Experimental and Molecular Pathology 107 (2019) 23–31

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Triptolide inhibits the growth and migration of colon carcinoma cells by down-regulation of miR-191

Yuxi Qi[a,b], Jinliang Li[a,*]

[a] Department of Anorectal Surgery, Jining No.1 People's Hospital, Jining 272011, China
[b] Affiliated Jining No.1 People's Hospital of Jining Medical University, Jining Medical University, Jining 272067, China

ARTICLE INFO

Keywords:
Colorectal cancer (CRC)
Triptolide (TPL)
miR-191
HT-29 cell
SW480 cell
NF-κB and Wnt/β-catenin pathways

ABSTRACT

Background: Triptolide (TPL) is the active component of *Tripterygium wifordii* Hook F, which has been reported to exert anti-tumor efficacies. Herein, we aimed to examine the efficacy of TPL in colorectal cancer (CRC) cells and to reveal its underlying mechanisms.

Methods: Human CRC cell lines HT-29 and SW480 were treated by TPL for 24 h in the presence or absence of epithelial-to-mesenchymal transition (EMT) inducer TGF-β1. The expression of miR-191 in cell was over-expressed by miRNA transfection. Thereafter, cell viability, migration, apoptosis, EMT-related protein expressions, and the activation of NF-κB and Wnt/β-catenin pathways were respectively assessed. Moreover, a mouse model of CRC was established and the effects of TPL on the growth of primary tumors were tested.

Results: TPL (50 and 100 nM) significantly reduced cell viability and migration, but increased apoptotic cell rate. TPL up-regulated Bax, increased the cleavage of caspase-3 and -9, and neutralized TGF-β1-induced alterations of EMT indicators, including E-cadherin, N-cadherin, Vimentin, and Snail. At the meantime, TPL treatment down-regulated miR-191 expression, and the effect of TPL on miR-191 expression was mediated by EZH2. More interestingly, anti-CRC properties and the inhibitory effects of TPL on NF-κB and Wnt/β-catenin pathways were reversed by miR-191 up-regulation. In vivo data showed that, TPL treatment decreased the growth of primary tumor xenografts and the expression of miR-191.

Conclusion: TPL is effective in killing CRC cells and suppressing the migratory capacity. TPL exerts anti-CRC properties possibly via down-regulating miR-191, and blocking the activation of NF-κB and Wnt/β-catenin pathways.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and one of the leading causes of cancer-related death worldwide. Despite the incidence of CRC is declined by 3% per year from 2004 through 2013, there are still > 1 million new cases diagnosed worldwide annually (Siegel et al., 2017). Surgery combined with chemo- and radio-therapy is the major therapeutic strategy for CRC. While unfortunately, CRC is less likely to be cure when it is detected at later stages, for which metastases are present (Cunningham et al., 2010). Further research is warranted on the innovative drugs and therapeutic targets to improve the prognosis of CRC.

Triptolide (TPL, $C_{20}H_{24}O_6$) is one of the active components of *Tripterygium wifordii* Hook F. In the past three decades, TPL has been used clinically for treating inflammatory and immunological disorders in China (Tao and Lipsky, 2000). At present, TPL was found to have multiple pharmacology efficacies, like anti-inflammation, immunity suppression, cardioprotection, and anti-osteoporosis (Cui et al., 2017). Besides, the anti-tumor activities of TPL in various kinds of cancers were reported, such as prostate cancer (Tamgue and Lei, 2017), myeloma (Kim and Park, 2017), pituitary corticotroph tumor (Li et al., 2017), pancreatic cancer (Zhang et al., 2017), lung cancer (Song et al., 2017), and liver cancer (Sun et al., 2017). In the regard of CRC, TPL has been suggested as a potential agent for CRC treatment through inhibiting tumor cells proliferation, migration and invasion (Johnson et al., 2011; Liu et al., 2012a; Liu et al., 2012b). However, the mechanisms by which TPL inhibits CRC are still unclear.

microRNAs (miRNAs) are a class of non-coding RNAs that play vital regulatory roles in multiple malignant cancers. Expression patterns of miRNAs are systematically altered in CRC, and some of them have been considered as potential biomarkers for the diagnosis and prognosis of CRC, like miR-21 (Schetter et al., 2008), miR-148a, miR-625-3p

* Corresponding author at: Department of Anorectal Surgery, Jining No.1 People's Hospital, No.6, Jiankang Road, Jining 272011, China.
E-mail address: lijinliang94@sina.com (J. Li).

https://doi.org/10.1016/j.yexmp.2019.01.008
Received 12 October 2018; Received in revised form 16 January 2019; Accepted 21 January 2019
Available online 23 January 2019
0014-4800/

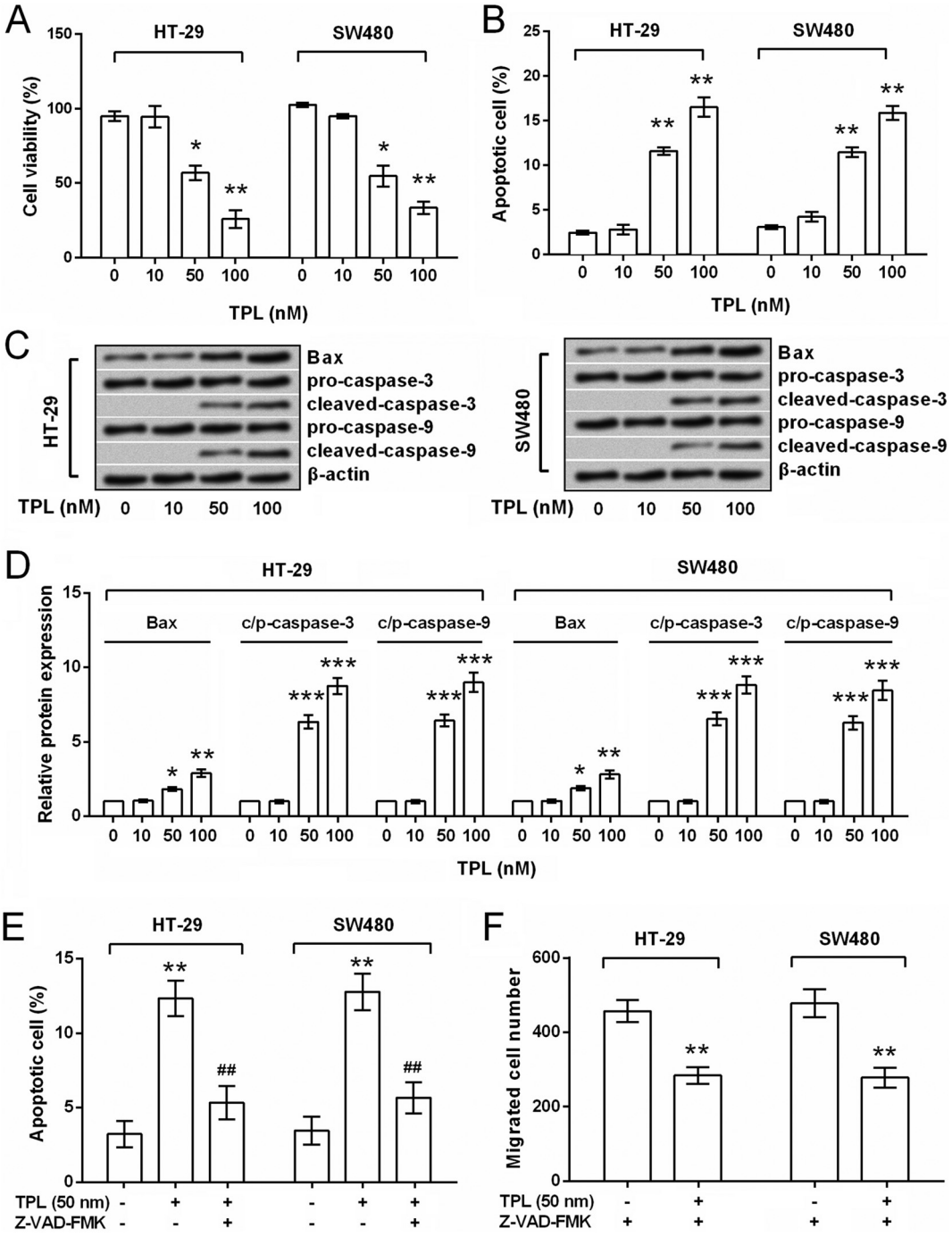

Fig. 1. Triptolide (TPL) inhibits HT-29 and SW480 cells viability and migration, but induces apoptosis. (A) Viability, (B) the rate of apoptotic cells, and (C–D) protein expressions of apoptosis-related factors were measured after treating with various dose of TPL (10, 50 and 100 nM) for 24 h. The cultured tumor cells were treated with 20 μM *Z*-VAD-FMK for 1 h to prevent TPL-induced cell death. (E) The rate of apoptotic cells and (F) the number of migrated cells were respectively measured. * $P < .05$, ** $P < .01$, *** $P < .001$ compared with control group. ## $P < .01$ compared with TPL (50 nM) group.

(Baltruskeviciene et al., 2017), miR-29a, miR-200b, miR-203, and miR-31 (Yuan et al., 2017). In addition, miRNA-based therapies have been proposed as efficiency strategies in CRC treatment (Tokarz and Blasiak, 2012). miR-191 is one of the highly expressed miRNAs in human CRC (Cummins et al., 2006; Xi et al., 2006; Zhang et al., 2015). Functional assay results indicated that, inhibition of miR-191 led to the decreases of cell growth, proliferation and tumorigenicity in a xenograft model of CRC (Zhang et al., 2015). Likewise, silence of miR-191 attenuated the invasiveness, suppressed proliferation and induced apoptosis in CRC SW620 cells (Qin et al., 2014). These authors suggested miR-191 as a tumor-promoting miRNA in CRC.

In the present study, we examined the efficacy of TPL in two human CRC cell lines (HT-29 and SW480). The mechanisms underlined TPL's anti-tumor properties were studied by investigating the relationship between TPL and miR-191 expression. The findings of this study will be helpful for better understand the anti-tumor functions of TPL.

2. Materials and methods

2.1. Cell culture

Human CRC cell lines HT-29 (HTB-38) and SW480 (CCL-228) were

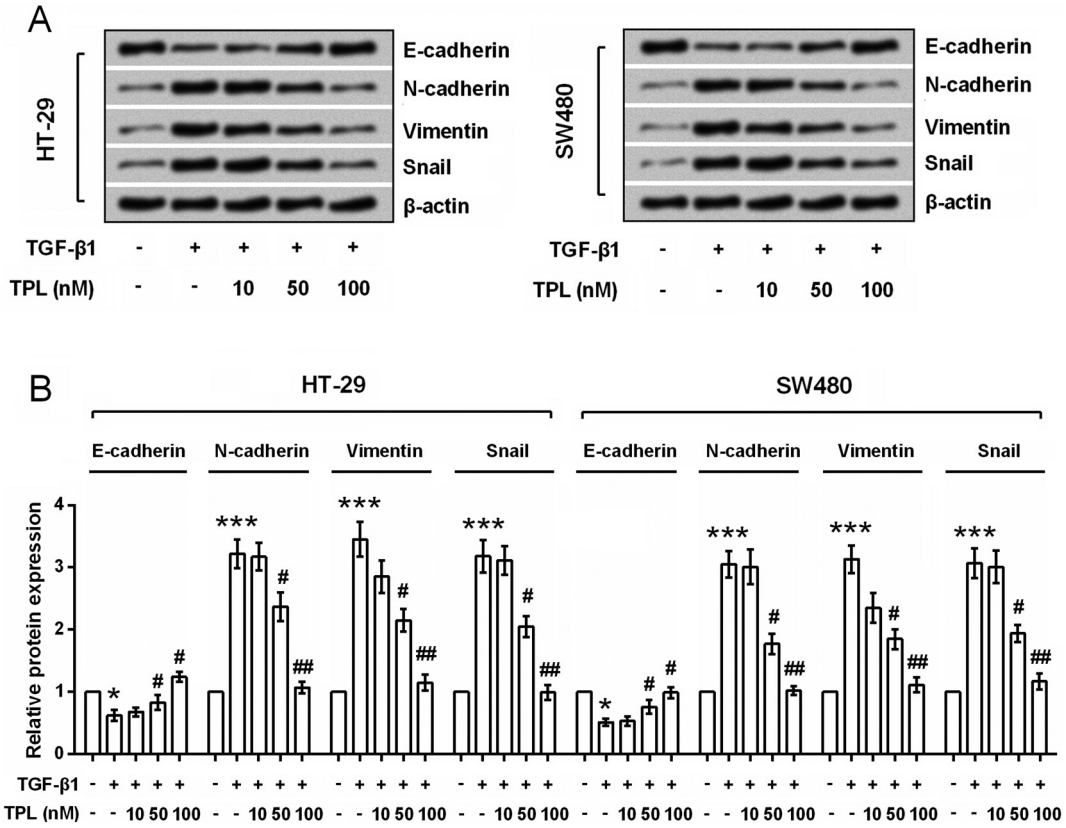

Fig. 2. Triptolide (TPL) inhibits the epithelial-to-mesenchymal transition (EMT) process of HT-29 and SW480 cells. (A) Protein expressions of EMT-related factors were detected after HT-29 and SW480 cells were treated with various dose of TPL (10, 50 and 100 nM) for 24 h in the presence or absence of 10 ng/mL TGF-β1. (B) Semi-quantitative analysis based on the results from western blotting. * $P < .05$, *** $P < .001$ compared with control group. # $P < .05$, ## $P < .01$ compared with TGF-β1 group.

purchased from the American Type Culture Collection (ATCC, Manassas, VA), and cultured in Dulbecco's modied Eagle medium (DMEM, Hyclone, Logan, UT) supplemented with 10% (*v*/v) fetal bovine serum (FBS, Gibco, Grand Island, NY). The cells were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

2.2. TPL treatment

TPL derived from *Tripterygium wilfordii* with purity higher than 98% was purchased from Sigma-Aldrich (St. Louis, MO). TPL was dissolved in DMSO (Sigma-Aldrich) for extended storage, and made up with the culture medium so that the final concentration of DMSO was < 0.1%. The cells were treated with 10, 50 and 100 nM TPL for 24 h.

For induction of epithelial-to-mesenchymal transition (EMT), TGF-β1 (Sigma-Aldrich) with concentration of 10 ng/mL was used to treat cells for 24 h.

2.3. Cell transfection

The mimic and scrambled control (miR-NC) specific for hsa-miR-191 were purchased from GenePharma Co. (Shanghai, China). The sequences for oligonucleotides were as follows: miR-191 mimic sense, 5'-CAACGGAAUCCCAAAAGCAGCUG-3', miR-191 mimic anti-sense, 5'-GCUGCUUUUGGGAUUCCGUUGUU-3'; miR-NC, 5'-UCACAACCUCCUA GAAAGAGUAGA-3'. siRNA specific for EZH2 (si-EZH2) was synthesized by GenePharma and transfected into cell to silence the expression of EZH2. A non-targeting sequence (GenePharma) served as negative control (si-NC), Cell transfections were conducted by using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. At 48 h of transfection, cells were collected for use in the following experiments.

2.4. Cell viability assay

HT-29 or SW480 cells were planted in 96-well plates with a density of 5000 cells/well for adherence. Following by TPL treatment, the culture medium was removed, cells were washed twice with PBS, and then 10 μL Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kyushu, Japan) solution was added into each well. The plates were incubated in the humidified incubator at 37 °C for 4 h, after which the absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

2.5. Apoptosis assay

The Annexin V-FITC Apoptosis Detection Kit (Beyotime) was utilized in the present work to detect cell apoptosis. HT-29 or SW480 cells were seeded in 6-well plates with a density of 5×10^5 cells/well for adherence. The cells were treated with TPL for 24 h, after which cells were collected and resuspended in 195 μL binding buffer. Then, 5 μL Annexin V-FITC and 10 μL PI was added, and the samples were incubated in the dark on ice for 30 min. A sample of 300 μL binding buffer was added, and cells were analyzed by the FACS can (Beckman Coulter, Fullerton, CA). The early apoptotic cells were calculated, which characterized by FITC-positive and PI-negative.

2.6. Wound healing assay

HT-29 or SW480 cells (5×10^5) were seeded in 6-well plates. After 12 h of incubation, a scratch was made by a 200-μL pipette tip. The cells

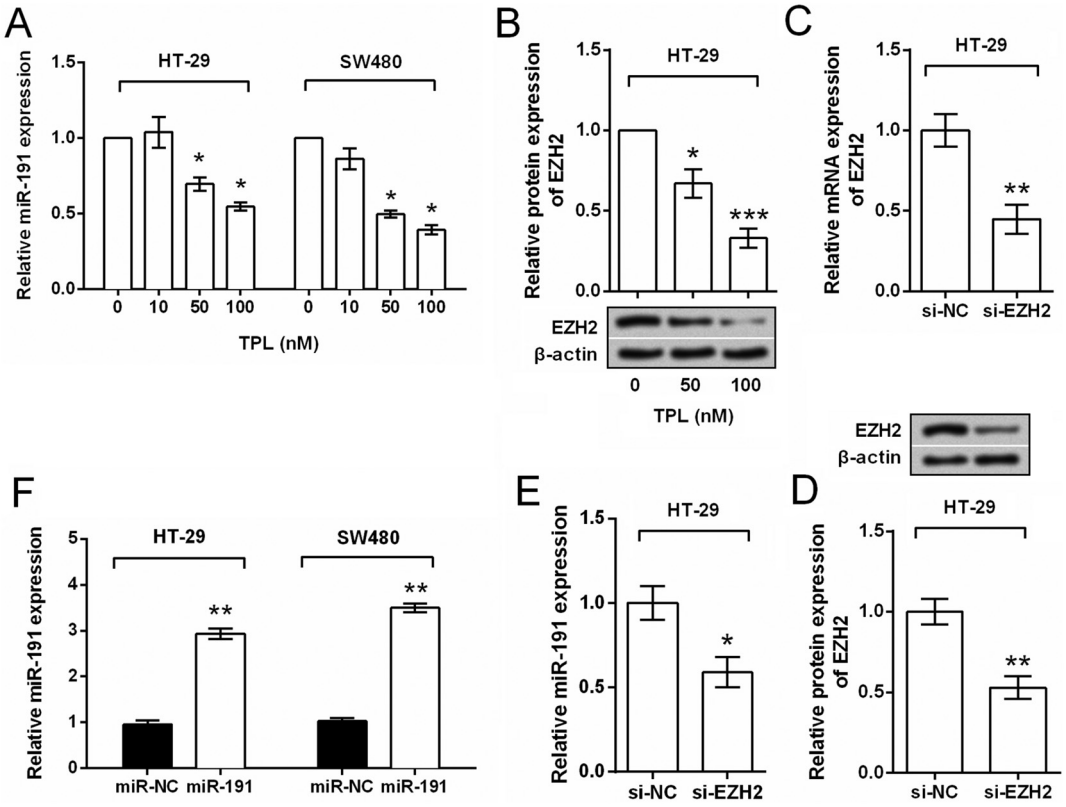

Fig. 3. Triptolide (TPL) represses the expression of miR-191 via EZH2. (A) The RNA level of miR-191 was measured in HT-29 and SW480 cells treated with various dose of TPL (10, 50 and 100 nM) for 24 h. (B) The protein level of EZH2 was measured after treating with TPL. siRNA specific for EZH2 (si-EZH2) or its non-targeted negative control (si-NC) were transfected into HT-29 cells, after which (C) mRNA and (D) protein levels of EZH2, as well as (E) the expression of miR-191 were measured. (F) The RNA level of miR-191 was measured after transfection with miR-191 mimic or scrambled control (miR-NC). * $P < .05$, ** $P < .01$, *** $P < .001$ compared with control group.

were then washed with PBS for twice, and incubated in serum-free medium containing 50 nM TPL for 24 h. Prior to TPL treatment, cells were treated with 20 μM *Z*-VAD-FMK (Sigma-Aldrich) for 1 h to prevent TPL-induced cell death. The number of migrated cells was counted microscopically.

2.7. qRT-PCR

The miRNAs were extracted from HT-29 and SW480 cells by miRNeasy Mini Kit (Qiagen, Shenzhen, China). Reverse transcription was performed with the stem-loop primer for hsa-miR-191 (5'-GTCGT ATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCAG CUG-3') under the catalysis of PrimeScript Reverse Transcriptase (Takara, Dalian, China). qRT-PCR was conducted by using Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA) with the specific primer for hsa-miR-191 (Fw: 5'-GGGCAACGGAA TCCCAAAAG-3' and Rv: 5'-CAGTGCGTGTCGTGGAGT-3'). Human U6 (Fw: 5'-CTCGCTTCGGCAGCACA-3' and Rv: 5'-AACGCTTCACGAATTT GCGT-3') was used as a reference control. Total mRNAs were extracted from cell by using Trizol reagent (Invitrogen). For the test of EZH2, reverse transcription and qPCR were carried out by using PrimeScript™ RT Master Mix and TB Green Fast qPCR Mix (both from Takara). The primary sequences for EZH2 were as follows. Fw: 5'-TGCAGTTGCTTC AGTACCCATAAT-3' and Rv: 5'-ATCCCCGTGTACTTTCCCATCATAAT-3'. Human β-actin (Fw: 5'-TTGCCGACAGGATGCAGAA-3' and Rv: 5'-CTCCTGCTTGCTGATCCACAT-3') was used as a reference control for calculating the relative expression of EZH2. Data were analyzed with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.8. Western blot

After the indicated treatments, proteins in HT-29 and SW480 cells were extracted by the RIPA lysis buffer (Beyotime). The proteins were quantified using the Bradford assay (Thermo, Hercules, CA). Equal amounts of the whole-cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and were transferred onto polyvinylidene difluoride (PVDF) membrane. The membranes were incubated with 5% non-fat dry milk for 1 h at room temperature, followed by incubation with primary antibodies overnight at 4 °C. The antibodies against Bax (ab32503), caspase-3 (ab13586), caspase-9 (ab32539), E-cadherin (ab1416), N-cadherin (ab18203), Vimentin (ab8978), Snail (ab53519), p-p65 (ab16502), p65 (ab86299), p-IκBα (ab92700), IκBα (ab32518), Wnt3a (ab28472), Wnt5a (ab72583),β-catenin (ab32572), EZH2 (ab228697) and β-actin (ab8226) were all purchased from Abcam (Cambridge, MA). The membranes were then incubated with the secondary antibodies at room temperature for 1 h, after which blots were visualized by enhanced chemiluminescence method. The intensity of bands was quantified by Image Lab™ Software (Bio-Rad, Hercules, CA).

2.9. In vitro experiments

A total of 90 SPF grade BALB/c nude mice (all male, 8–10 weeks) were purchased from Vital River Laboratories (Beijing, China). All the animal experiments of this study were approved by the Animal Ethics Committee of Jining No.1 People's Hospital and performed according to the instruction of the institute. To establish xenografts of CRC in mice, 5×10^6 HT-29 cells were injected subcutaneously into the flanks of mice. After growing for 3 days, the mice were randomly divided into

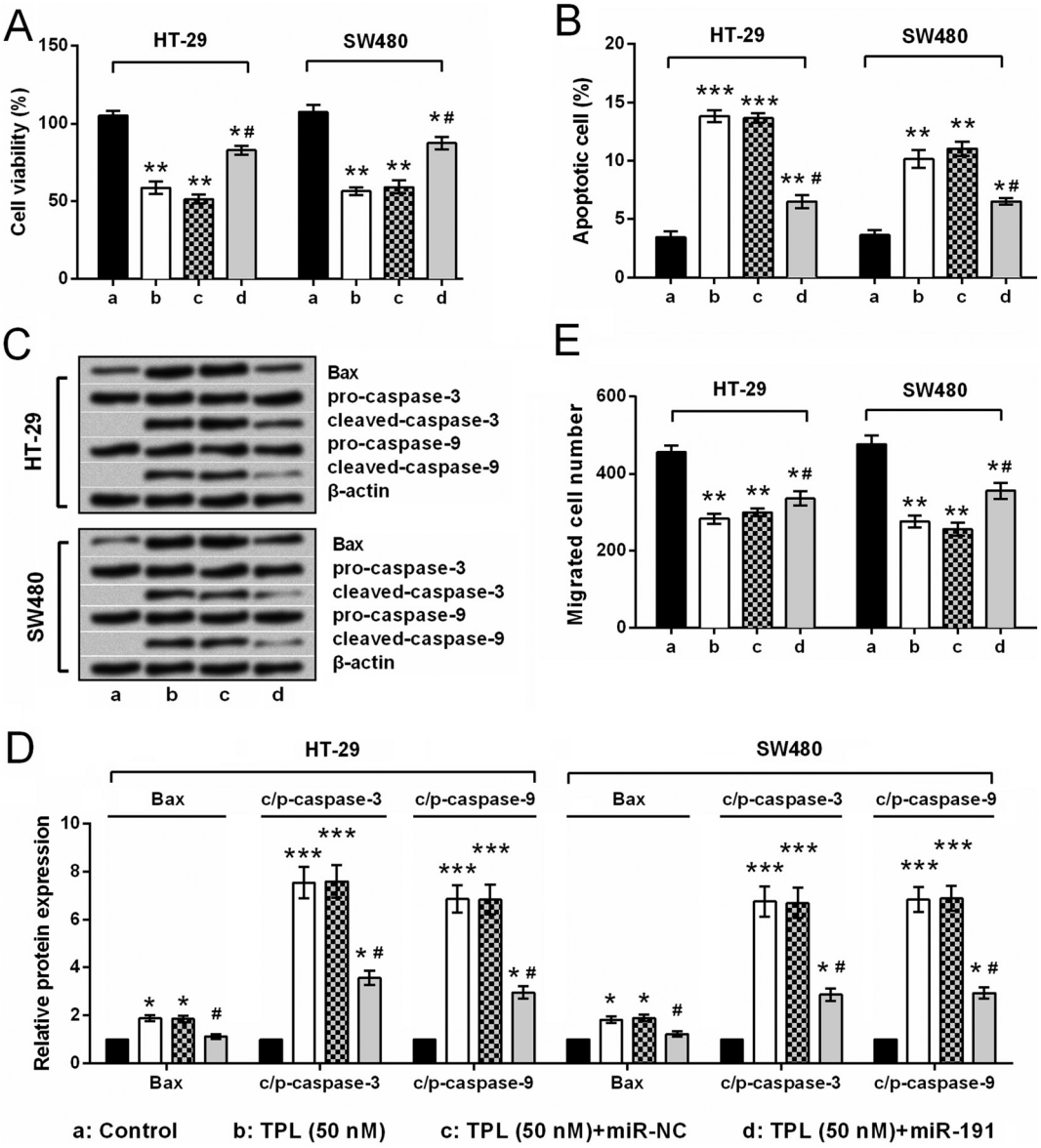

Fig. 4. The anti-viability, anti-migratory, and pro-apoptotic properties of Triptolide (TPL) are alleviated by miR-191 up-regulation. (A) Viability, (B) apoptotic cells rate, (C–D) protein expressions of apoptosis-related factors, and (E) migratory capacity of HT-29 and SW480 cells were measured respectively, after the cells were pre-transfected with miR-191 mimic or miR-NC, and then treated with 50 nM of TPL. * $P < .05$, ** $P < .01$, *** $P < .001$ compared with control group. # $P < .01$ compared with TPL (50 nM) + miR-NC group.

five groups (18 mice per group): Control, vehicle, TPL (0.1 mg/kg), TPL (0.3 mg/kg), and TPL (1 mg/kg). The mice in TPL groups received 0.1, 0.3 or 1 mg/kg/day TPL treatment orally for continuous 4 weeks. The mice in vehicle group received the same volume of 0.9% saline. Non-treated mice served as control. Mice were sacrificed, and tumor xenografts were removed. Tumor volume was measured every three day, and tumor weight was measured at the termination of the study. The expression of miR-191 in tumor tissues was detected by qRT-PCR, according to the abovementioned method.

2.10. Statistical analysis

Results were expressed as mean ± SD from three independent experiments. Differences between groups were analyzed by the SPSS version 13.0 program (SPSS Inc., Chicago, IL) using Student *t*-test or one-way analysis of variance (ANOVA). A *P*-value of < 0.05 was considered to indicate a statistically significant result.

3. Results

3.1. TPL inhibited the growth and migration of cultured CRC cells

Various doses of TPL (10, 50 and 100 nM) were used to treat two CRC cell lines (HT-29 and SW480) for 24 h. As a result, 50 and 100 nM of TPL exhibited cytotoxic effects on HT-29 cells, resulting in the viability reduced to 56.9 ± 8.6% and 25.9 ± 10.2% ($P < .05$ and $P < .01$), and the ratio of apoptotic cells shifting from 2.5% to 11.6% and 16.5% (both $P < .01$) (Fig. 1A–B). Same trends were observed in SW480 cells. TPL with doses of 50 and 100 nM significantly reduced the viability of SW480 cells to 54.7 ± 12.2% and 31.6 ± 8.1% ($P < .05$ and $P < .01$), and increased the apoptotic cells rate from 3.1% to 11.5% and 15.9% (both $P < .01$) (Fig. 1A–B). Western blot analytical results further confirmed the apoptosis induced by TPL, as Bax was remarkably accumulated ($P < .05$ and $P < .01$), and caspase-3 and -9 were remarkably cleaved (all $P < .001$) by addition of 50 and 100 nM TPL (Fig. 1C–D).

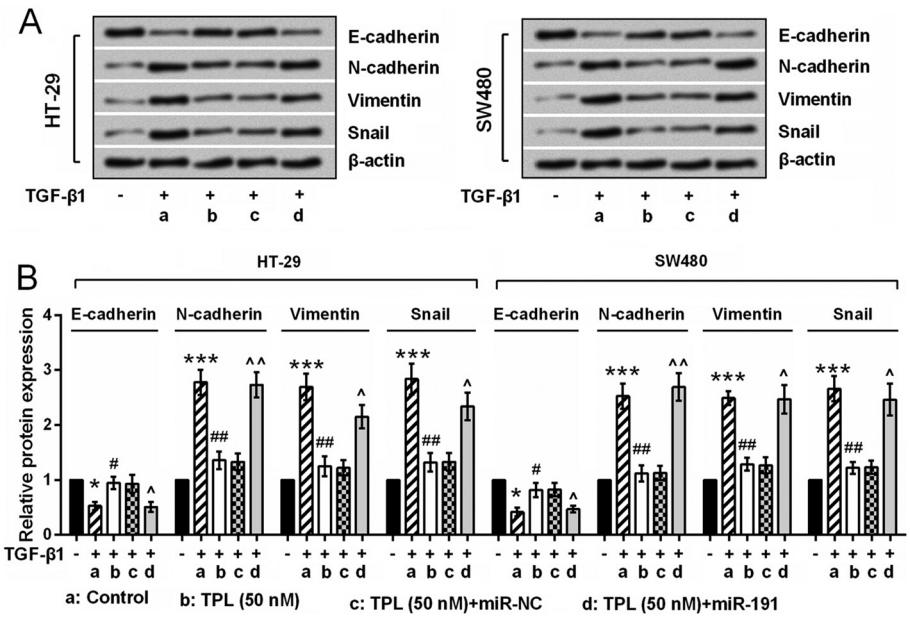

Fig. 5. The inhibitory effects of Triptolide (TPL) on epithelial-to-mesenchymal transition (EMT) process are alleviated by miR-191 up-regulation. (A) Protein expressions of EMT-related factors were detected after HT-29 and SW480 cells were pre-transfected with miR-191 mimic or miR-NC, and then treated with 50 nM TPL for 24 h in the presence or absence of 10 ng/mL TGF-β1. (B) Semi-quantitative analysis based on the results from western blotting. * $P < .05$, *** $P < .001$ compared with non-treated group. # $P < .05$, ## $P < .01$ compared with TGF-β1 group. ^ $P < .05$, ^^ $P < .01$ compared with TGF-β1 + TPL (50 nM) + miR-NC group.

The impacts of TPL treatment on HT-29 and SW480 cells migration were also evaluated. Considering TPL provokes potent cytotoxicity, the migratory capacity of HT-29 and SW480 cells was measured with the presence of *Z*-VAD-FMK, for ruling out the influence of apoptosis on the tested migration. Firstly, apoptosis of HT-29 and SW480 cells was evaluated following Z-VAD-FMK treatment. As results shown in Fig. 1E, the apoptosis induced by TPL was significantly inhibited by Z-VAD-FMK treatment ($P < .01$), indicating Z-VAD-FMK was capable of reducing TPL's cytotoxic effects. Then, wound healing assay was conducted to test cell migration. Data in Fig. 1F showed that, the number of migrated cells was significantly reduced by 50 nM TPL with the presence of Z-VAD-FMK ($P < .01$), suggesting the anti-migratory function of TPL.

Next, the expression changes of indicators of EMT were measured by western blot analysis to reveal if the inhibited migration by TPL was associated with EMT process. As results given in Fig. 2A–B, the protein level of E-cadherin was down-regulated ($P < .05$), while the protein levels of N-cadherin, Vimentin and Snail were up-regulated (all $P < .001$) in response to TGF-β1, indicating EMT was induced in HT-29 and SW480 cells. In contrast, 50 and 100 nM of TPL remarkably up-regulated the protein level of E-cadherin ($P < .05$), while down-regulated the protein levels of N-cadherin, Vimentin, and Snail ($P < .05$ or $P < .01$). The result suggested that TPL suppressed CRC cells migration possibly associated with EMT process.

3.2. TPL inhibited cultured CRC cells via down-regulating miR-191

It has been shown that miR-191 was frequently up-regulated in CRC cancer and the elevated expression of miR-191 contributed to CRC cells proliferation and tumorigenicity (Zhang et al., 2015). Herein, we found that the expression of miR-191 was significantly decreased by 50 and 100 nM TPL ($P < .05$, Fig. 3A). The results indicated that TPL conferred its anti-CRC properties possibly via down-regulating the expression of miR-191. In order to further reveal how TPL down-regulated the expression of miR-191, the relationship between TPL and EZH2 expression was studied. EZH2, a well-known histone methyltransferase, is involved in establishing and maintaining gene repression through cell division (Tan et al., 2014). Fig. 3B showed that, protein level expression of EZH2 was significantly repressed by TPL treatment ($P < .05$ in 50 nM group; $P < .001$ in 100 nM group). Besides, by silencing the expression of EZH2 (Fig. 3C–D), the down-regulation of miR-191 was observed ($P < .05$, Fig. 3E). These data collectively suggested the down-regulated miR-191 by TPL was via EZH2.

Then, the expression of miR-191 in HT-29 and SW480 cells were overexpressed by miRNA transfection ($P < .01$, Fig. 3F), to examine whether miR-191 was involved in the anti-CRC functions of TPL. We found that, the impacts of TPL on HT-29 and SW480 cells growth and migration were all alleviated by miR-191 up-regulation, as cell viability and migration were increased ($P < .05$, Fig. 4A and E), apoptotic cell rate was reduced ($P < .05$ or $P < .01$, Fig. 4B), as well as Bax, cleaved caspase-3 and -9 were down-regulated ($P < .05$, Fig. 4C–D) by miR-191 up-regulation.

As expected, miR-191 up-regulation could also alleviate the inhibitory effects of TPL on EMT process, as the protein level of E-cadherin was down-regulated ($P < .05$), and the protein levels of N-cadherin, Vimentn and Snail were up-regulated ($P < .01$, $P < .05$ and $P < .05$) by miR-191 up-regulation (Fig. 5A–B).

3.3. TPL blocked NF-κB and Wnt/β-catenin pathways via down-regulating miR-191

Next, we focused on NF-κB and Wnt/β-catenin pathways to further explain the anti-tumor effects of TPL. Western blot analytical results in Fig. 6A–D showed that, TPL treatment significantly inhibited the phosphorylation levels of p65 and IκBα, and the protein levels of Wnt3a, Wnt-5a and β-catenin (all $P < .05$), indicating TPL treatment blocked NF-κB and Wnt/β-catenin pathways. However, TPL induced these alterations were all reversed by miR-191 up-regulation ($P < .05$ or $P < .01$, Fig. 6A–D).

3.4. TPL treatment inhibited the growth of primary tumors in nude mice

Finally, in vivo experiments were performed to further confirm the anti-CRC properties of TPL. Fig. 7A showed that, oral administration with TPL significantly reduced tumor volume in both dose- and time-dependent manner. Also, tumor weight was reduced by TPL with a dose-dependent manner ($P < .05$ or $P < .01$, Fig. 7B), indicating the inhibitory effects of TPL on the growth of primary tumor xenografts. More interestingly, the expression of miR-191 in tumor tissues was significantly reduced by TPL treatment ($P < .05$ or $P < .01$, Fig. 7C). These in vivo data were consistent with the results observed from in vitro experiments, suggesting the anti-CRC properties of TPL via miR-191.

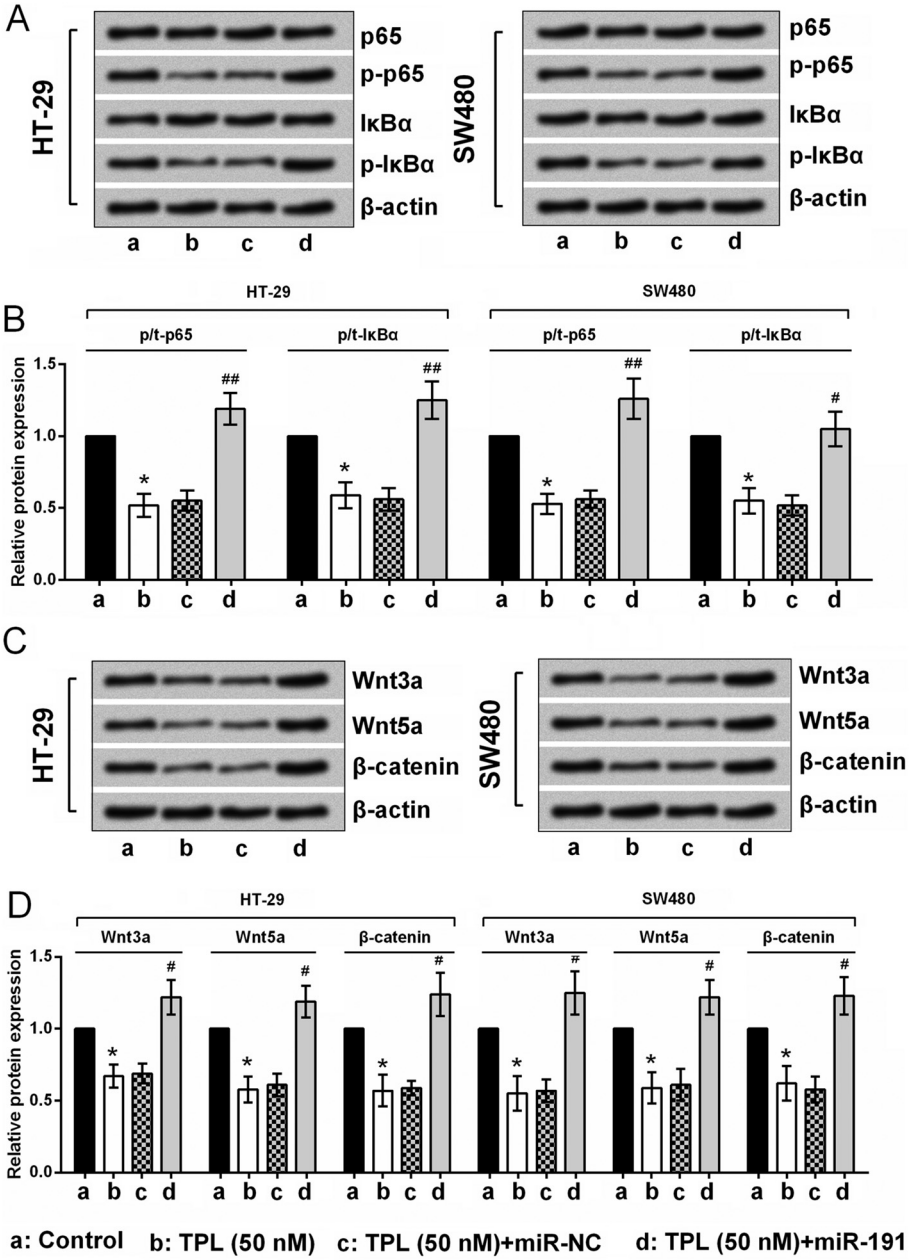

Fig. 6. NF-κB and Wnt/β-catenin pathways are blocked by Triptolide (TPL) while are activated by miR-191 up-regulation. Protein expressions of core factors in (A) NF-κB and (C) Wnt/β-catenin pathways were detected after HT-29 and SW480 cells were pre-transfected with miR-191 mimic or miR-NC, and then treated with 50 nM TPL for 24 h. (B and D) Semi-quantitative analysis based on the results from western blotting. * $P < .05$, *** $P < .001$ compared with non-treated group. # $P < .05$, ## $P < .01$ compared with TPL (50 nM) + miR-NC group.

4. Discussion

Tripterygium wilfordii, a traditional Chinese medicinal plant, has been used in China for centuries to treat a variety of inflammatory and autoimmune diseases. TPL is one of the major components of *Tripterygium wilfordii*. Its structure has been characterized early in 1972 by Kupchan and his colleagues (Kupchan et al., 1972). In the following four decades, its pharmacology efficacies including anti-tumor are widely reported (Cui et al., 2017; Kim and Park, 2017; Li et al., 2017; Song et al., 2017; Sun et al., 2017; Tamgue and Lei, 2017; Zhang et al., 2017). Herein, TPL was found to be effective in inhibiting the growth and migration of two CRC cell lines (HT-29 and SW480), as cell viability and migration were decreased, apoptosis was induced and EMT process was impeded by addition of 50 and 100 nM TPL. The inhibitory effects of TPL on the growth of primary tumors were also verified in a mouse model of CRC. Moreover, we found that miR-191 expression was reduced in response to TPL administration, and the anti-CRC actions of TPL were alleviated by miR-191 up-regulation. And also, the blockage of NF-κB and Wnt/β-catenin pathways induced by TPL was reversed by miR-191 up-regulation.

It has been previously reported that TPL exerted anti-CRC properties (Johnson et al., 2011; Liu et al., 2012a; Liu et al., 2012b; Oliveira et al., 2015). For instance, a previous study reported that 50 and 100 nM TPL potently inhibited HT29 and HCT116 cells growth (Johnson et al., 2011). The findings were similar to our results, suggested that TPL inhibited HT29 and SW480 cells viability in a dose-dependent fashion. We additionally demonstrated that, TPL increased the expression of Bax, and sequential activation of caspase-3 and -9. The result was consistent with the findings described elsewhere (Oliveira et al., 2015), indicating TPL induced HT29 and SW480 cells apoptosis in a

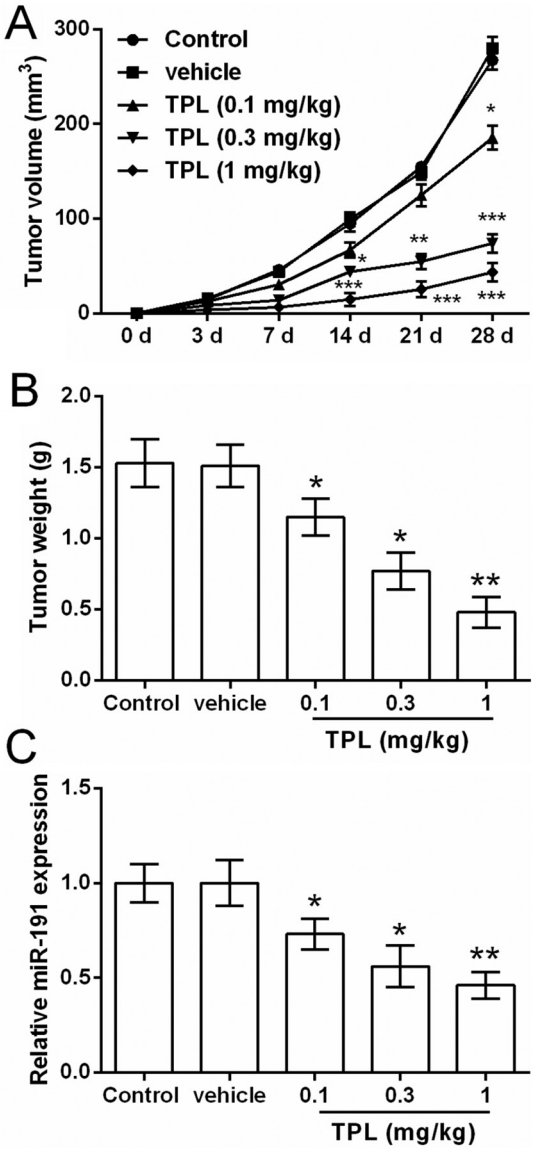

Fig. 7. Triptolide (TPL) treatment inhibited the growth of primary tumors in nude mice. HT-29 cells were injected subcutaneously into the flanks of mice to establish xenografts of colorectal cancer (CRC). After growing for 3 days, the mice were orally administrated with 0.1, 0.3 or 1 mg/kg/day TPL for continuous 4 weeks. Non-treated mice served as control, and the mice received same volume of vehicle (0.9% saline) served as negative control. (A) Tumor volume, (B) tumor weight as well as (C) the expression of miR-191 in tumor tissues were measured. * $P < .05$, ** $P < .01$, *** $P < .001$ compared with vehicle group.

mitochondria-dependent pathway.

Metastasis, the main cause of CRC mortality, requires the migration of cancer cells towards blood or lymphatic vessels (Mehlen and Puisieux, 2006). EMT endows cells with migratory and invasive properties, and EMT has been found to be involved in the migratory fronts of CRC (Tanaka et al., 2016). During EMT, cells lose their epithelial characteristics, down-regulating E-cadherin expression, while up-regulating N-cadherin, Vimentin, Snail, Slug, Twist, and ZEB1 expressions (Thiery et al., 2009). It has been reported that, TPL inhibited the migration of CRC cells via decreasing the expression of VEGF and COX-2, and inhibiting the expression of cytokine receptors such as thrombin receptor, CXCR4, TNF receptors and TGF-β receptors (Johnson et al., 2011). Our data showed that TPL up-regulated the expression of E-cadherin, and down-regulated the expressions of N-cadherin, Vimentin and Snail, providing the first in vitro evidence that TPL inhibited CRC cells migration through regulating EMT-related proteins.

It has been widely accepted that miRNA regulation is implicated in many cancers, including CRC (Tokarz and Blasiak, 2012). A growing number of studies reported that miR-191 acted as a multifunctional miRNA, with important roles in human diseases, such as acute myocardial infarction (Bilal et al., 2016), traumatic brain injury (Yang et al., 2016) and autoimmune vitiligo (Shi et al., 2014). Besides, miR-191 and cancer abnormalities have been reported in more than twenty different malignancies, marking miR-191 as a ubiquitously notorious miRNA like miR-21 or miR-155 (Nagpal and Kulshreshtha, 2014). In CRC tissues, miR-191 was found to be highly expressed compared to normal colorectal tissues (Xi et al., 2006), indicating the potential use of miR-191 as a biomarker for CRC diagnose. In vivo inhibition of miR-191 led to decreased tumorigenicity, and in vitro overexpression of miR-191 in CRC lines induced the G1-to-S cell-cycle transition and promoted cell resistance to 5-Fu (Zhang et al., 2015). Also, another study revealed miR-191 as an oncogene due to its overexpression in treatment-resistant CRC (Zhou et al., 2010). In line with these previous findings, we found that miR-191 exerted tumor-promoting functions in HT29 and SW480 cells, as its overexpression alleviated TPL-reduced cell growth, migration and EMT. We additionally demonstrated that miR-191 expression was suppressed in response to TPL administration, indicating miR-191 down-regulation might contribute to the anti-CRC actions of TPL. So far, the pharmacologic effects of TPL via regulating other miRNAs, like miR-137 (Han et al., 2017), miR-16-1 (Hou et al., 2017) and miR-21 (Li et al., 2016) have been reported. To the very best of our knowledge, the present study is the first to suggest miR-191 is implicated in the anti-tumor activities of TPL.

The comprehension of cancer-related mechanisms involved in alteration of methyltransferases is nowadays a matter of intense investigation. Overexpression of methyltransferases can drive malignant transformation through the silencing of tumor suppressive genes and non-coding RNAs (Stamato et al., 2017). EZH2, a well-known histone methyltransferase, catalyzes the methylation of histone H3 and leads to the silencing of tumor suppressive miRNAs (Ihira et al., 2017; Stamato et al., 2017). Besides, it has been revealed that EZH2 was a target for TPL in several types of cancers, like prostate cancer (Tamgue et al., 2013) and myeloma (Zhao et al., 2010). Thus, in order to further reveal how TPL down-regulated the expression of miR-191, the relationship between TPL and EZH2 expression was studied. Our data showed that the protein expression of EZH2 was down-regulated by TPL, and down-regulation of EZH2 was capable of suppressing miR-191 expression, which suggested that TPL down-regulated miR-191 via EZH2.

NF-κB and Wnt/β-catenin pathways play critical roles in many cellular processes, such as cell survival, inflammation and differentiation (Manigandan et al., 2015; Schon et al., 2014). NF-κB pathway is activated through degradation of IκBα and then triggering the translocation of various heterodimers, predominantly p65/p50, to the nucleus (Habibi et al., 2016). Both of these two pathways are pivotal in the regulation of colon tissue regeneration and the development of colon tumors (Moreau et al., 2011; Schon et al., 2014). The abnormal activation of Wnt/β-catenin pathway is a frequent early event in intestinal epithelial cells during the development of CRC (Bienz and Clevers, 2000). More interestingly, β-catenin regulates NF-κB activity in CRC cell lines and primary CRC (Schon et al., 2014), that β-catenin siRNA-treated cells exhibited NF-κB nuclear accumulation (Moreau et al., 2011). Herein, we observed that NF-κB and Wnt/β-catenin pathways were remarkably blocked by TPL, while the blockages were neutralized by miR-191. Our data provide the evidence that TPL inhibited CRC cells growth, migration and EMT via down-regulating miR-191, and thereby repressing the activation of NF-κB and Wnt/β-catenin pathways.

5. Conclusion

In summary, we demonstrate that TPL is effective in killing CRC cells by suppressing tumor cells viability, migration, EMT, and inducing apoptosis. The anti-CRC effects of TPL may be associated with the down-regulation of miR-191, and the activation of NF-κB and Wnt/β-catenin pathways. Further studies are required to confirm this hypothesis.

Acknowledgments

None.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest

All authors declare that they have no conflict of interest.

References

Baltruskeviciene, E., et al., 2017. Down-regulation of miRNA-148a and miRNA-625-3p in colorectal cancer is associated with tumor budding. BMC Cancer 17, 607.
Bienz, M., Clevers, H., 2000. Linking colorectal cancer to Wnt signaling. Cell 103, 311–320.
Bilal, M., et al., 2016. Circulation of miR-26a, miR-191, and miR-208b in plasma of patients with acute myocardial infarction. J. Pak. Med. Assoc. 66, 125.
Cui, J., et al., 2017. Advanced progress of main pharmacology activities of triptolide. Zhongguo Zhong Yao Za Zhi. 42, 2655–2658.
Cummins, J.M., et al., 2006. The colorectal microRNAome. Proc. Natl. Acad. Sci. U. S. A. 103, 3687–3692.
Cunningham, D., et al., 2010. Colorectal cancer. Lancet 375, 1030–1047.
Habibi, F., et al., 2016. Alteration in inflammation-related miR-146a expression in NF-KB signaling pathway in diabetic rat hippocampus. Adv. Pharm. Bull. 6, 99–103.
Han, F., et al., 2017. Triptolide Prevents Extracellular Matrix Accumulation in Experimental Diabetic Kidney Disease by Targeting microRNA-137/Notch1 Pathway.
Hou, H.W., et al., 2017. Triptolide exerts protective effects against fibrosis following ileocolonic anastomosis by mechanisms involving the miR-16-1/HSP70 pathway in IL-10-deficient mice. Int. J. Mol. Med. 40, 337–346.
Ihira, K., et al., 2017. EZH2 inhibition suppresses endometrial cancer progression via miR-361/twist axis. Oncotarget 8, 13509–13520.
Johnson, S.M., et al., 2011. Triptolide inhibits proliferation and migration of colon cancer cells by inhibition of cell cycle regulators and cytokine receptors. J. Surg. Res. 168, 197–205.
Kim, J.H., Park, B., 2017. Triptolide blocks the STAT3 signaling pathway through induction of protein tyrosine phosphatase SHP-1 in multiple myeloma cells. Int. J. Mol. Med. 40, 1566–1572.
Kupchan, S.M., et al., 1972. Triptolide and tripdiolide, novel antileukemic diterpenoid triepoxides from Tripterygium wilfordii. J. Am. Chem. Soc. 94, 7194–7195.
Li, X., et al., 2016. Triptolide reduces proliferation and enhances apoptosis of human non-small cell lung cancer cells through PTEN by targeting miR-21. Mol. Med. Rep. 13, 2763–2768.
Li, R., et al., 2017. Triptolide suppresses growth and hormone secretion in murine pituitary corticotroph tumor cells via NF-kappaB signaling pathway. Biomed. Pharmacother. 95, 771–779.
Liu, J., et al., 2012a. Triptolide inhibits colon-rectal cancer cells proliferation by induction of G1 phase arrest through upregulation of p21. Phytomedicine 19, 756–762.
Liu, Y., et al., 2012b. Triptolide inhibits colon cancer cell proliferation and induces cleavage and translocation of 14-3-3 epsilon. Cell Biochem. Funct. 30, 271–278.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.
Manigandan, K., et al., 2015. Taxifolin curbs NF-kappaB-mediated Wnt/beta-catenin signaling via up-regulating Nrf2 pathway in experimental colon carcinogenesis. Biochimie 119, 103–112.
Mehlen, P., Puisieux, A., 2006. Metastasis: a question of life or death. Nat. Rev. Cancer 6, 449–458.
Moreau, M., et al., 2011. Beta-catenin and NF-kappaB cooperate to regulate the uPA/uPAR system in cancer cells. Int. J. Cancer 128, 1280–1292.
Nagpal, N., Kulshreshtha, R., 2014. miR-191: an emerging player in disease biology. Front. Genet. 5, 99.
Oliveira, A., et al., 2015. Triptolide abrogates growth of colon cancer and induces cell cycle arrest by inhibiting transcriptional activation of E2F. Lab. Investig. 95, 648–659.
Qin, S., et al., 2014. MicroRNA-191 correlates with poor prognosis of colorectal carcinoma and plays multiple roles by targeting tissue inhibitor of metalloprotease 3. Neoplasma 61, 27–34.
Schetter, A.J., et al., 2008. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. JAMA 299, 425–436.
Schon, S., et al., 2014. Beta-catenin regulates NF-kappaB activity via TNFRSF19 in colorectal cancer cells. Int. J. Cancer 135, 1800–1811.
Shi, Y.L., et al., 2014. Serum miRNA expression profiles change in autoimmune vitiligo in mice. Exp. Dermatol. 23, 140–142.
Siegel, R.L., et al., 2017. Cancer statistics, 2017. CA Cancer J. Clin. 67, 7–30.
Song, J.M., et al., 2017. Triptolide suppresses the in vitro and in vivo growth of lung cancer cells by targeting hyaluronan-CD44/RHAMM signaling. Oncotarget 8, 26927–26940.
Stamato, M.A., et al., 2017. Inhibition of EZH2 triggers the tumor suppressive miR-29b network in multiple myeloma. Oncotarget 8, 106527–106537.
Sun, Y.Y., et al., 2017. Triptolide inhibits viability and induces apoptosis in liver cancer cells through activation of the tumor suppressor gene p53. Int. J. Oncol. 50, 847–852.
Tamgue, O., Lei, M., 2017. Triptolide promotes senescence of prostate cancer cells through histone methylation and heterochromatin formation. Asian Pac. J. Cancer Prev. 18, 2519–2526.
Tamgue, O., et al., 2013. Triptolide inhibits histone methyltransferase EZH2 and modulates the expression of its target genes in prostate cancer cells. Asian Pac. J. Cancer Prev. 14, 5663–5669.
Tan, J.Z., et al., 2014. EZH2: biology, disease, and structure-based drug discovery. Acta Pharmacol. Sin. 35, 161–174.
Tanaka, S., et al., 2016. Snail1 expression in human colon cancer DLD-1 cells confers invasive properties without N-cadherin expression. Biochem. Biophys. Rep. 8, 120–126.
Tao, X., Lipsky, P.E., 2000. The Chinese anti-inflammatory and immunosuppressive herbal remedy Tripterygium wilfordii Hook F. Rheum. Dis. Clin. N. Am. 26, 29–50 (viii).
Thiery, J.P., et al., 2009. Epithelial-mesenchymal transitions in development and disease. Cell 139, 871–890.
Tokarz, P., Blasiak, J., 2012. The role of microRNA in metastatic colorectal cancer and its significance in cancer prognosis and treatment. Acta Biochim. Pol. 59, 467–474.
Xi, Y., et al., 2006. Prognostic values of microRNAs in colorectal cancer. Biomark. Insights 2, 113–121.
Yang, T., et al., 2016. Elevated serum miR-93, miR-191, and miR-499 are noninvasive biomarkers for the presence and progression of traumatic brain injury. 137, 122–129.
Yuan, Z., et al., 2017. Dynamic plasma microRNAs are biomarkers for prognosis and early detection of recurrence in colorectal cancer. Br. J. Cancer 117, 1202–1210.
Zhang, X.F., et al., 2015. miR-191 promotes tumorigenesis of human colorectal cancer through targeting C/EBPbeta. Oncotarget 6, 4144–4158.
Zhang, C., et al., 2017. Effect of the natural product triptolide on pancreatic cancer: a systematic review of preclinical studies. Front. Pharmacol. 8, 490.
Zhao, F., et al., 2010. Triptolide alters histone H3K9 and H3K27 methylation state and induces G0/G1 arrest and caspase-dependent apoptosis in multiple myeloma in vitro. Toxicology 267, 70–79.
Zhou, J., et al., 2010. 5-Fluorouracil and oxaliplatin modify the expression profiles of microRNAs in human colon cancer cells in vitro. Oncol. Rep. 23, 121–128.