

Luteolin suppresses colorectal cancer cell metastasis via regulation of the miR-384/pleiotrophin axis

YUANYUAN YAO¹, CHUNHUI RAO², GANG ZHENG³ and SAISAI WANG¹

¹Department of Colorectal Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine;

²Department of Colorectal Surgery, Hangzhou Hospital of Traditional Chinese Medicine, Guangxing Hospital Affiliated to Zhejiang University of Traditional Chinese Medicine, Hangzhou, Zhejiang 310003;

³Department of Cardiology, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310009, P.R. China

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Abstract. Luteolin (3,4,5,7-tetrahydroxyflavone) is a natural flavonoid that has been found to exhibit anticancer properties in certain types of cancers. In the present study, the role of luteolin and its underlying mechanisms were explored in colorectal cancer (CRC) cells. First, the effects of luteolin on CRC cells proliferation, migration and invasion were examined by CCK-8, wound healing and Transwell assays, respectively. It was demonstrated that luteolin had no effects on CRC cells proliferation while inhibited cells migration and invasion both *in vitro* and *in vivo*. Then, expression of pleiotrophin (PTN) and miR-384 was detected in cells and CRC tissues by qPCR. Luteolin was found to upregulate miR-384 and downregulate PTN expressions both in CRC cells and tissues. miR-384 inhibition and PTN overexpression partially reversed the inhibition of HT-29 cells migration and invasion induced by luteolin. Target analysis revealed that miR-384 directly regulates PTN expression. The correlation analysis between PTN expression and clinical characteristics revealed that PTN expression was positively related to cancer progression. The present study demonstrated that luteolin exerts anticancer effects against CRC cells by modulating PTN via miR-384 expression suggested that PTN may serve as a promising candidate for therapeutic applications in CRC treatment.

Introduction

Luteolin (3,4,5,7-tetrahydroxyflavone) is a natural flavonoid abundant in various fruits and vegetables. Luteolin has been

reported to display a wide range of bioactive properties, such as anti-virus (1,2), hepatoprotective (3), anti-inflammatory (4) and anti-diabetic effects (5). Recently, the antitumor effects of luteolin have been extensively investigated in breast and gastric cancer (6), melanoma (7), esophageal carcinoma (8), glioblastoma (9), hepatocellular carcinoma (10), placental choriocarcinoma (11) and lung adenocarcinoma (12). The role of luteolin in colorectal cancer (CRC) has also been investigated. Luteolin has been found to inhibit CRC cells proliferation, induce cells apoptosis and cell cycle perturbation (13-15). Liu *et al* also reported that luteolin was able to inhibit CRC cells epithelial to mesenchymal transition by suppressing CREB1 expression (16). Luteolin was demonstrated to play the protective roles in 1,2-dimethylhydrazine and azoxymethane (AOM)-induced experimental colon carcinogenesis (17,18). Moreover, luteolin increased the sensitivity of CRC cells to chemotherapy (19,20).

MicroRNAs (miRNAs) are a category of small, non-coding, single-stranded RNAs of 18-25 nucleotides in length which bind to the 3'-untranslated regions (3'-UTRs) of their target mRNAs. miRNAs regulate the expressions of target genes through post-transcriptional silencing or induction of degradation (21,22). Recent studies have revealed critical functions of miRNAs in tumor biological processes, including cell proliferation, differentiation, metastasis and chemosensitivity (23,24). By regulating a variety of target genes in cancer cells, miRNAs act as oncogenes or tumor suppressors.

Several studies have reported the involvement of miRNAs in the effects induced by luteolin. In prostate cancer cells, luteolin treatment inhibited prostate cancer cells proliferation and induced apoptosis through downregulation of miR-301 by triggering DEDD2 expression (25). Zhou *et al* found that luteolin upregulated miR-34 expression in gastric cancer cells and upregulation of miR-34 enhanced the susceptibility of cells to luteolin (6). Luteolin also inhibited the tumorigenesis and induced the apoptosis of non-small cell lung cancer cells by upregulation of miR-34a-5p by targeting MDM4 (26). Thus, we hypothesized that miRNAs play significant roles during luteolin treatment in CRC cells and this has not yet been investigated.

Correspondence to: Dr Saisai Wang, Department of Colorectal Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, 79 Qingchun Road, Hangzhou, Zhejiang 310003, P.R. China
E-mail: saisaiwang@zju.edu.cn

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In the present study, it was observed that luteolin inhibited CRC cells migration and invasion both *in vitro* and *in vivo* while had no effects on cells proliferation. miR-384 expression was markedly upregulated and inhibition of miR-384 partly reversed the inhibitory effects induced by luteolin. However, the expression of pleiotrophin (PTN) was found to be downregulated after luteolin treatment and was confirmed to be a direct target of miR-384. By analyzing CRC tissues, we found that miR-384 expression was downregulated while PTN expression was upregulated compared with the paired-matched adjacent tissues. The correlation analysis between PTN expression and clinical characteristics revealed that PTN expression is positively related to the cancer progression. Hence, the present study demonstrated that luteolin exerts anticancer effects against CRC cells by modulating PTN via miR-384 expression suggesting that PTN may serve as a promising candidate for therapeutic applications in CRC treatment.

Materials and methods

Cells and reagents. HT-29, SW480, SW620 and LoVo human CRC cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The normal human mucosal epithelial cell line NCM460 was purchased from the National Institute of Cells (Shanghai, China). All cells were maintained in RPMI-1640 medium (HyClone; GE Healthcare, Chicago, IL, USA) supplemented with 10% fetal bovine serum (FBS; Gibco™, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin-streptomycin in a humidified incubator containing 5% CO₂ at 37°C. Luteolin was purchased from Sigma-Aldrich/Merck (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO).

CRC tissues and relative patient clinical information. We collected a total of 50 cases of CRC and matched normal tissues from patients who underwent surgery at Hangzhou Hospital of Traditional Chinese Medicine from May 2017 to May 2018. All postoperative pathology was determined to be colorectal adenocarcinoma. The following patient characteristics were recorded: age, sex, histologic differentiation, TNM stage and serum levels of carcinoembryonic antigen (CEA) before surgery and all the data are listed in Table I. Approval of the present study was obtained from the Ethics Committee of Hangzhou Hospital of Traditional Chinese Medicine and informed consent was acquired from all the patients.

Cell viability assay. Cell proliferation ability was detected by Cell Counting Kit-8 (CCK-8) assay kit (Beyotime Institute of Biotechnology, Shanghai, China). In brief, CRC cells were plated in a 96-well plate at the initial density of 1,000 cells/well and cultured in medium without or with luteolin at concentrations of 0, 10, 50 and 100 μM for 24, 48 and 72 h. CCK-8 solutions were added into the cell cultures 2 h before harvesting. The optical density at 450 nm was measured using an ultra-microplate reader (EMax; Molecular Devices, Sunnyvale, CA, USA).

In vivo nude mouse study. Thirty-nine BALB/c nude mice (17-20 g) were utilized to evaluate the effects of luteolin

on tumor growth and tumor metastasis *in vivo*. Five- to six-week-old female BABL/c nude mice were purchased from Shanghai Slac Animal (Shanghai, China) and maintained in the animal facility at the Zhejiang University (Hangzhou, China). Mice were provided with water and food *ad libitum* and kept under standard conditions (temperature 24±2°C, humidity, 50-70%, 12-h light/dark cycle). The study received ethical approval from the Animal Care and Use Committee of Zhejiang University, and experiments and animal care were performed according to the approved protocols.

For the tumor growth assay, HT-29 cells (1×10⁶) were subcutaneously injected into the right flanks of the nude mice. When the volumes of xenograft tumors reached an average of 100 mm³, the mice were randomly divided into two groups: the control group and the luteolin group with 10 mice in each group (20 mice in total). Mice received luteolin were intragastrically administered with luteolin at 100 mg/kg every 2 days for 30 days. The control group mice received the same volume of phosphate-buffered saline (PBS). Tumor measurements were performed with a caliper by measuring the largest diameter and its perpendicular length every 3 days. According to the rules of our ethics committee, humane endpoints set for the tumor experiments were as follows: the tumor weight was >10% of the original body weight or the average tumor diameter was >20 mm in mice. Thus, when the tumor almost reached 20 mm, the experiment was terminated. The mice were sacrificed by cervical dislocation, and tumors were harvested and weighed on day 30. Tumor volume was calculated using the following formula: $V = \frac{1}{2} \times (\text{length} \times \text{width}^2)$. To note, we repeated this experiment for the same setting for three times (8 mice for the first experiment, and (6 mice for the other two experiments).

For liver metastasis assay, mice (n=19) were anesthetized with 75 mg/kg pentobarbital by intraperitoneal injection. Mice were fixed and then a small left abdominal flank incision (1 cm) was made and the spleen was exteriorized for the intrasplenic injection. HT-29 cells (5×10⁶) suspended in 100 μl ice-cold PBS were injected into the spleen with a 27-gauge needle. A sterile cotton was held over the site of injection for 30 sec to prevent tumor cell leakage and bleeding. The injected spleen was returned to the abdomen and the wound was sutured with 5-0 black silk. Mice receiving luteolin were intragastrically administered with luteolin at 100 mg/kg every 2 days for 30 days. The control mice received the equal volume of PBS. After 3 weeks, the mice were sacrificed by cervical dislocation, spleen and liver were removed and observed. The liver metastatic tumors on the surface were calculated and H&E staining was performed. H&E staining was performed at Servicebio Co., Ltd. (Wuhan, China). To note, we also repeated this experiment three times (6 mice for the first two experiments and 7 mice for the third trial).

Wound healing assay. HT-29 cells were seeded in 24-well plate until reaching 80% confluency in the complete medium. The monolayer was wounded using a sterile 200-μl micropipette tip. The cells were washed with PBS three times to rinse off the detached cells. Cells were incubated for 48 h in 2% FBS medium containing the indicated concentrations of luteolin. Images of the wound morphology were acquired under a light microscopy at x100 magnification.

Table I. Clinicopathological characteristics of PTN expression in the human CRC cases.

Characteristics	Expression of PTN		P-value
	Low (n=25)	High (n=25)	
Age (years)			0.7761
>60	15	13	
≤60	10	12	
Sex			0.3961
Male	14	10	
Female	11	15	
Differentiation			0.1963
Well	15	14	
Moderate	10	8	
Poor	0	3	
T factor			0.0477 ^a
1+2	16	9	
3+4	9	16	
N factor			0.0449 ^a
0	18	11	
1+2	7	14	
M factor			0.0414 ^a
0	19	12	
1	6	13	
CEA (ng/ml)			0.3686
>5	13	9	
≤5	12	16	

^aP<0.05, significant results. PTN, pleiotrophin; CRC, colorectal cancer; CEA, carcinoembryonic antigen.

Transwell migration and invasion assays. Cell migration and invasion potential were assessed using 8- μ m Transwell chambers (Corning Costar, Corning, NY, USA). Briefly, 8x10⁴ CRC cells suspended in 200 μ l serum-free medium treated with luteolin or not were seeded in the upper chambers with the lower chamber filled with 600 μ l of medium supplemented with 20% FBS. For the invasion assay, the upper surfaces of the membranes were coated with 50 μ l Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) 6 h before cells were seeded. Cells were allowed to migrate for 36 h before being fixed with ice-cold methanol and stained with 0.1% crystal violet for 30 min. Cells that passed through the membrane were photographed and counted with an Olympus CKX41 light microscope (Olympus, Shanghai, China; magnification, x100).

Western blotting. Cells or CRC tissues for protein extraction were lysed with RIPA buffer (Beyotime Institute of Biotechnology). Protein concentration was determined by BCA assay (Cwbiotech, Beijing, China). Total proteins (30 μ g) were separated by 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes.

The membranes were incubated with primary antibodies against human MMP-16 (cat. no. E-AB-32127), MMP-2 (cat. no. 10373-2-AP), MMP-3 (cat. no. 17873-1-AP), MMP-9 (cat. no. 10375-2-AP), PTN (cat. no. 27117-1-AP) (all diluted 1:1,000) and GAPDH (cat. no. 60004-1-Ig) (dilution 1:5,000) at 4°C overnight. Subsequently, the membranes were incubated with the peroxidase (HRP)-conjugated goat anti-rabbit (dilution 1:5,000; cat. no. SA00001-2) or goat anti-mouse (dilution 1:5,000; cat. no. SA00001-1) secondary antibody at room temperature for 2 h. MMP-16 was purchased from Elabscience (Shanghai, China). All the other antibodies were purchased from Proteintech Group Inc. (Chicago, IL, USA). The protein bands were detected with FluorChem E System (ProteinSimple, Santa Clara, CA, USA).

RNA isolation and quantitative real-time PCR (qPCR). Total RNA was extracted from the cells or the surgically resected CRC and adjacent non-tumor tissues using TRIzol reagent (Thermo Fisher Scientific, Inc.). Then, 1 μ g of total RNA was reverse transcribed using the HiFiScript cDNA Synthesis kit or miRNA cDNA Synthesis kit (Cwbiotech) according to the manufacturer's instructions. The PTN expression levels were determined with UltraSYBR Mixture (Cwbiotech) and the miR-384 levels were determined with miRNA qPCR Assay Kit (Cwbiotech) on CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA, USA). The miR-384 and U6 primers were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China), and the PTN and β -actin primers were synthesized by Thermo Fisher Scientific, Inc. The primers were as follows: miR-384 forward, 5'-TGTTAAATCAGGAATTTTAA-3' and reverse, 5'-TGT TACAGGCATTATGAA-3'; U6 forward, 5'-CTCGTTCCG CAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGC GT-3'; PTN forward, 5'-ACCAAGTGAGTCATCCGTCCA-3' and reverse, 5'-TGCAAATTTTCGACGCTGCT-3'; β -actin forward, 5'-GTATCCTGACCCTGAAGTACC-3' and reverse, 5'-TGAAGGTCTCAAACATGATCT-3'. The thermocycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Relative fold-change in expression of miR-384 and PTN was normalized to U6 and β -actin expression, respectively. The relative expression was analyzed using the 2^{- $\Delta\Delta$ Cq} (27) method.

Plasmid, miR-384 mimic, miR-384 inhibitor and transfection. miR-384 mimic, miR-384 inhibitor and its relative control miRNAs were synthesized by Shanghai GenePharma Co., Ltd. The sequences are as follows (28): miR-384 mimic: 5'-AUU CCUAGAAAUUGUUCAUA-3', miR-384 inhibitor: 5'-UAU GAACAAUUCUAGGAAU-3', miR-384 mimic NC: 5'-UUC UCCGAACGUGUCACGU-3', miR-384 inhibitor NC: 5'-CAG UACUUUUGUGUAGUACAA-3'. Full length clone DNA of human pleiotrophin was obtained from Sino Biological Inc. (Shanghai, China). Transfections were performed using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Mapping of prediction algorithm data. Target genes for miR-384 were predicted using miRDB (<http://www.mirdb.org>) and TargetScan v.5.1 (<http://www.targetscan.org/>). From

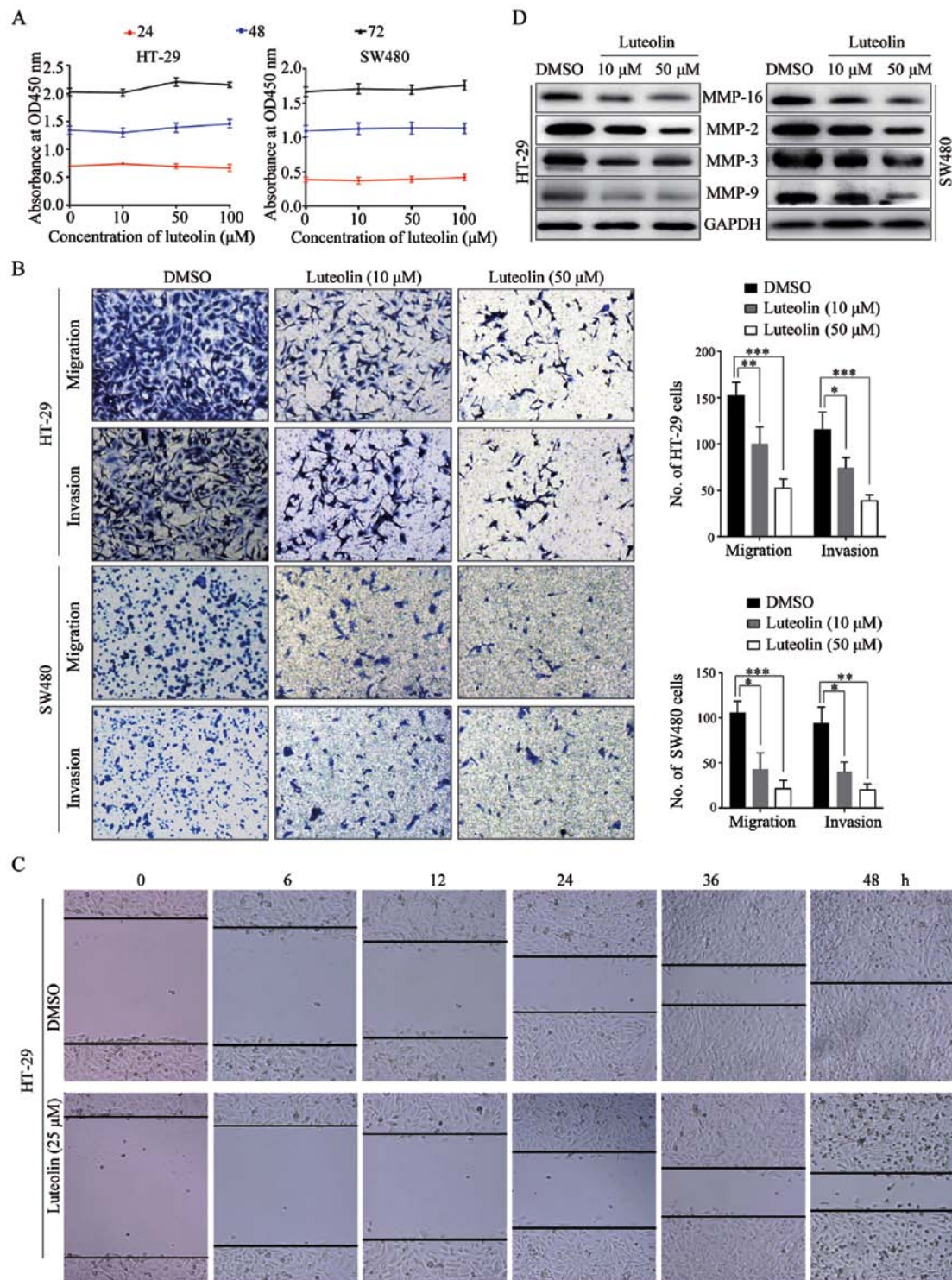


Figure 1. Luteolin inhibits CRC cells migration and invasion *in vitro* and *in vivo*. (A) HT-29 and SW480 cells were treated with luteolin at the concentrations of 10, 50 and 100 μ M. CCK-8 assay was performed after culturing for 24, 48 and 72 h. (B) Migration and invasion capacity of HT-29 and SW480 cells after treated with luteolin were determined and representative images were taken. Original magnification, x100. (C) Monolayers of HT-29 cells were wounded by a tip and treated with the indicated concentrations of luteolin. The representative images of the wound healing were taken at 0, 6, 12 and 24 h after the scratch was created. Original magnification, x100. (D) Expressions of MMP-2, MMP-3, MMP-9 and MMP-16 in HT-29 and SW480 cells were detected by western blotting after stimulation with luteolin for 24 h. Results were obtained from 3 independent experiments and are expressed as the means \pm SEM. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test. * P <0.05, ** P <0.01, *** P <0.001. CRC, colorectal cancer; CCK-8, Cell Counting Kit-8; DMSO, dimethyl sulfoxide.

the TargetScan, we found that PTN ranked the top 2 genes that was the targets of miR-384. We then confirmed it in the miRDR database and found that PTN ranked 86. Thus, we chose PTN as one candidate of miR-384 target genes.

Luciferase reporter assay. Wild-type (WT) or the mutated type (MUT) of PTN-3'-UTR containing miR-384 binding sites was synthesized by Shanghai GenePharma Co., Ltd., and cloned downstream of the luciferase reporter gene. HT-29

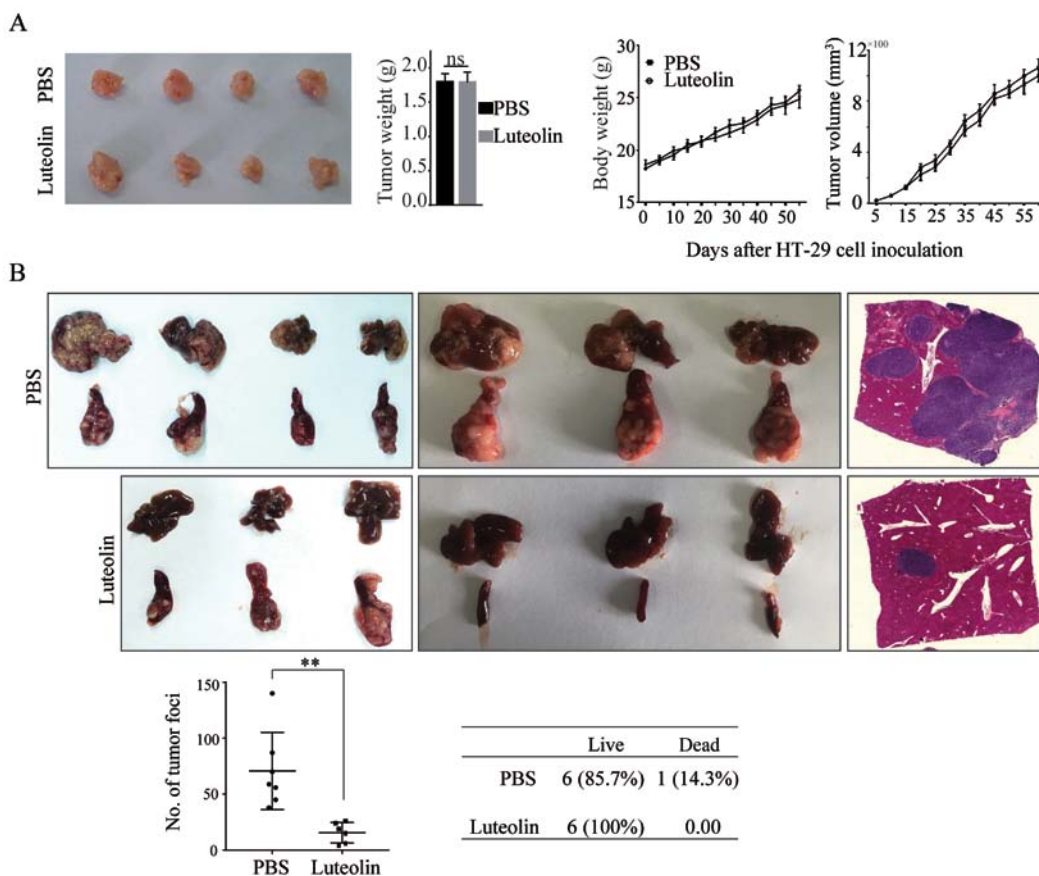


Figure 2. Luteolin inhibits CRC cells metastasis *in vivo*. (A) Nude mice were inoculated subcutaneously with HT-29 cells followed by PBS or luteolin treatment. The volume of the tumor and the body weight was monitored. Tumors were photographed at the end of the experiment. (B) Nude mice were intraspinaly injected with HT-29 cells followed by PBS and luteolin treatment. At the end of the experiment, tumor lesions in the liver were photographed and counted. Original magnification, x4. Results were obtained from 3 independent experiments and are expressed as the means \pm SEM. ** $P < 0.01$; ns, not significant. CRC, colorectal cancer; PBS, phosphate-buffered saline.

cells were seeded into 24-well plates (5×10^4 cells/well) one day before and then co-transfected with the WT PTN-3'-UTR or the MUT PTN-3'-UTR vectors (500 ng) and the miR-384 mimics or negative controls with Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Inc.). After 48 h of transfection, cells were harvested, and the luciferase activity was detected with the Dual-Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA).

Oncomine database. Oncomine database (<http://www.oncomine.org>) was selected to search the PTN expression levels between the CRC and normal groups.

Statistical analysis. Data are expressed as means \pm SEM. Statistical significance was determined by Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's test. The relationship between the PTN expression and the CRC clinical features was analyzed with the Chi-square test. P-value < 0.05 was considered as indicative of statistical significance.

Results

Luteolin inhibits CRC cells migration and invasion both *in vitro* and *in vivo*. In order to explore the functions of

luteolin in CRC, we first detected HT-29 and SW480 cells proliferation after luteolin treatment by CCK-8 assay. The results showed that luteolin had no effects on CRC cells proliferation (Fig. 1A). We then examined cells migration and invasion by Transwell and wound healing assays. As shown in Fig. 1B, we observed that luteolin-treated HT-29 and SW620 cells displayed significantly reduced cells migration and invasion. Consistently, luteolin treatment induced a slower closing of scratch wounds in the HT-29 cells (Fig. 1C), indicating that luteolin inhibited cells migratory ability. We also detected the expressions of several matrix metalloproteinases (MMPs), which are strongly related with tumor migration and invasion and overexpressed in human CRCs. As shown in Fig. 1D, luteolin obviously downregulated MMP-2, MMP-3, MMP-9 and MMP-16 expressions.

We then performed the *in vivo* assay to observe the tumor growth and tumor metastasis by injecting HT-29 cells into the nude mice subcutaneously and into the spleen, respectively. In accordance with the *in vitro* results, intake of luteolin did not impact the tumor growth as evidenced by no differences in tumor volume and tumor weight (Fig. 2A). However, luteolin significantly inhibited HT-29 cells metastasis from the spleen to the liver (Fig. 2B). The number of metastatic nodules in the liver was significantly decreased. Taken together, all these data suggest that luteolin plays a significant role in suppressing the

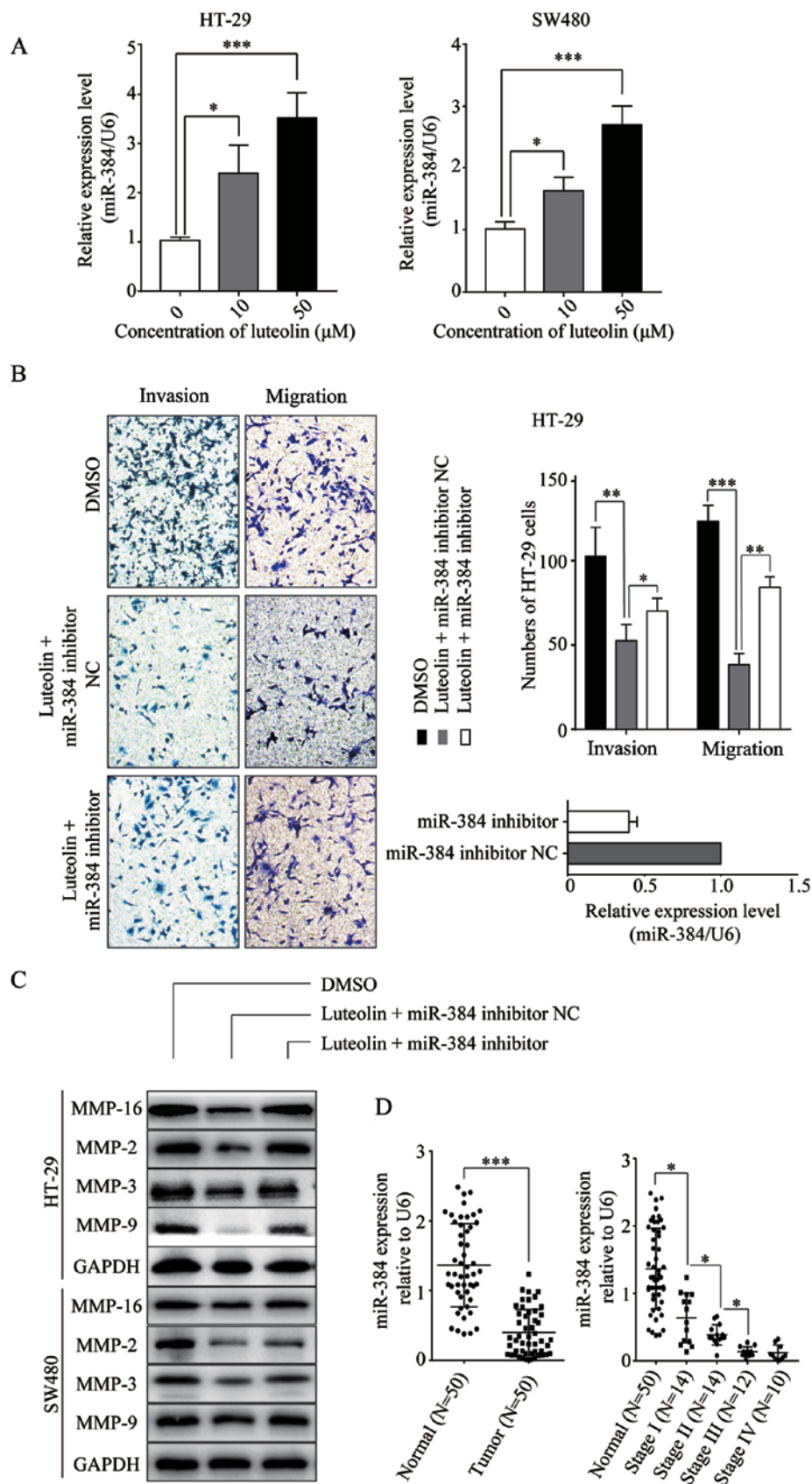


Figure 3. Luteolin inhibits CRC cells migration and invasion by upregulating miR-384. (A) Total RNA was extracted from HT-29 and SW480 cells treated with luteolin (10 and 50 μM) for 24 h and miR-384 expression was determined by qPCR. (B) HT-29 and SW480 cells were transfected with the miR-384 inhibitor NC or miR-384 inhibitor for 24 h, and then treated with 50 μM luteolin and cell migration and invasion abilities were determined by Transwell assay. (C) HT-29 and SW480 cells were transfected with the miR-384 inhibitor NC or miR-384 inhibitor for 24 h and cells were treated with luteolin for 24 h before protein was extracted for western blotting to determine MMP-2, MMP-3, MMP-9 and MMP-16 expression. (D) Total RNA was extracted from 50 pairs of CRC tissues and the corresponding adjacent normal tissues and qPCR was performed to determine the miR-384 expression. Results are representative of 3 independent experiments and are expressed as mean \pm SEM. Statistical significance in (A and B) was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test and statistical significance in D was determined by Student's t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. CRC, colorectal cancer; DMSO, dimethyl sulfoxide.

migration and invasion of CRC cells both *in vitro* and *in vivo* while has no effects on cells proliferation.

Luteolin inhibits CRC cells migration and invasion by upregulating miR-384. To identify the specific miRNA involved in the luteolin-induced inhibition of CRC cells migration and invasion, the expression level of miR-384 was determined in CRC cells treated with luteolin. It was found that the expression of miR-384 was significantly elevated in the CRC cell lines following treatment with luteolin in a dose-dependent manner compared with no-luteolin stimulation (Fig. 3A). Then, we constructed the miR-384 inhibitor and transfected into the CRC cells before luteolin treatment. As shown in Fig. 3B, the miR-384 inhibitor partially reversed the inhibition of cells migration and invasion induced by luteolin. Simultaneously, the expression levels of MMP-2, MMP-3, MMP-9 and MMP-16 which were decreased by luteolin treatment showed an increase compared to the miR-384 inhibitor NC group (Fig. 3C). All these results imply that miR-384 is involved in the anticancer effects of luteolin. We then detected the miR-384 expression level in CRC tissues. Downregulation of miR-384 expression was noted in the CRC tissues compared with that observed in the non-tumor tissues ($P < 0.001$; Fig. 3D). Moreover, with the progression of the tumor, the expression was decreased significantly. The expression of miR-384 was lowest in patients with TNM stage IV.

PTN is a direct target of miR-384. miRNAs exert their function by regulating the expression of their target genes. Thus, we took advantage of the prediction algorithms (miRDB and TargetScan v.5.1) to identify the potential targets of miR-384. Among the potential target genes, pleiotrophin (PTN) caught our interest. Thus, we detected the PTN expression after luteolin stimulation. The results showed that PTN expression was downregulated in a dose-dependent manner (Fig. 4A). Overexpression of PTN was able to significantly reverse the luteolin-mediated inhibition of cell migration and invasion as well as MMP expressions (Fig. 4B-D). This finding suggests that luteolin inhibited CRC cells migration and invasion by downregulating PTN expression. We next detected the relationship between PTN and miR-384. The expression of PTN was decreased when cells transfected with miR-384 mimic (Fig. 4E). Then miR-384 mimic or miR-384 mimic NC and PTN were co-transfected into CRC cells and the luciferase activity was detected. Compared with the control, a decrease in relative luciferase activity was observed when the WT PTN 3'-UTR was co-transfected with miR-384. However, the miR-384 mimic did not affect luciferase activity in the mutant construct (Fig. 4F). These data indicate that miR-384 directly modulates PTN expression by binding to its 3'-UTR. We also detected PTN expression after cells transfected with the miR-384 inhibitor followed by treatment with luteolin. Results showed that miR-384-inhibitor rescued the PTN downregulation induced by luteolin (Fig. 4G), confirming that luteolin-induced suppression of PTN expression was mediated by miR-384.

Expression of PTN in CRC tissues and its relationship with clinical characteristics. Data from Oncomine database were analyzed to examine the differential expression levels of PTN

DNA between CRC and normal tissues. As shown in Fig. 5A, PTN was upregulated in tumor tissues ($P < 0.001$). We also collected 50 cases of human CRC tissues and the paired adjacent normal tissues. The relative expression level of PTN was measured by qPCR (Fig. 5B) and western blotting (Fig. 5C). Results showed that PTN was highly expressed in CRC tissues. Furthermore, as the tumor progressed, the expression level of PTN increased as well. We also measured the PTN expression level in four CRC cell lines (SW480, SW620, LoVo and HT-29) and one normal colon epithelial cell (NCM460). The expression level of PTN was markedly higher in CRC cell lines than that noted in the NCM460 cells (Fig. 5D). Then, we analyzed the expression levels of miR-384 and PTN in tumor tissues, and we found that the Pearson's correlation coefficient was -0.58 , demonstrating a negative correlation between miR-384 and PTN (Fig. 5E).

We next divided the patients into two groups according to the PTN expression level (25 patients in each group). The relationship between PTN expression and the clinical characteristics including age, sex, tumor differentiation, TNM stage and serum CEA level was determined. The results are shown in Table I. The differential expression of PTN was significantly associated with tumor size, N stage and M stage of CRC patients (all $P < 0.05$).

Discussion

Currently, the prognosis or therapy for patients with advanced stage of colorectal cancer (CRC) is poor. Treatment includes chemotherapy, radiotherapy and surgery. Recently, traditional Chinese medicine has attracted much attention in the treatment of cancer due to their extensive tumor-suppressive activity, safety and inexpensiveness. One such reagent is luteolin. In the present study, luteolin was found to inhibit CRC cells migration and invasion both *in vitro* and *in vivo*, but it did not affect the proliferation of CRC cells. Further experiments revealed the important role of the miR-384/PTN axis in the luteolin-induced effects.

Many reports have reported that luteolin can induce cell cycle arrest and apoptosis in CRC cells. For example, Krifa *et al* reported that luteolin induced cytotoxicity in human CRC cell line BE and the IC_{50} value was 90.13 and 48.32 μ M for 24 and 48 h, respectively (13). Kang *et al* showed that luteolin inhibited HT-29 cells proliferation in a dose-dependent manner from 5 to 100 μ g/ml (29). Luteolin also decreased the cell viability of human colon carcinoma cell line Caco-2 (30). In the present study, we found that luteolin had no effects on HT-29 and SW480 cells proliferation. We hypothesized that the CRC cells used were different and thus the sensitivity to luteolin may be varied. Thus, the concentrations of 0-100 μ M of luteolin in this study had no effects on cells proliferation.

Pleiotrophin (PTN) is a small heparin-binding cytokine which can be induced during tumorigenesis (31). Levels of PTN have been found to be increased in several cancer cell lines and primary tumors, including pancreatic cancer (32), hepatocellular carcinoma (33), breast (34) and papillary thyroid cancer (35). PTN promotes tumor progression either through direct effects on tumor cells or through stimulation of angiogenesis and remodeling of the tumor microenvironment (36-38). In CRC, Yamakawa *et al* first reported that the

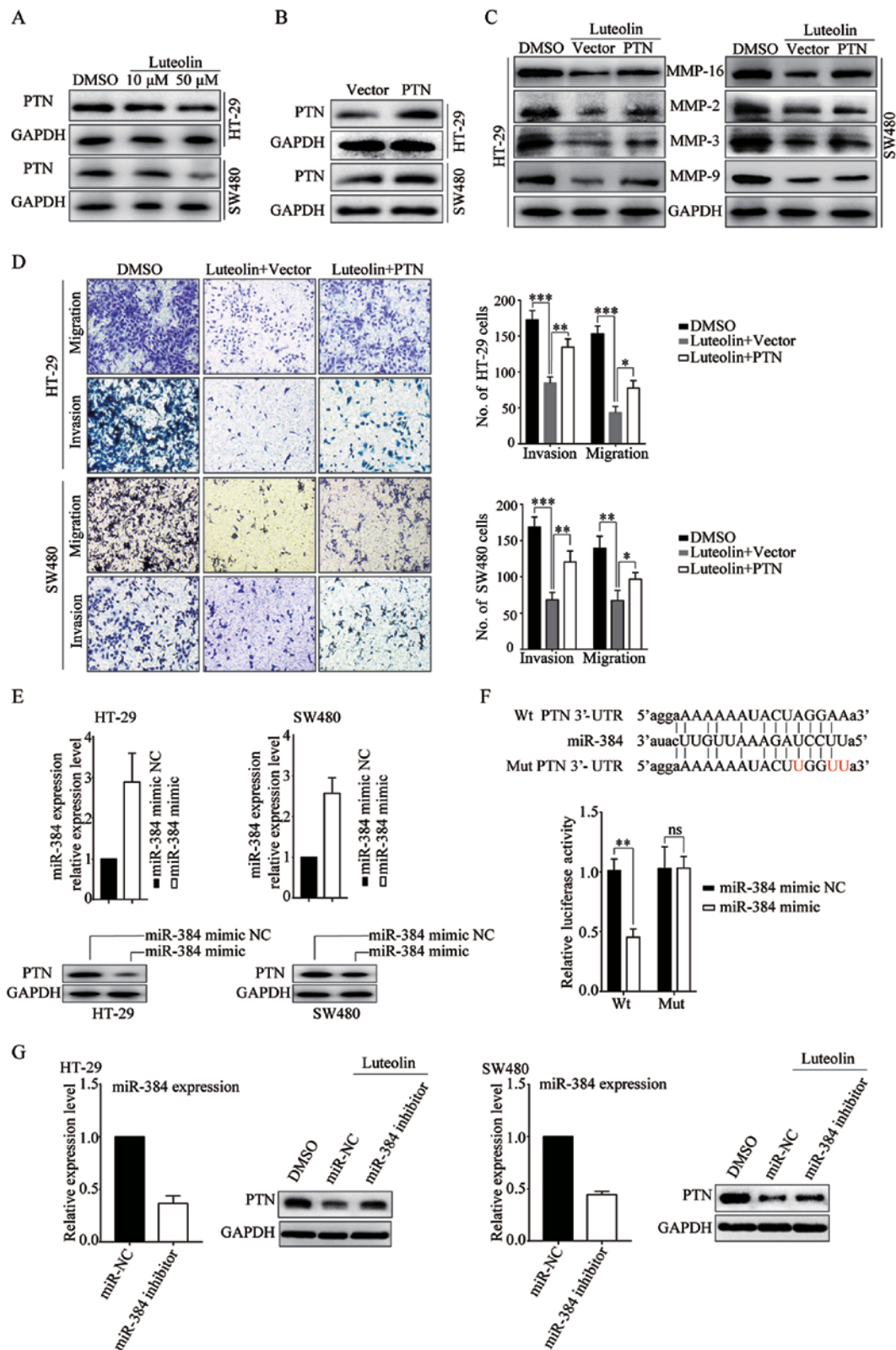


Figure 4. PTN is a direct target of miR-384. (A) The protein level of PTN in HT-29, and SW480 cells treated with luteolin after 24 h was determined by western blotting. (B) HT-29 and SW480 cells were transfected with the vector or PTN overexpression plasmid for 24 h, and the transfection efficiency was determined by western blotting. (C) Transfected cells were treated with luteolin for 24 h before protein was extracted for western blotting to determine MMP-2, MMP-3, MMP-9 and MMP-16 expression. (D) Transfected cells were treated with luteolin for 24 h and cell migration and invasion abilities were determined by Transwell assay. Original magnification, x100. (E) HT-29 and SW480 cells were transfected with miR-384 mimic NC or miR-384 mimic for 24 h before proteins were extracted for western blotting to determine PTN expression. (F) Putative Wt and Mut miR-384 binding sites in the 3'-UTR of PTN. Relative luciferase activities were analyzed in HT-29 and SW480 cells co-transfected with Wt or Mut reporter plasmids and miR-384 mimic or miR-384 mimic NC. (G) HT-29 and SW480 cells were transfected with miR-384 inhibitor NC or miR-384 inhibitor for 24 h before cells were treated with luteolin for 24 h and then protein was extracted for western blotting to determine PTN expression. Results are representative of 3 independent experiments and are expressed as mean \pm SEM. Statistical significance in D was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test and statistical significance in F was determined by Student's t-test. ns, no significant; * P <0.05, ** P <0.01, *** P <0.001; ns, not significant. PTN, pleiotrophin; DMSO, dimethyl sulfoxide; Wt, wild-type; Mut, mutant.

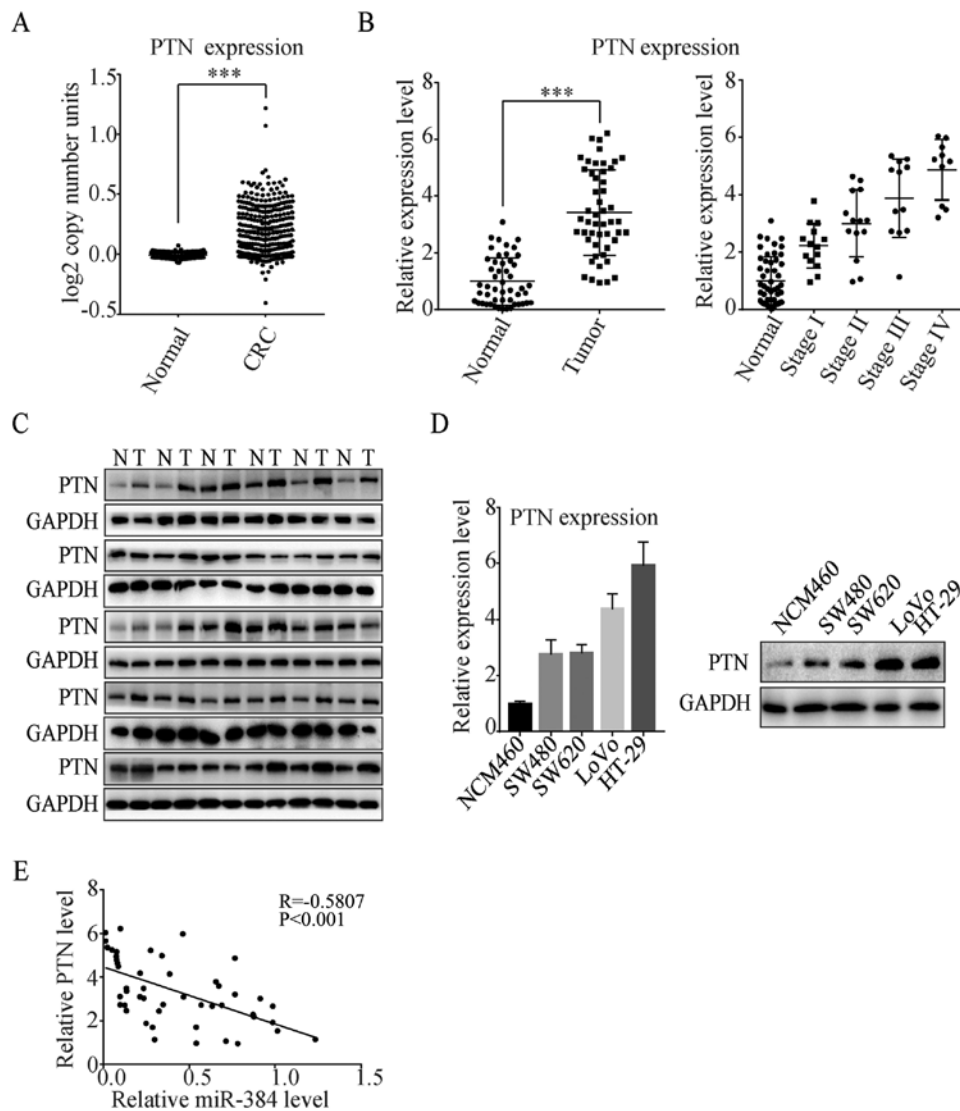


Figure 5. PTN expression is downregulated in CRC tissues and cell lines. (A) Comparison of DNA expression of PTN in CRC and normal tissues from Oncomine database. (Normal, n=445; CRC, n=302) (B) The PTN mRNA expression in 50 pairs of CRC tumor tissues and the corresponding adjacent normal tissues were determined by qPCR (Normal, n=50; Stage I, n=14; Stage II, n=14; Stage III, n=12; Stage IV, n=10). (C) The PTN protein expression in 30 pairs of CRC tissues (T) and the corresponding adjacent normal tissues (N) were determined by western blotting. (D) The mRNA and protein level of PTN in four CRC cell lines (SW480, SW620, LoVo and HT-29) and one normal human colon epithelium cell line NCM460 were determined by qPCR and western blotting, respectively. Results are representative of 3 independent experiments. (E) Pearson's correlation analysis of the negative correlation between the expression of miR-384 and PTN in colorectal cancer tissues. Results are expressed as mean \pm SEM. ***P<0.001. PTN, pleiotrophin; CRC, colorectal cancer.

PTN expression level was decreased in CRC compared with those in normal adjacent mucosae (39). Kong *et al* reported that PTN was highly expressed in CRC tissues and its expression was related to CRC differentiation and TNM staging, and a high level of PTN is a predictor of poor prognosis (40). In this study, by analyzing the data from the Oncomine database and clinical samples, we also found that PTN expression was much higher in CRC tissues than that in the normal tissues and its expression was related to TNM staging while PTN had no relationship with tumor differentiation (Table I). We speculate that the probable reason is that the sample size in the present study was too small. Further research with an increased number of samples is needed.

Growing evidence suggests that miR-384 is a potent tumor-suppressor molecule. The functions of miR-384 have been studied in various types of cancers including papillary

thyroid (41), pancreatic (42) and non-small cell lung cancer (43). Astrocyte elevated gene-1 (43,44), PTN (33), piwi-like RNA-mediated gene silencing 4 (PIWIL4) (45), KRAS and CDC42 (28) have been identified as target genes of miR-384. Meanwhile, miR-384 was found to be negatively regulated by long non-coding RNA CRNDE (45,46). In CRC, miR-384 was downregulated and closely related with the progression. Inhibition of miR-384 promoted the invasive and metastatic abilities of CRC cells (28). Sun *et al* reported that in papillary thyroid cancer cells PTN was found to be a downstream target of miR-384 (41). We also confirmed by luciferase reporter assay that in CRC cells PTN is a direct target of miR-384.

In summary, our results demonstrated the antitumor role of luteolin in CRC and the mechanism of which is partly mediated via the miR-384/PTN axis. Thus, miR-384 or PTN can be developed as a therapeutic target in the treatment of CRC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YY, CR and SW participated in the conception and design of the study. YY, CR and GZ performed the statistical analysis and were involved in the preparation of the figures. YY and SW reviewed the results and participated in the discussion of the data. YY, CR and SW prepared the manuscript and revised it. All authors read and approved the manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Approval of present study was obtained from the Ethics Committee of the Hangzhou Hospital of Traditional Chinese Medicine and informed consent was acquired from all the patients. The animal study received ethical approval from the Animal Care and Use Committee of Zhejiang University, and experiments and animal care were performed according to the approved protocols.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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