

# Osteopontin regulates the growth and invasion of liver cancer cells via DTL

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**Abstract.** Osteopontin (OPN), a secreted phosphoglycoprotein, has important roles in tumor growth, invasion and metastasis in numerous types of cancers. Denticleless E3 ubiquitin protein ligase homolog (DTL), one of the CUL4-DDB1-associated factors (DCAFs), has also been associated with the invasion and metastasis of cancer cells. In the present study, OPN was found to induce DTL expression in liver cancer cells, and the results obtained using luciferase activity assays demonstrated that OPN could transcriptionally activate DTL expression in liver cancer cells. Furthermore, the results of the present study demonstrated that OPN could increase the expression of DTL via PI3K/AKT signaling. In conclusion, the present study demonstrated that OPN, as an extracellular matrix protein, is able to promote the growth and invasion of liver cancer cells through stimulation of the expression of DTL via the PI3K/AKT signaling pathway.

## Introduction

Osteopontin (OPN) is a secreted arginine-glycine-aspartic acid-containing phosphoprotein which exists mainly as a soluble cytokine and can bind to certain integrins or CD44 variants, which further mediate diverse biological functions (1-3). OPN is involved in the pathogenesis of numerous disease states, including cancer and chronic inflammatory

diseases (4,5). OPN levels have been reported to be markedly increased in both numerous types of human cancers and in the plasma of patients with cancer (6-8). Previous studies have reported that OPN is associated with tumor metastasis and progression (9,10), and OPN has been reported to promote cell survival through inhibition of apoptosis (11). It has been reported that OPN has important roles in mediating the growth, metastasis and immune response of hepatocellular carcinoma (HCC) (12,13). OPN has also been shown to be a promising serum biomarker of HCC (14). Activation of the mitogen activated protein kinase (MAPK), NF- $\kappa$ B and PI3K/Akt signaling pathways in HCC cells may be involved in mediating the effects of OPN on liver cancer cells (15-17). Furthermore, OPN has been reported to promote the expression of metalloproteinases (MMPs), including inducing the expression of long non-coding RNAs, such as HOTAIR, during the invasion of liver cancer cells (18,19). However, other potential mechanisms that may be involved in the effects of OPN on liver cancer cells require further elucidation.

Denticleless E3 ubiquitin protein ligase homolog [DTL, also known as CDT2, CUL4-DDB1-associated factor (DCAF2) or RAMP] belongs to the DCAF protein family, contains WD40 repeats, and exerts a crucial role in the regulation of the degradation of CDT1 in the DNA damage response. DTL has been reported to be involved in DNA damage repair, the cell cycle and DNA replication, processes intimately involved with chromosomal separation and cell division, and DTL is a key regulator of cell cycle progression and genome stability (20-22). A previous study reported that DTL might affect genome stability by modulating the non-homologous end joining repair pathway. The role of DTL in genome stability suggests that DTL may be associated with tumorigenesis and a previous study reported that the expression level of DTL is increased in numerous types of cancer (23).

Although it is well established that DTL fulfills important roles in numerous biological processes, to the best of our knowledge the mechanism underlying the regulation of its expression in cancer has yet to be fully elucidated. Previous reports have suggested that SB743921, a selective inhibitor of kinesin spindle protein (KSP), and the microRNAs (miRs) miR-490-5p and miR-30a-5p may be associated with the

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expression of DTL in cancer cells (24-26). In the present study, it was demonstrated that OPN was able to induce the expression of DTL in a dose dependent manner. Moreover, DTL expression was found to be regulated by OPN siRNAs and a vector expressing OPN. In addition, the results of the present further demonstrated that the AKT signaling pathway, which is activated by OPN, may be involved in mediating the effects of OPN on the expression of DTL in liver cancer cells. Finally, using luciferase activity assays, it was demonstrated that both OPN and the AKT signaling pathway were able to transcriptionally affect the expression of DTL in liver cancer cells.

## Materials and methods

**Cell culture and transfection.** The Huh7 liver cancer cell line was purchased from the American Type Culture Collection and the HepG2 liver cancer cell line was purchased from Guangzhou Saiku Biotechnology Co., Ltd. STR profiling was performed to confirm the authenticity of the HepG2 cell line. The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with heat-inactivated fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Gibco; Thermo Fisher Scientific, Inc.) in a tissue-culture incubator containing 5% CO<sub>2</sub> at 37°C. The transfection of the plasmids and siRNAs for the purposes of altering the expression of DTL was performed using Invitrogen® Lipofectamine 3000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions.

**Plasmid construction and siRNA sequences.** Total RNA was extracted from Huh7 cells by TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), and then 1 µg total RNA was subjected to reverse transcription (RT) to generate cDNA using the reagent for RT (GoScript™ Reverse Transcription Mix; cat. no. A2790; Promega Corporation). This cDNA was used as a template to amplify the coding sequence of OPN by PCR using PrimeSTAR® HS (cat. no. R040A; Takara Bio, Inc.). The primers used to amplify the full length of OPN were as follows: 5'-GTAGGTACCATGAGAATTGCAGTGATTTG-3' and 5'-GTACTCGAGTTAATTGACCTCAGAAGATG-3'. The product of the PCR was purified by the Kit for DNA purification (cat. no. B110092, Sangon Biotech, China), and digested by *Kpn*I and *Xho*I (cat. nos. 1068A and 1094A; Takara Bio, Inc.). The full length OPN was further inserted into pcDNA3.1 vector by using a DNA Ligation Kit (cat. no. 6022Q; Takara Bio, Inc.). The recombinant vector was verified by sequencing (Invitrogen; Thermo Fisher Scientific, Inc.). For western blotting and Transwell assay, 3 µg plasmids (OPN expressing vector or control vector) were transfected into each well of a 6-well plate containing Huh7 cells or HepG2 cells (2x10<sup>5</sup> cells/well) for 24 h using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). The mixture containing the plasmid and Lipofectamine 3000 was incubated at room temperature for 20 min before transfection. A total of 24 h post-transfection, cells were harvested or seeded into the Transwell chamber. For the cell viability assay, 0.2 µg corresponding plasmids were transfected into cells in each well of a 96-well plate at a density of 2,000 cells per well for the indicated time (0, 24, 48 and 72 h), the mixture containing

the plasmid and Lipofectamine 3000 was incubated at room temperature for 20 min, and then the mixture was added to the wells of 96-well plate. The 96-well plate was incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub> for the indicated time (0, 24, 48 and 72 h) before viability assay. The working concentration of siRNA was 20 nM. Similar to the plasmid transfection protocol, the mixture containing the siRNA and Lipofectamine 3000 was incubated at room temperature for 20 min before transfection. GFP was separately inserted into pcDNA3.1 and this GFP-expressing vector was used as a control for OPN overexpression. siRNAs were synthesized by GenePharma Inc, Shanghai, China. For siRNA transfection, two OPN siRNAs or two DTL siRNAs were used; the two siRNAs were mixed together for transfection. The sequences of the siRNAs used for knockdown were as follows: OPN sense (S), 5'-GUGGGUUGGUCAGUUAUGATT-3' and antisense (AS), 5'-UCAUAACUGUCCUCCACTT-3'; and S, 5'-GUCUCACCAUUCUGAUGAATT-3' and AS, 5'-UUC AUCAGAAUGGUGAGACTT-3'; DTL, S, 5'-CUUCUUAUG GAGAAACAGGTT-3' and AS, 5'-CCUGUUUCUCCAUA GAAGTT-3'; and S, 5'-AAUAUGGAACAUGUACUAGTT-3' and AS, 5'-CUAGUACAUGUCCAUAUUTT-3'; and control S, 5'-ACGCAUGCAUGCUUGCUUUTT-3' and AS, 5'-AAA GCAAGCAUGCAUGCGUTT-3'. The putative 2,000 bp promoter region of human DTL was determined by UCSC genome browser (<http://genome.ucsc.edu/>). The genomic DNA of Huh7 cells was extracted using a kit from Beyotime Institute of Biotechnology (cat. no. D0061). The genomic DNA was used as a template to amplify the promoter region of DTL by PCR using PrimeSTAR® HS (cat. no. R040A; Takara Bio, Inc.). The forward and reverse primers used were as follows: 5'-GGA GAACCGTTTGAACCTCGGG-3' and 5'-GGGAGAACTCAG AAGCTGAG-3'. Thermocycling conditions were as follows: Initial denaturation at 98°C for 3 min, followed by 29 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 20 sec and elongation at 72°C for 2 min; and a final extension step at 72°C for 5 min. The PCR product was inserted into the pGL3-Basic luciferase-reporter vector (Promega Corporation).

**Cell viability assay.** The liver cancer cells were seeded in 96-well plates at a density of 2,000 cells per well in 150 µl of culture medium. After 24 h, the siRNAs or OPN overexpression vector were transfected into the corresponding wells in triplicate. The plates were then incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub> for the indicated time (0, 24, 48 and 72 h). The medium was subsequently removed and the cells were washed twice with PBS. DMEM (90 µl) containing 10 µl CCK8 solution (Beyotime Institute of Biotechnology) was then added to each well, and the plates were incubated at 37°C for an additional 2 h. The absorbance values at 450 nm were assessed using a microplate reader spectrophotometer (Tecan Group, Ltd.). All experiments were repeated at least 3 times.

**Chemicals and antibodies.** The primary antibodies used in the present study were as follows: Anti-OPN (1:1,000; cat. no. SAB5700738, Sigma-Aldrich; Merck KGaA), anti-DTL (1:1,000; cat. no. ab174385, Abcam), phosphorylated (p)-AKT (Ser473; 1:500; cat. no. AA329; Beyotime Institute of Biotechnology), AKT (1:500; cat. no. AA326; Beyotime Institute of Biotechnology) and actin (1:500; cat. no. AA128;

Beyotime Institute of Biotechnology). HRP conjugated goat anti-rabbit IgG (1:2,000; cat. no. A0208) and goat anti-mouse IgG (1:2,000; cat. no. A0216) secondary antibodies were purchased from Beyotime Institute of Biotechnology. The PI3K/AKT inhibitors LY294002 and wortmannin were purchased from Beyotime Institute of Biotechnology, and recombinant human OPN (rhOPN) was purchased from ProPepTech, Inc.

**Luciferase reporter assay.** Huh7 and HepG2 cells were seeded at a density of  $2 \times 10^3$  cells per well in 96-well plates on the day before transfection. The cells were co-transfected with 0.1  $\mu$ g firefly luciferase reporter construct containing the DTL promoter region (Promega Corporation), 0.01  $\mu$ g pRL-TK Renilla luciferase reporter plasmid (Promega Corporation) and the pcDNA3.1-OPN vector (0.2  $\mu$ g) using Lipofectamine™ 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 24 h of transfection, the luciferase activity was assessed using a dual-luciferase reporter assay system (cat. no. E1910; Promega Corporation) according to the manufacturer's instructions, and the signal was normalized to that of the internal *Renilla* control in order to assess the transfection efficiency.

**Transwell assay.** Cell invasion assays were performed using the Transwell (Corning, Inc.) system, which allows cells to invade through a Matrigel™-coated polycarbonate membrane with a pore size of 8  $\mu$ m. The aforementioned Huh7 and HepG2 cells transfected with DTL siRNAs were trypsinized using 0.25% trypsin at 37°C for 5 min. The cells were seeded into the upper chambers in serum-free DMEM with or without rhOPN, and were incubated at 37°C for 24 h. DMEM supplemented with 10% FBS was added to the lower chambers. The membranes were then washed with PBS, cells above the membrane were gently removed using a cotton swab, and cells that had migrated across the membrane were fixed with cold methanol for 15 min at room temperature. Finally, the cells were stained using crystal violet for 10 min at room temperature. Cells beneath the membrane were counted in five fields of view using an inverted light microscope. Each experiment was repeated three times.

**Western blotting.** Huh7 and HepG2 cells were exposed to various experimental conditions, such as transfection and treatment with rhOPN, prior to being harvested and lysed for protein extraction using RIPA buffer (cat. no. P0013B; Beyotime Institute of Biotechnology). Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc.). The samples were then subjected to 12% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked using 5% milk in TBS-0.1% Tween (TBST) buffer at room temperature for 30 min, and then probed with the aforementioned primary antibodies at 4°C overnight. After washing with TBST three times, each for ten min, membranes were further incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. The western blots were visualized using an enhanced chemiluminescence detection system (Tiangen Biotech Co., Ltd.).  $\beta$ -actin was used as the sample loading control. Semi-quantification was performed using ImageJ (V1.8.0; National Institute of Health).

**RT-quantitative (q)PCR.** Total RNA was extracted using TRIzol reagent and RT was performed using oligo(dT) 20 (Takara Bio, Inc.) as primer and M-MLV reverse transcriptase (Promega Corporation) at 42°C for 30 min. The primer sequences for qPCR amplification were as follows: DTL forward (F), 5'-CCAGTATCTCAGAGCCTCCG-3' and reverse (R), 5'-TGGATTCTCAGCCTTCCGTT-3'; and  $\beta$ -actin F, 5'-CCCACACTGTGCCCATCTAC-3' and R, 5'-GGAACC GCTCATTGCCAATG-3'.  $\beta$ -actin was used as the loading control. The qPCR reactions were performed using 20  $\mu$ l 1:1 diluted iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) with three replicates. The thermocycling conditions were as follows: Initial heat activation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec and 55°C for 20 sec for. The transcript level of the DTL mRNA was further analyzed by RT-qPCR using an ABI-7500 Sequence Detector System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative expression levels of the eighteen selected genes were calculated using the  $2^{-\Delta\Delta Cq}$  method (27).

**Statistical analysis.** GraphPad Prism 5 (Dotmatics) was used to analyze the data. Statistical analyses were performed using the unpaired Student's t-test for comparisons of 2 groups and one-way ANOVA followed by Tukey's post hoc test for comparisons of  $\geq 3$  groups. All experiments were performed at least 3 times.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**DTL mediates OPN-induced proliferation and invasion by liver cancer cells.** OPN and DTL have previously been reported to be associated with both the proliferation and invasion of liver cancer cells (13,28). Therefore, in the present study, it was hypothesized that OPN may be able to stimulate both the proliferation and invasion of liver cancer cells, at least in part, via DTL. First, the constructed vector expressing OPN, and the efficacy of the siRNAs against OPN and DTL, were validated. OPN was significantly overexpressed in both Huh7 and HepG2 cells transfected with the vector expressing OPN compared with the control and treating the cells with the siRNAs led to significantly decreased protein expression levels of both OPN and DTL in both of the liver cancer cell lines compared with the control (Fig. 1A and B). The viability of the two cell lines was subsequently assessed using a CCK8 assay following transfection with OPN expressing vector and/or DTL siRNA for 24, 48 or 72 h. The OPN-induced growth of the Huh7 and HepG2 cells was demonstrated to be inhibited notably by treatment with DTL siRNA compared with the negative control (Fig. 1C and D). The Transwell assays (Fig. 2A) demonstrated that though DTL knockdown by siRNA itself only slightly affected invasion by liver cancer cells compared with the negative control, the reduced expression level of DTL caused a significant inhibition of the rhOPN-induced invasion of Huh7 cells (fold change,  $2.60 \pm 0.33$  vs.  $1.38 \pm 0.25$ ) compared with the negative control + rhOPN group. This indicated that DTL may participate in rhOPN-induced invasion by Huh7 cells. Knockdown of DTL likewise led to a significant decrease in OPN-induced invasion by HepG2

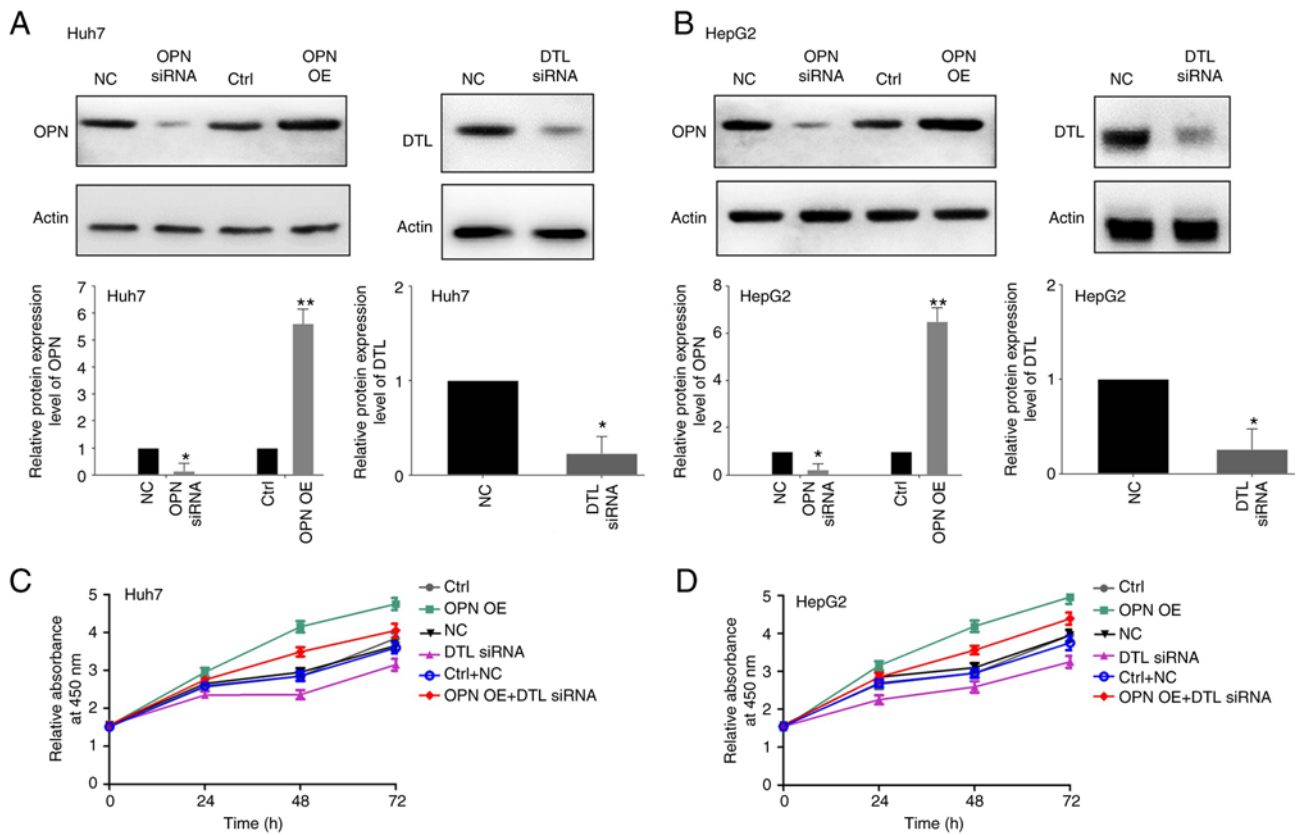


Figure 1. DTL mediates the effects of OPN on the proliferation of liver cancer cells. (A and B) The levels of OPN and DTL were significantly decreased following transfection with the corresponding siRNAs, whereas the OPN level was significantly increased after transfection with the vector overexpressing OPN in liver cancer cells. The relative protein expression level was semi-quantified for each group. \* $P < 0.05$  and \*\* $P < 0.01$  vs. NC. (C and D) DTL knockdown attenuated the effects of OPN-induced proliferation in liver cancer cells. Data are presented as the mean  $\pm$  SD of 3 independent experiments performed in quadruplicate.  $P < 0.05$  for the OPN OE vs. OPN OE + DTL siRNA for the 48 and 72 h time points. OPN, osteopontin; DTL, denticleless E3 ubiquitin protein ligase homolog; OE, overexpression; NC, negative control; Ctrl, control.

cells (fold change,  $3.15 \pm 0.53$  vs.  $1.56 \pm 0.31$ ) compared with the negative control + rhOPN group. To further evaluate the role of DTL in OPN-induced invasion of liver cancer cells, a vector expressing OPN was used to assess the effect of DTL on OPN-induced invasion. Knockdown of DTL significantly decreased OPN-induced invasion by both Huh7 cells (fold change,  $2.36 \pm 0.28$  vs.  $1.32 \pm 0.16$ ) and HepG2 cells (fold change,  $2.83 \pm 0.36$  vs.  $1.62 \pm 0.28$ ) compared with the negative control + OPN overexpression group (Fig. 2B).

**OPN regulates DTL expression.** To assess the effect of OPN on DTL protein expression levels, western blotting was performed using the Huh7 and HepG2 cells. Treatment with rhOPN led to a marked increase in the expression of DTL in a dose-dependent manner (Fig. 3A and B). Apparent increases in the protein expression levels of DTL in Huh7 and HepG2 cells were observed following treatment with 5 or 20 nM rhOPN for 6 h compared with those in the untreated group (Fig. 3A and B). Knockdown of OPN using siRNAs led to significantly decreased protein expression levels of DTL in HepG2 and Huh7 cells compared with the control (Fig. 3C and D). Moreover, it was demonstrated that OPN overexpression could significantly increase the expression of DTL compared with the negative control. It was also demonstrated that knockdown of DTL by siRNA could markedly reduce the OPN-induced expression of DTL

compared with the OPN overexpression group in both Huh7 cells and HepG2 cells. However, knockdown of DTL demonstrated little effect on OPN expression in both Huh7 cells and HepG2 cells. Taken together, the above data suggested that OPN was able to regulate the expression of DTL in liver cancer cells.

*The PI3K/AKT signaling pathway is involved in the regulation of DTL expression by OPN.* It has been previously reported that OPN is able to affect the PI3K/AKT pathway in cancer cells (29,30). Therefore, it was hypothesized that the PI3K/AKT signaling pathway may participate in the regulation of DTL expression that is mediated by OPN. The upregulation of DTL protein expression induced by rhOPN was significantly attenuated when Huh7 cells were pretreated with the PI3K/AKT inhibitors LY294002 ( $5 \mu\text{M}$ ) or wortmannin ( $5 \mu\text{M}$ ) for 3 h (Fig. 4A). Similar effects were also demonstrated in the HepG2 cells (Fig. 4B). Inhibition of the AKT signaling pathway in liver cancer cells by LY294002 and wortmannin was assessed using p-AKT expression levels. The protein expression level of p-AKT was significantly downregulated after treatment with LY294002 or wortmannin in both Huh7 cells and HepG2 cells. Collectively, these results suggested that the PI3K/AKT signaling pathway may be involved in the OPN-induced upregulation of DTL expression in liver cancer cells.

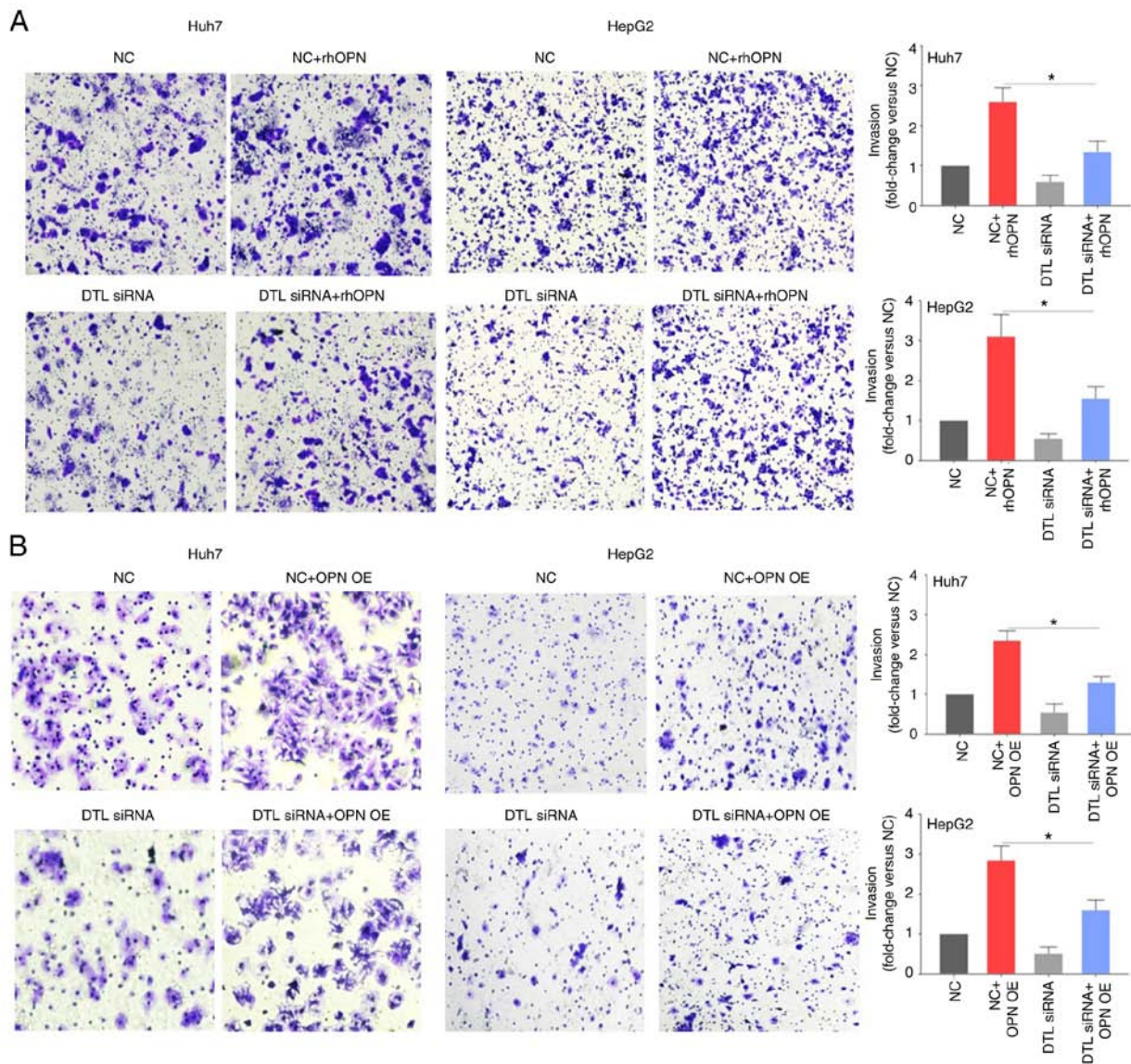


Figure 2. DTL is involved in the OPN-induced invasiveness of liver cancer cells. Cells were transfected with DTL siRNA or OPN expressing vector or treated with rhOPN for 24 h and then the cells were assessed using the Transwell assay. (A) DTL knockdown slightly inhibited invasion by both Huh7 cells and HepG2 cells, but markedly decreased the rhOPN-induced invasiveness of both Huh7 cells and HepG2 cells. These results indicated that DTL is also involved in the rhOPN-induced promotion of the invasiveness of both Huh7 cells and HepG2 cells. The data are presented as the mean  $\pm$  SD of three independent experiments. (B) Similar effects were also demonstrated in the groups transfected with the vector expressing OPN. The results further indicated DTL is involved in the invasiveness of both Huh7 cells and HepG2 cells, serving a vital role in OPN-induced invasiveness of both Huh7 cells and HepG2 cells. \* $P < 0.05$ . OPN, osteopontin; DTL, denticleless E3 ubiquitin protein ligase homolog; NC, negative control; rh, recombinant human.

*OPN transcriptionally regulates DTL expression in liver cancer cells via the PI3K/AKT signaling pathway.* It was hypothesized that OPN may transcriptionally influence the expression of DTL in liver cancer cells. To evaluate whether DTL may be transcriptionally inhibited by OPN, luciferase activity assays were performed on extracts from Huh7 or HepG2 cells that were co-transfected with the luciferase-reporter plasmid in combination with vectors expressing either OPN or OPN siRNAs and the respective control groups. OPN knockdown led to a significant decrease in the DTL promoter activity compared with the control groups (Fig. 5A). Moreover, the promoter activity of DTL was significantly increased by OPN overexpression in liver cancer cells compared with the control. rhOPN was also used in subsequent experiments to further assess the aforementioned effects. Treatment with

rhOPN led to a marked increase in the promoter activity of DTL compared with the control and the PI3K/AKT inhibitor LY294002 strongly attenuated this effect in liver cancer cells (Fig. 5B). Furthermore, the mRNA expression level of DTL in liver cancer cells treated with rhOPN was quantified. rhOPN induced a significant increase in the expression of DTL mRNA compared with the control and the PI3K/AKT inhibitor LY294002 was able to markedly reduce this effect of rhOPN in liver cancer cells (Fig. 5C).

### Discussion

It has been reported previously that elevated levels of OPN in the plasma of patients with certain types of cancer are closely associated with cancer relapse or that the elevated level of OPN

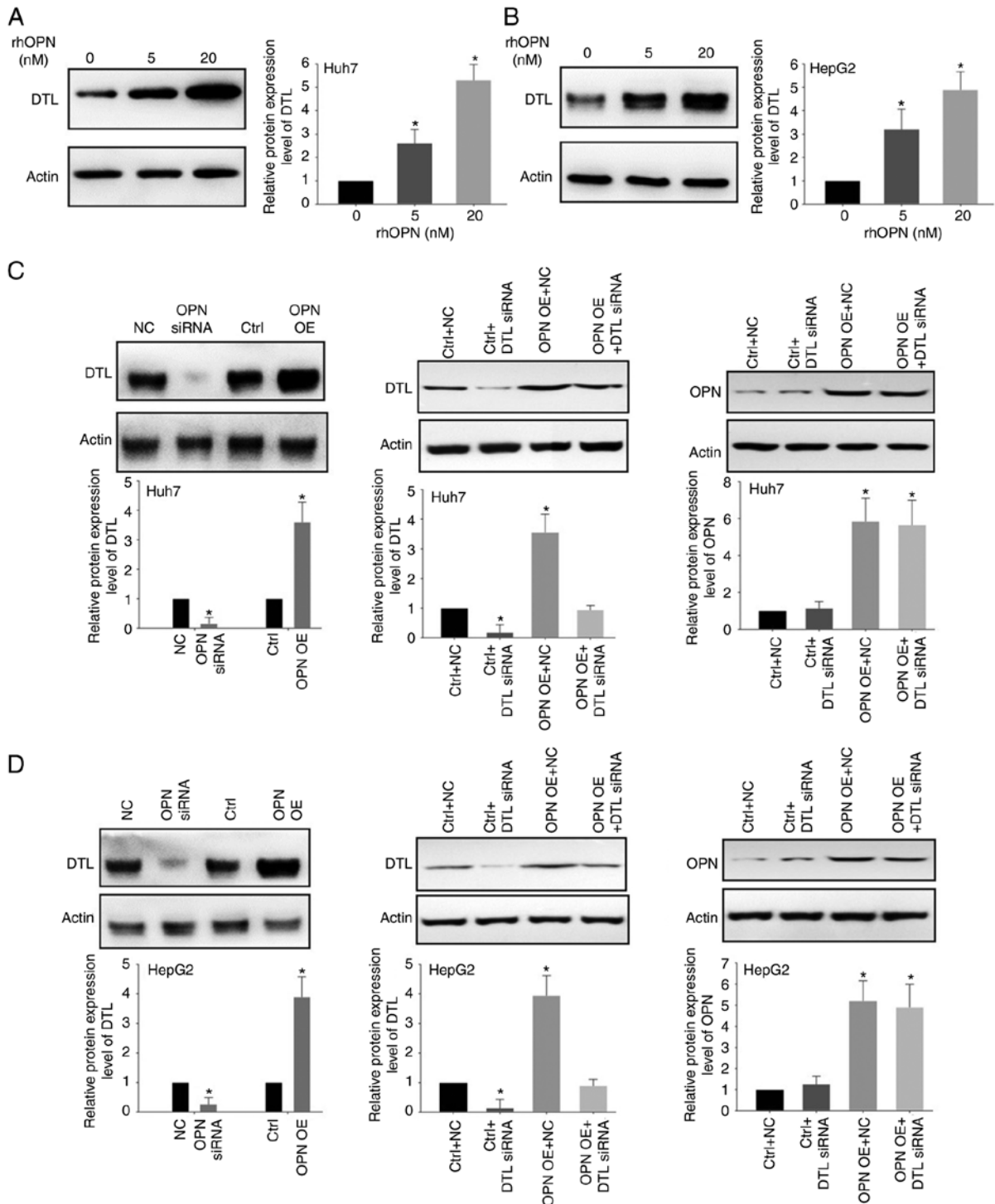


Figure 3. OPN mediates the protein expression level of DTL in liver cancer cells. (A) Treatment of Huh7 cells with rhOPN induced the expression of DTL in a dose-dependent manner. After 6 h of treatment with 5 and 20 nM rhOPN, DTL expression was significantly upregulated in Huh7 cells. (B) Similar effects were demonstrated in HepG2 cells; rhOPN also significantly induced the expression of DTL in the HepG2 cells. (C) An OPN-overexpression vector and OPN siRNAs were used to regulate the OPN level in Huh7 cells. The DTL level was then detected using western blotting at 48 h after transfection of the cells, which demonstrated that OPN knockdown by OPN siRNAs led to significant downregulation of the expression of DTL, whereas OPN overexpression led to a significant increase in the DTL protein expression level. Western blotting was also performed to assess the effects of knockdown of DTL on OPN-induced elevation of DTL level and the effect of knockdown of DTL on OPN expression. (D) Similar effects were observed in HepG2 cells. Data are presented as the mean  $\pm$  SD of three independent experiments. \* $P < 0.05$  vs. control. OPN, osteopontin; DTL, denticleless E3 ubiquitin protein ligase homolog; OE, overexpression; NC, negative control; Ctrl, control; rh, recombinant human.

might decrease the efficacy of treatment (31). Previous studies have reported that the level of OPN is upregulated in a liver cancer model (32,33). Furthermore, OPN has been reported to be involved in the regulation of certain signal transduction

pathways, including the PI3K/AKT signaling pathway, which mainly function in stimulating the migration, invasion and metastasis of cancer cells (15-17). The AKT signaling pathway has been reported to be induced by OPN (29,30,34). Previous

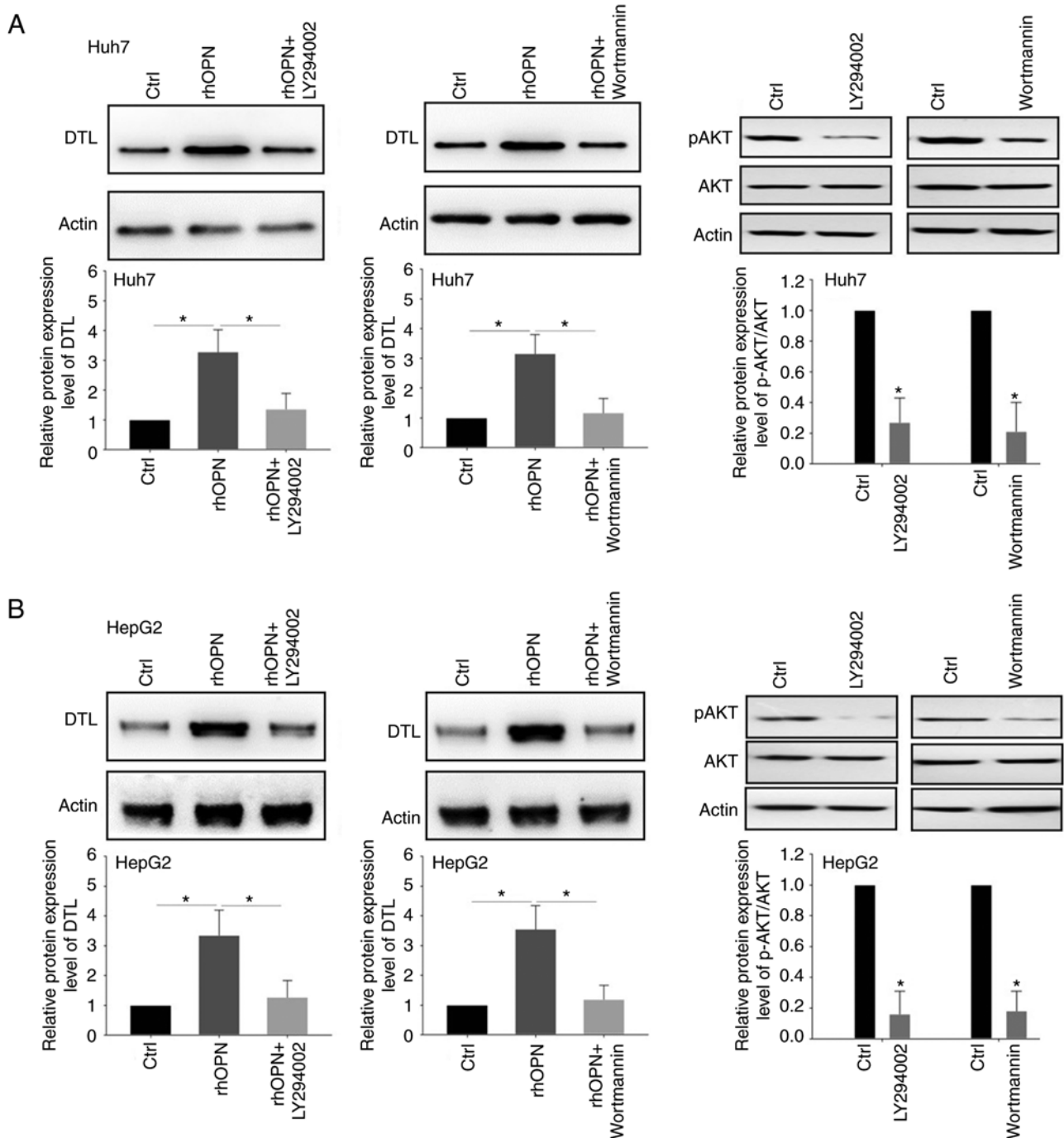


Figure 4. PI3K/AKT signaling is involved in the regulation of DTL by OPN in liver cancer cells. (A) Western blotting indicated that treatment with the PI3K/AKT inhibitors LY294002 and wortmannin led to a significant decrease in the expression of DTL induced by rhOPN (5 nM) in Huh7 cells. Western blotting was also performed to assess the effects of LY294002 and wortmannin on the AKT pathway in Huh7 cells. The relative protein expression level has been semi-quantified for each group. (B) Similar effects were observed in HepG2 cells, and the rhOPN-induced enhancements of DTL expression were inhibited by treatments with LY294002 (5  $\mu$ M) and wortmannin (5  $\mu$ M). \*P<0.05 vs. Ctrl or as indicated. OPN, osteopontin; DTL, denticleless E3 ubiquitin protein ligase homolog; OE, overexpression; NC, negative control; Ctrl, control; rh, recombinant human.

studies reported that OPN induction of Collagen-I occurred via integrin  $\alpha(v)\beta(3)$  engagement and activation of the PI3K/pAkt/NF $\kappa$ B signaling pathway. OPN could also induce activation of phosphatidylinositol 3-kinase and Akt by binding to the CD44 receptor, an effect which can even affect the chemoresistance of certain cancer cells (29,30,34). Therefore, in the present study, whether the AKT signaling pathway was involved in the relationship between OPN and DTL was evaluated. As an extracellular cytokine, OPN-mediated signaling

has been previously reported to lead to resistance to apoptosis in cancer cells. Therefore, the identification of new factors associated with OPN-mediated signaling could be beneficial in terms of increasing understanding of OPN function.

DTL is one member of the DCAF family that fulfills critical roles in the cell cycle and DNA repair. The dysregulation of DTL expression has previously been reported in different types of cancer (23,35). Previous studies have also reported that DTL was involved in proliferation and invasion by cancer

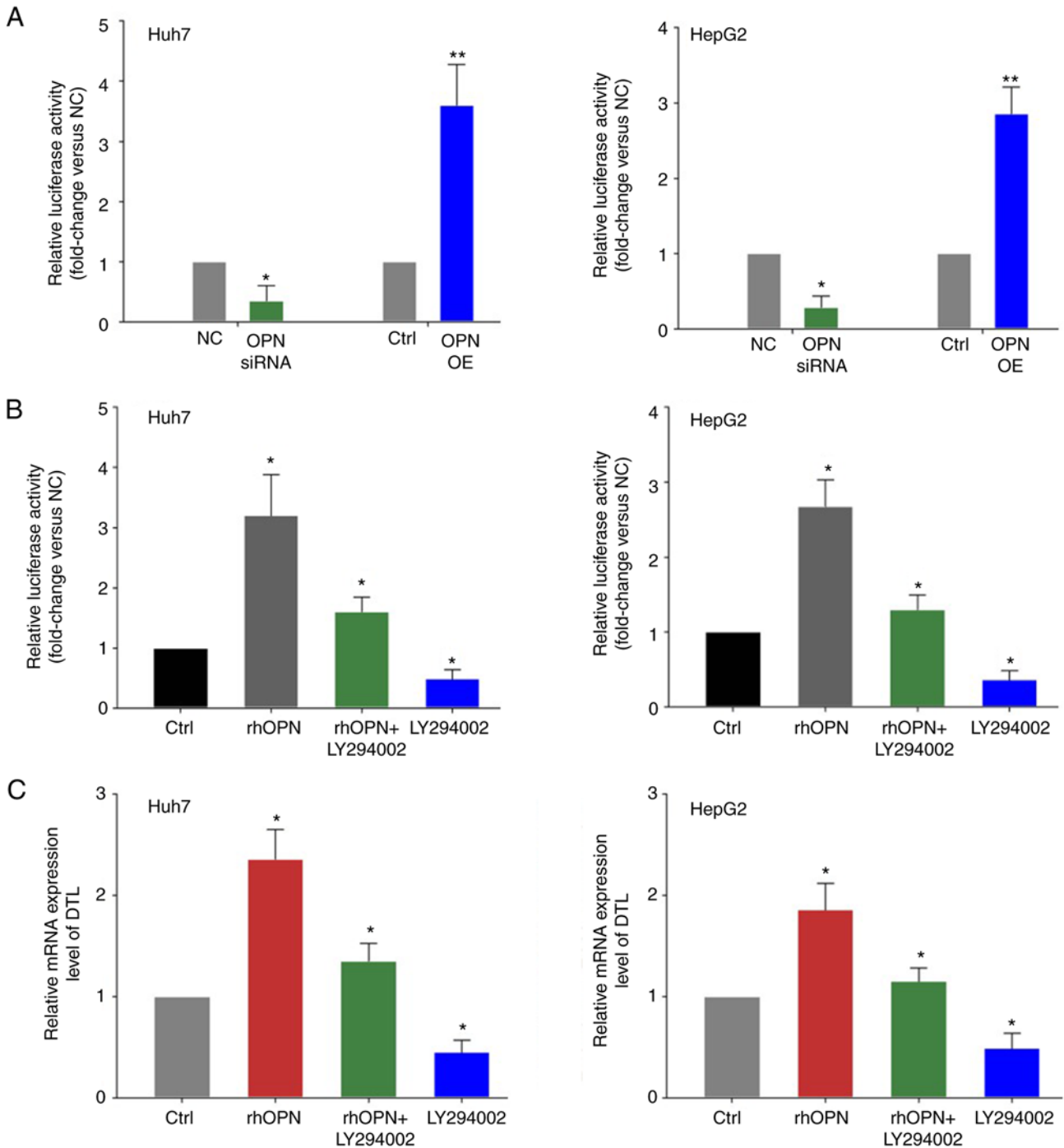


Figure 5. OPN and the PI3K/AKT signaling pathway are transcriptionally associated with DTL expression in liver cancer cells. (A) The effects of OPN on DTL promoter activity in Huh7 and HepG2 liver cancer cells were evaluated. Luciferase reporter assays used to assess the DTL promoter activity were performed 24 h after co-transfection with constructs derived from the DTL promoter region and either OPN-overexpressing vector or OPN siRNAs. The promoter activity is presented relative to the luciferase activity. Independent experiments were repeated three times. Data are presented as the mean  $\pm$  SD, normalized against the activity of the internal control vector pRL-TK. (B) Effects of rhOPN and the PI3K/AKT inhibitor LY294002 on DTL promoter activity in Huh7 and HepG2 liver cancer cells. (C) rhOPN and PI3K/AKT inhibitor LY294002 affect the expression level of DTL mRNA. Treatment with LY294002 eliminated the effects of OPN on the DTL mRNA level. The experiments were repeated three times. \* $P < 0.05$  and \*\* $P < 0.01$  vs. control. OPN, osteopontin; DTL, denticleless E3 ubiquitin protein ligase homolog; OE, overexpression; NC, negative control; Ctrl, control; rh, recombinant human.

cells (28). An increase in the protein expression level of DTL was demonstrated to both accelerate the growth of liver cancer cells and increase their invasive capabilities. Although the function of DTL in biological processes has already been reasonably well defined, many other important aspects still need to be investigated, including its regulation and identifying its protein interactions with DTL. It has been reported that

KSP inhibitor SB743921, miR-490-5p and miR-30a-5p may affect the expression of DTL (24-26). However, the manner in which DTL expression is regulated by extracellular proteins, or their associated signal transduction pathways, in cancer cells has yet to be fully elucidated.

Little is known about the associations between OPN, the PI3K/AKT signaling pathway and DTL in cancer cells.

Therefore, the present study evaluated whether OPN acted as a regulator of the AKT signaling pathway and DTL in liver cancer cells. The effects of OPN on the AKT signaling pathway and DTL were first assessed using an OPN-overexpressing vector and siRNAs against OPN. The results demonstrated that both rhOPN and overexpression of OPN could increase the protein expression level of DTL in liver cancer cells, whereas knock-down of OPN by siRNA treatment led to a decrease in the protein expression level of DTL. As OPN mainly functions as an extracellular protein associated with certain signaling processes, the data obtained suggested that OPN may serve to maintain the DTL level in liver cancer cells. To further assess the association between the AKT signaling pathway and the DTL level, liver cancer cells were treated with the PI3K/AKT pathway inhibitors LY294002 and wortmannin, which demonstrated that both LY294002 and wortmannin were capable of reducing the protein expression level of DTL. Furthermore, using a luciferase reporter assay, it was demonstrated that OPN could transcriptionally induce the expression of DTL via the AKT signaling pathway. These results indicated that OPN could regulate expression of DTL, but DTL couldn't affect the OPN level in liver cancer cells.

In conclusion, the results of the present study have established a link between OPN and DTL in liver cancer cells, which indicated that OPN was able to transcriptionally increase the level of DTL. To the best of our knowledge, this is the first study to have reported such an effect. Furthermore, the results suggested that the AKT signaling pathway was involved in mediating the effects of OPN on the expression of DTL. However, the current data were mainly derived from liver cancer cells; therefore, the relationship between OPN and DTL in an animal model of cancer requires further investigation.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

ZL and LY designed the study. ZL, GY, XY and SZ performed the experiments. ZF, FC and XC performed the data analysis. ZL and FC confirm the authenticity of all the raw data, and ZL and LY drafted the manuscript. All authors have read and approved the final version of the manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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