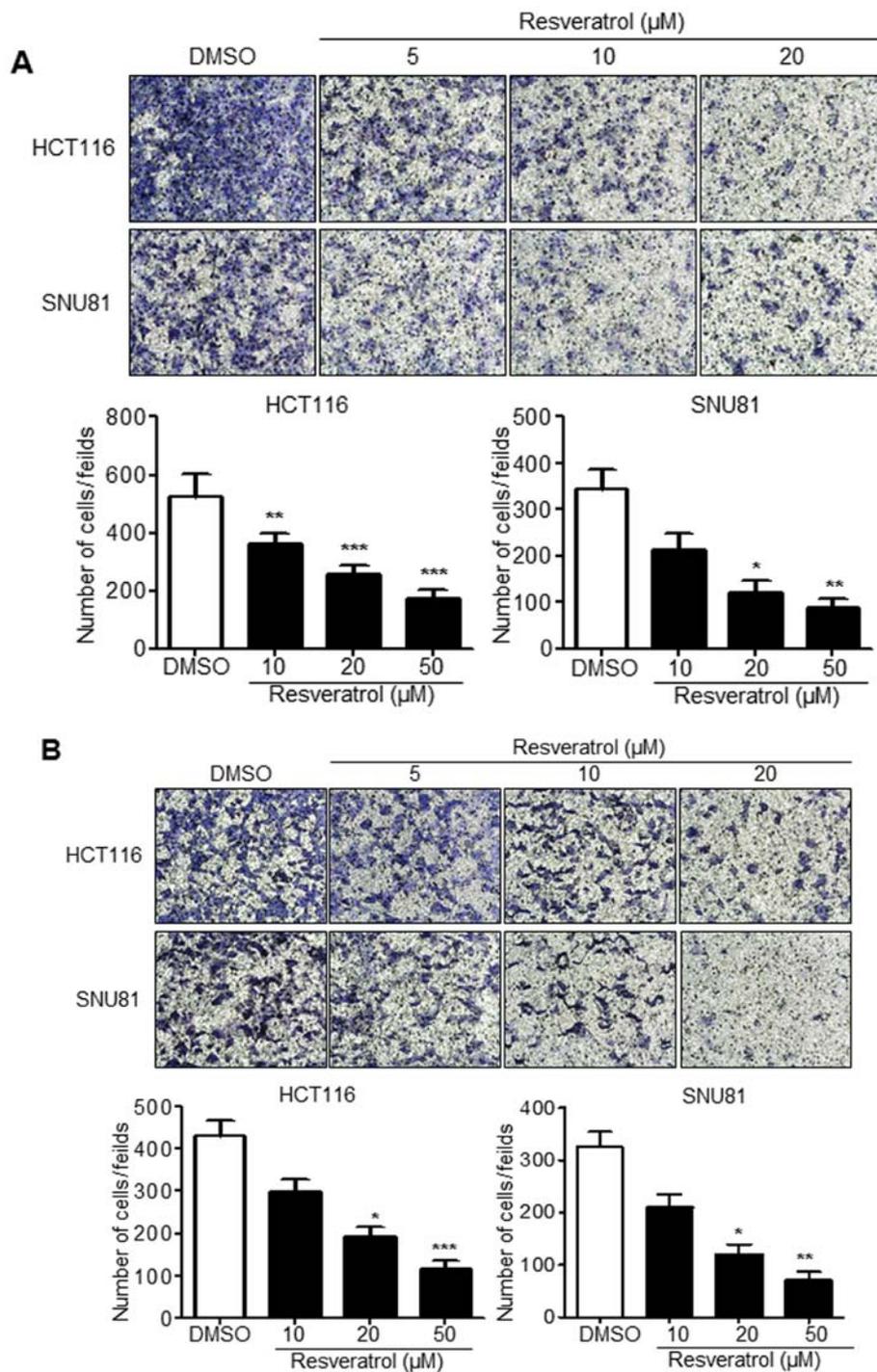
Figure 2. RSV inhibits the invasion and metastasis of colorectal cancer cells. (A) Invasion assay. HCT116 and SNU81 cells were treated with RSV, following which the percentage of invasive and migratory cells was determined. TTP suppressed the invasion and migration of colon cancer cells following treatment with RSV in a dose-dependent manner, as observed in the Boyden chamber assay. Graphical representations of the results for HCT116 and SNU81 cells are presented. (B) Migration assay. The *in vitro* invasive properties of HCT116 and SNU81 cells were assessed using Boyden chambers. Invading cells were visualized by membrane staining using Diff-Quik solution and counted (five fields/well). Graphical representation of the results for HCT116 and SNU81 cells are presented. Magnification, x100. *P<0.05; **P<0.01; ***P<0.001 vs. DMSO. DMSO, dimethyl sulfoxide; TTP, tristetraprolin; RSV, resveratrol.

SNU81 cells (Fig. 5A). Given a previous finding (41-44) that TTP reduced cIAP2, E2F1, LATS2 and Lin28 expression, and inhibited cancer cell growth, the present study subsequently investigated whether RSV regulates the promoter activities of cIAP2, E2F1, LATS2 and Lin28, which bind with TTP in colorectal cancer cells. HCT116 and SNU81 cells were transfected with luciferase reporter constructs that incorporated the 3'UTRs of cIAP2, E2F1, LATS2 and Lin28 mRNA (psiCHECK2/cIAP2, psiCHECK2/E2F1, psiCHECK2/ LATS2 and psiCHECK2/Lin28). Each transfected cell was treated with RSV (20 μM), and the luciferase activity was measured 24 h post-treatment. Consistent with previous studies, the induction of TTP expression reduced the luciferase activities of cIAP2, E2F1, LATS2 and Lin28 in HCT116 and SNU81 cells (Fig. 5B). RSV enhanced the inhibitory activity of TTP on target genes in HCT116 and SNU81 cells (Fig. 5B).
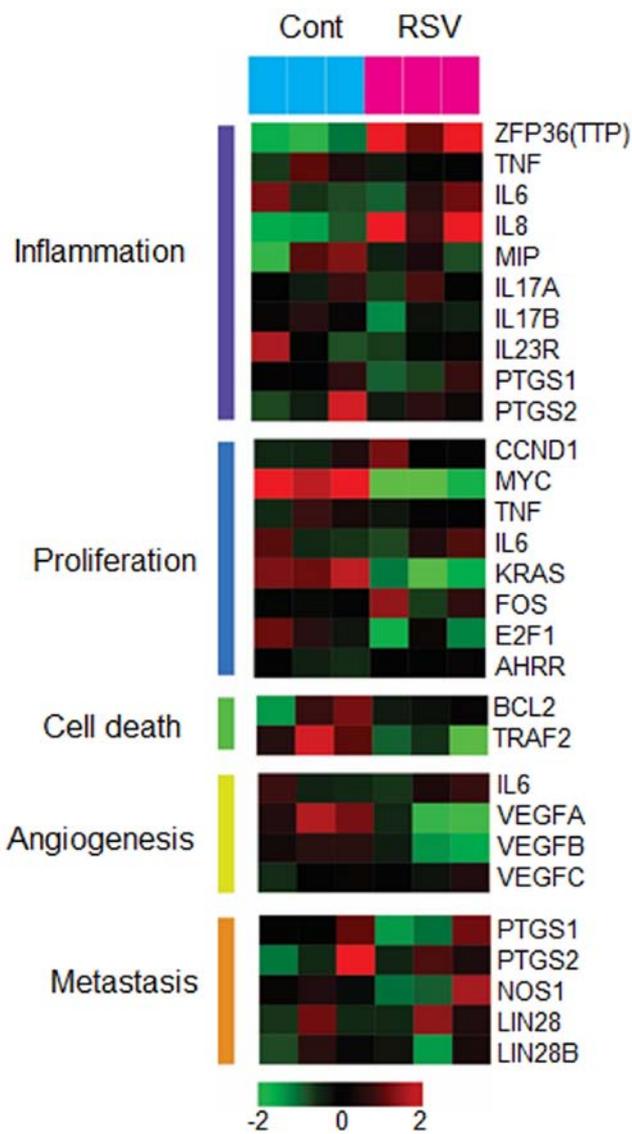
Figure 3. Microarray heat map demonstrating the effect of RSV on genes associated with inflammation, proliferation, cell death, angiogenesis and metastasis in HCT116 cells. Data are presented in matrix format with rows indicating the individual gene and columns indicating each sample. Red and green represent upregulated and downregulated gene expression levels, respectively, as indicated in the scale bar (log 2 transformed). RSV, resveratrol; Cont, control; ZFP36, ZFP36 ring finger protein; TNF, tumor necrosis factor; IL, interleukin; MIP, major intrinsic protein of lens fiber; PTGS, prostaglandin-endoperoxide synthase; CCND1, cyclin D1; MYC, Myc proto-oncogene; KRAS, KRAS proto-oncogene GTPase; FOS, Fos proto-oncogene AP-1 transcription factor subunit; E2F transcription factor 1; AHRR, aryl-hydrocarbon receptor repressor; BCL2, apoptosis regulator Bcl-2; TRAF2, TNF receptor associated factor 2; VEGF, vascular endothelial growth factor; NOS1, nitric oxide synthase 1; LIN28, lin-28 homolog A; LIN28B, lin-28 homolog B.

In addition, siTTP increased mRNA and protein levels of E2F1 in HCT116 and SNU81 cells (Fig. 5C). Therefore, our results indicated that RSV induces TTP expression and its target gene mRNA-decaying activity in colorectal cancer cells.

Discussion

Despite the fact that RSV has been studied in various human cancers, its biological effects on colorectal cancer have not been fully elucidated. The present study aimed to investigate the effects of RSV in human colorectal cancer cells and to elucidate its effect on regulating TTP expression. Although TTP serves key roles in cancer cells, TTP regulation by RSV in colorectal cancer cells is yet to be clearly investigated. The present study demonstrated that RSV inhibited cell proliferation and invasion/metastasis by activating TTP in human colorectal cancer cells.

Phytochemicals are promising therapeutic agents for cancer treatment. RSV, a natural compound occurring in grapes, peanuts and berries, has anticancer properties against a number of types of cancer, including colorectal cancer (28,47,48). RSV induces cellular apoptosis, and decreases migration and invasion, by regulating a number of mechanisms in colorectal cancer cells (28,29,47,48). Du *et al* (47) accordingly reported that the inhibitory effect of RSV on colorectal cancer cells was mediated by the hedgehog/GLI family zinc finger 1 signaling pathways. Feng *et al* (48) demonstrated that treatment with RSV inhibits proliferation and induces apoptosis in human colon cancer cells. Furthermore, Buhrmann *et al* (29) demonstrated that RSV suppresses the tumorigenesis of colorectal cancer cells by targeting sirtuin 1 and suppressing nuclear factor-κB activation. In addition, RSV has been comprehensively investigated with respect to its role in cell cycle regulation in colorectal cancer. RSV upregulated cyclin dependent kinase inhibitor 1A, causing cell-cycle arrest at the G_0/G_1 and G_2/M phases, and the activation of the caspase-dependent cyclin/cyclin dependent kinase pathway in colon cancer cells (49,50). Therefore, RSV appears to have antitumor properties in colorectal cancer cells by targeting various signaling pathways. Consistent with previous studies, the present results indicated that RSV inhibited the viability of HCT116 and SNU81 cells in a dose- and time-dependent manner. RSV also significantly inhibited the colony formation of HCT116 and SNU81 cells in a dose-dependent manner. These observations are in accordance with previous studies (28,29,47,48) that demonstrated that RSV negatively regulates the growth of colorectal cancer cells. In addition, RSV significantly reduced the invasion and metastasis of colorectal cancer cells (HCT116 and SNU81 cells) in a dose-dependent manner. These results regarding RSV regulation of cell mobility and adhesion, and the inhibition of invasion and metastasis in colorectal cancer cells, were consistent with those of previous studies (28,29,47,48).

An increasing number of studies have validated the importance of TTP expression in human cancer (51-54). The loss of TTP gene expression has been reported in various cancer types (51,53), and reduced TTP gene expression has been demonstrated to lead to cancer development (32,33,55). Therefore, TTP overexpression may be a novel strategy for cancer prevention. TTP also serves an important role in a number of cancer types, including colon cancer. TTP inhibits IL-23 expression (56) and downregulates vascular endothelial growth factor (VEGF) and COX-2 expression in human colon cancer (38,57). Accordingly, TTP expression may be controlled via the aforementioned signaling pathways. Therefore, examining whether RSV regulates TTP expression in colorectal cancer cells is essential. To investigate the association between RSV and TTP gene expression in colorectal cancer cells (HCT116), gene expression profiling was performed. The microarray experiment demonstrated that RSV significantly
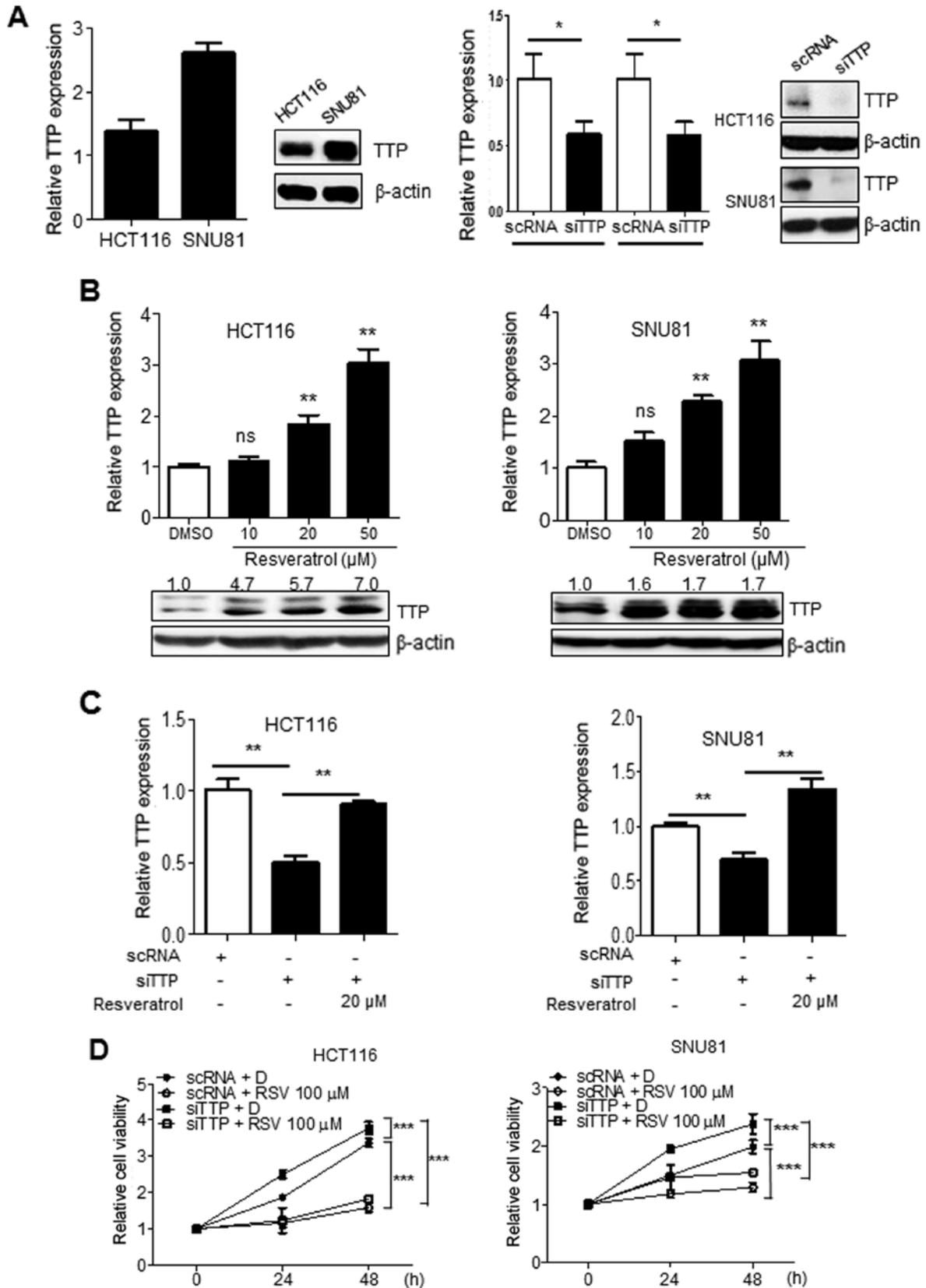
Figure 4. RSV induces TTP expression in colorectal cancer cells. (A) Endogenous mRNA and protein expression levels of TTP in colon cancer cells (HCT116 and SNU81). *P<0.05. (B) RSV induced the mRNA and protein expression of TTP in a dose-dependent manner in HCT116 and SNU81 cells. β-actin was detected as the loading control for RT-qPCR and western blotting. The relative protein expression level was calculated as a ratio of the control (DMSO), using ImageJ software to detect the intensity of the protein band. **P<0.01 vs. DMSO. (C) RSV restored TTP expression in cell lines whose TTP expression was reduced by TTP siRNA. HCT116 and SNU81 cells were transfected with scRNA or TTP siRNA (100 nM). After 24 h, DMSO or RSV (20 µM) was added to the cells, followed by RNA extraction after 24 h. β-actin was detected as the loading control for RT-qPCR. Each bar represents the mean ± SD of three independent experiments. **P<0.01. (D) Cell survival was assessed using the MTT assay. Cell viability relative to that of the control is expressed as the mean ± SD of three independent experiments. ***P<0.001. TTP, tristetraprolin; RSV, resveratrol; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; si, small interfering; DMSO, dimethyl sulfoxide; ns, not significant; SD, standard deviation; sc, scramble.
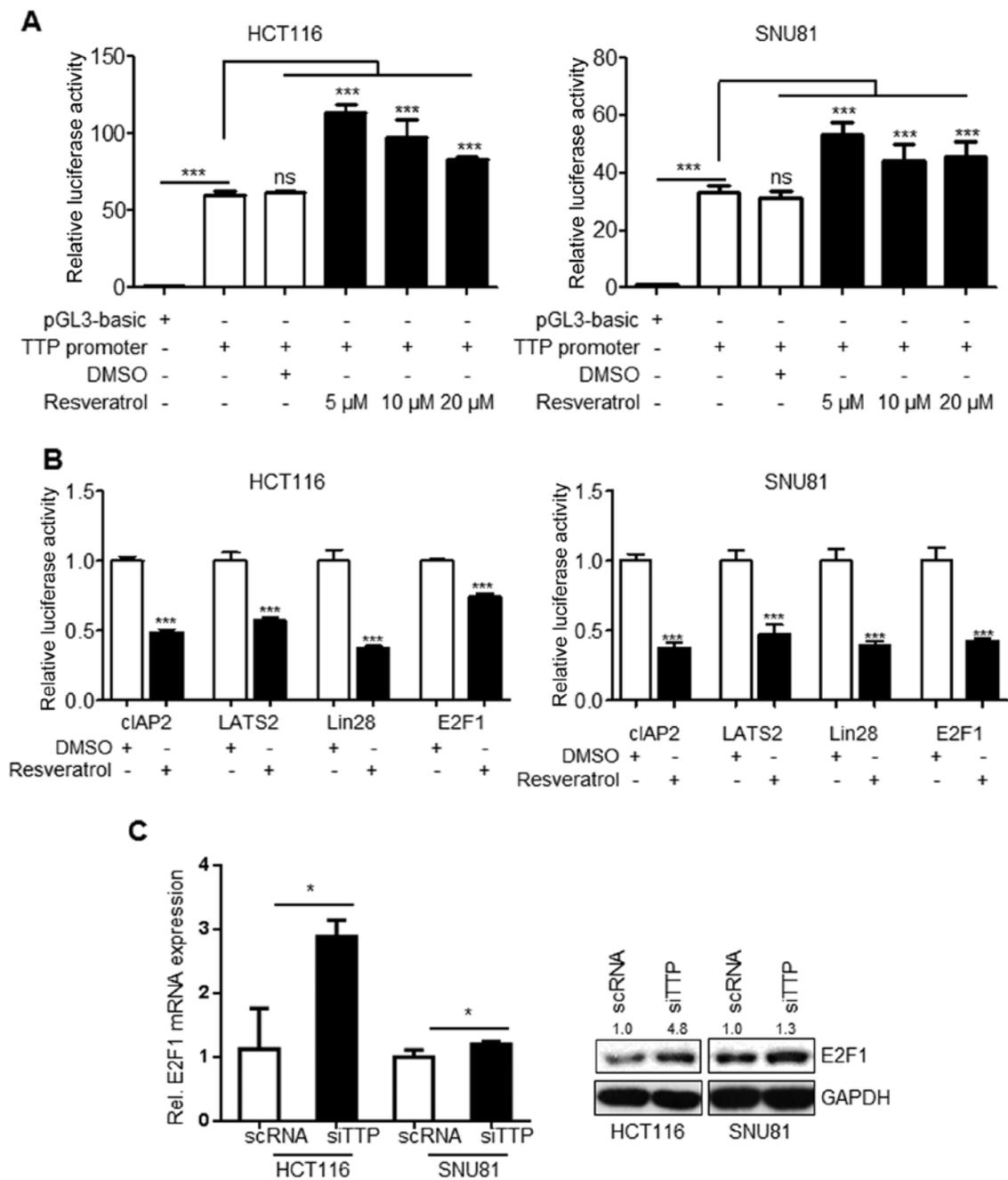
Figure 5. RSV controls the downstream target genes of TTP. (A) HCT116 and SNU81 cells were transfected with pGL3/TTPp-1411 containing the TTP promoter (-1343 to +68) for 24 h and treated with RSV at increasing doses for 24 h. Following treatment with RSV for 24 h, the luciferase activity was measured. The expression levels obtained from pGL3-basic (empty vector)-transfected cells without treatment with RSV were set to 1. Each bar represents the mean ± SD of three independent experiments. ***P<0.001. (B) RSV induced the mRNA-decaying activity of TTP. HCT116 and SNU81 cells were transfected with psiCHECK2-cIAP2, LATS2, Lin28 and E2F1 3'UTRs. At 24 h post-transfection, cells were treated with 20 μM RSV for 24 h, followed by determination of the luciferase activity. The *Renilla* luciferase activity was normalized to the firefly luciferase activity. The luciferase activity obtained from psiCHECK2-cIAP2, LATS2, Lin28, and E2F1 3'UTR-transfected and DMSO-treated cells was set to 1. Each bar represents the mean ± standard deviation of three independent experiments. ***P<0.001 vs. respective control. (C) TTP inhibits E2F1 expression in colorectal cancer cells. siTTP increased the mRNA and protein expression levels of E2F1 in HCT116 and SNU81 cells. Each bar represents the mean ± standard deviation of three independent experiments. *P<0.05 vs. respective control. TTP, tristetraprolin; RSV, resveratrol; DMSO, dimethyl sulfoxide; ns, not significant; SD, standard deviation; cIAP2, baculoviral IAP repeat containing 3; LATS2, large tumor suppressor kinase 2; Lin28, lin-28 homolog A; E2F1, E2F transcription factor 1; si, small interfering; sc, scramble.

increased TTP expression. Furthermore, RSV regulated genes associated with inflammation, cell proliferation, cell death, angiogenesis and metastasis, and suppressed Myc, KRAS, and FOS gene expression. The microarray experiment also indicated that RSV suppressed E2F1, a downstream target gene of TTP. Consistent with the microarray data, the RT-qPCR data indicated that RSV increased TTP expression

in HCT116 and SNU81 cells in a dose-dependent manner. Similar results were also found with respect to the western blot analysis, wherein RSV administration dose-dependently increased TTP protein expression. Furthermore, RSV restored TTP mRNA expression following TTP silencing in HCT116 and SNU81 cells. siRNA-induced TTP inhibition attenuated the effects of RSV on cell growth. These results suggested that

TTP may be involved in the effect of RSV on the inhibition of human colorectal cancer cell growth. RSV inhibited HCT116 and SNU81 cell proliferation by upregulating TTP. The present observations are in accordance with earlier studies wherein RSV increased TTP expression in glioma and breast cancer cells (31,39). RSV was demonstrated to inhibit MCF-7 cell proliferation by upregulating TTP via COX-2 and VEGF downregulation, and inducible nitric oxide synthase upregulation (31). Furthermore, RSV increased TTP expression to induce glioma cell apoptosis in U87MG human glioma cells (39). These results suggested that RSV upregulates TTP expression in colorectal cancer cells.

Given that TTP gene silencing triggers cancer development, it may be hypothesized that the loss of TTP function in cancer cells may induce transcriptional silencing through TTP promoter regulation. The present study indicated that RSV increased the mRNA and protein expression levels of TTP in human colorectal cancer cells. Further studies regarding whether RSV regulates TTP promoter activity in colorectal cancer cells demonstrated that RSV significantly increased TTP promoter activity in HCT116 and SNU81 cells. Furthermore, RSV significantly inhibited the promoter activities of cIAP2, E2F1, LATS2 and Lin28, which are downstream target genes of TTP in HCT116 and SNU81 cells. RSV enhanced the TTP inhibitory activity in HCT116 and SNU81 cells by negatively regulating cIAP2, E2F1, LATS2 and Lin28 expression. These findings are in accordance with earlier studies wherein TTP overexpression suppressed the stability of E2F1 and Lin28 mRNA (42,57), and controlled the stability of cIAP2 and LATS2 mRNA by binding to the 3'UTR of cIAP2 mRNA or promoting let-7 biogenesis (38,43), demonstrating that cIAP2, E2F1, LATS2, and Lin28 may be physiological targets of TTP. In addition, in agreement with previous studies, it was demonstrated that siTTP significantly increased the mRNA and protein expression levels of E2F1 in HCT116 and SNU81 cells. Likewise, other studies have reported that TTP inhibits the expression of LIN28, cIAP and LATS2 in human cancer cells (40,41,43,44). Therefore, it may be inferred that RSV suppresses the viability of colorectal cancer cells by regulating the stability of LIN28, cIAP and LATS2 mRNA, mediated via TTP regulation. In conclusion, the results of the present study suggested RSV inhibits the proliferation and invasion/metastasis of colorectal cancer cells by upregulating TTP, which is associated with the downregulation of TTP target genes, including cIAP2, E2F1, LATS2 and Lin28.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SRL and HJ conceived the study and drafted the manuscript. WTK and WZK performed the immunoassays and molecular studies. SZK performed the molecular studies and SHL participated in the statistical analysis and helped to draft the manuscript. SMK designed the research and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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