CTPS cytoophidia formation affects cell cycle progression and promotes TSN-induced apoptosis of MKN45 cells

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Abstract. Cytidine triphosphate synthase (CTPS) forms filamentous structures termed cytoophidia in numerous types of cell. Toosendanin (TSN) is a tetracyclic triterpenoid and induces CTPS to form cytoophidia in MKN45 cells. However, the effects of CTPS cytoophidia on the proliferation and apoptosis of human gastric cancer cells remain poorly understood. In the present study, CTPS-overexpression and R294D-CTPS mutant vectors were generated to assess the effect of CTPS cytoophidia on the proliferation and apoptosis of gastric cancer MKN45 cells. Formation of CTPS cytoophidia significantly inhibited MKN45 cell proliferation (evaluated using EdU incorporation assay), significantly blocked the cell cycle in G_1 phase (assessed using flow cytometry) and significantly decreased mRNA and protein expression levels of cyclin D1 (assessed by reverse transcription-quantitative PCR and western blotting, respectively). Furthermore, the number of apoptotic bodies and apoptosis rate were markedly elevated and mitochondrial membrane potential was markedly decreased. Moreover, mRNA and protein expression levels of Bax increased and Bcl-2 decreased markedly in MKN45 cells following transfection with the CTPS-overexpression vector. The proliferation rate increased, percentage of G_1/G_0-phase cells decreased and apoptosis was attenuated in cells transfected with the R294D-CTPS mutant vector and this mutation did not lead to formation of cytoophidia. The results of the present study suggested that formation of CTPS cytoophidia inhibited proliferation and promoted apoptosis in MKN45 cells. These results may provide insights into the role of CTPS cytoophidia in cancer cell proliferation and apoptosis.

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Introduction

Cytidine triphosphate synthase (CTPS) is a key enzyme responsible for *de novo* synthesis of CTP, which is an essential nucleotide and precursor for RNA and DNA synthesis (1); therefore, CTPS activity affects cell cycle progression. It has been reported that CTPS forms filamentous structures termed cytoophidia (Greek for 'cellular snakes') in Drosophila (2,3), bacteria (4), yeast (3), zebrafish (5), human and rat cells (6,7), which suggests that the cytoophidium is an evolutionarily conserved subcellular structure that may serve an essential role in regulating metabolism (8).

Cytoophidia are mesoscale, intracellular, filamentous structures that contain metabolic enzymes; they are not membrane-bound cell organelles. They comprise a type of intracellular compartment and are involved in cell metabolism (2). Certain studies have reported that cytoophidia may serve as metabolic stabilizers and a buffer system in response to environmental changes (5,9,10). Cytoophidia respond to nutrient stress by elongating following nutrient deprivation in Drosophila (11) and budding yeast (12). In *Schizosaccharomyces pombe*, cytoophidia formation decreases following cold or heat shock (13). Certain studies have reported that cytoophidium sequester the active binding sites of enzymes, thereby inhibiting CTPS activity in *Escherichia coli* and Drosophila tissue (14,15). However, Strochlic *et al* (16) reported that Drosophila CTPS within cytoophidia is catalytically active. These aforementioned studies suggest that CTPS activity following cytoophidia formation differs with cell type.

The changes in CTPS activity are reported to be associated with cancer progression (17,18). Significantly higher activity of CTPS has been reported in acute lymphocytic leukemia cells compared with lymphocytes of healthy controls (19). CTPS also promotes malignant progression of triple-negative breast cancer (20). Cytoophidia formed by CTPS have been reported in human hepatocellular carcinoma cells but not in adjacent non-cancerous hepatocytes (21). To the best of our knowledge, the potential association between CTPS cytoophidia and cancer cell proliferation is has not been previously elucidated.

Toosendanin (TSN) is a triterpenoid derivative extracted from the bark of *Melia toosendan* Sieb et Zucc and exerts anticancer effects on numerous types of human cancer cell,

such as colorectal cancer cells and glioma cells (22-25). Our previous studies demonstrated that TSN induces apoptosis of human gastric cancer MKN45 cells (26) and induces formation of CTPS cytoophidia. To the best of our knowledge, however, the association between formation of CTPS cytoophidia and apoptosis in MKN45 cells remains unknown. The present study evaluated whether the CTPS formed cytoophidia affected TSN-induced MKN45 cell proliferation or apoptosis. The results of the present study may facilitate further understanding of the role of CTPS cytoophidia in cancer cell apoptosis.

Materials and methods

Cell culture. The gastric cancer MKN45 cell line was purchased from the Beijing Beina Chuanglian Biotechnology Institute and cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Biological Industries) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37˚C in a 5% CO_2 humidified environment. All cells were cultured in 6-well plates for drug treatment or transfection with plasmids.

Generation of constructs. Total RNA was extracted from MKN45 cells using Trizol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For reverse transcription (RT), 1 μg RNA was used with ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo Life Science) at 37˚C for 15 min and denaturation at 98˚C for 5 min, cDNA was stored at -20˚C. The overexpression vector of CTPS (OE-CTPS) was constructed using the seamless cloning technique. The linearized pcDNA3.1(+) vector was PCR amplified with primers as follows: forward 5'-CATAAGCTTAAGTTTAAACGCTAGCCAGC-3' and reverse 5'-TACCCATACGATGTTCCAGATTACGCTTGA GGATCCACTAGTCCAGTGTGG-3'. The full-length coding sequences of human CTPS were amplified using PCR with primers as follows: forward 5'-GCGTTTAAACTTAAG CTTATGAAGTACATTCTGGTTACTGGTGGT-3' and reverse 5'-TGGAACATCGTATGGGTAGTCATGATT TATTGATGGAAACTTCAG-3'. The seamless cloning reaction was performed using ClonExpress II One Step Cloning kit C112 (Vazyme Biotech Co., Ltd.) according to the manufacturer's protocol. To generate the point mutation $CTPS^{R294D}$, site-directed mutagenesis was performed on the pcDNA3.1(+)-CTPS plasmid using primers as follows: Forward 5'-GACAGATATGATGACTTGCTGGAG-3' and reverse 5'-AGCCATCTCTTTCCATTTCATCA-3' (underlined section indicates the mutated site). The PCR products were digested with Dpn I (New England BioLabs, Inc.) at 37˚C for 1 h and ligated using Quick Ligation kit (New England BioLabs, Inc.) before transformation, as previously described (27). All PCR reactions were performed in a total volume of 50 μl using 2X PrimeSTAR Max Premix (Takara Bio). Empty pcDNA3.1(+) vector served as a negative control. The destination constructs were fully sequenced (Sangon Biotech Co., Ltd.) before use for transfection. The cells were seeded in 6-well plates and transfected with 4 μg of Empty pcDNA3.1(+) vector, pcDNA3.1(+)-CTPS, pcDNA3.1(+)-$CTPS^{R294D}$ at 37˚C for 6 h using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, then cultured for 48 h.

Immunofluorescence assay. MKN45 cells were treated with 0, 60, 80 and 120 nM TSN at 37˚C for 72 h or transfected with OE-CTPS and OE-$CTPS^{R294D}$ vectors. Cells were fixed using 4% paraformaldehyde for 30 min at 4˚C. After washing with PBST (1X PBS; 0.2% Triton X-100), the cells were blocked with 5% bovine serum albumin (BSA; 0.5% Triton X-100 in PBS) for 60 min at 37˚C. The cells were incubated with primary rabbit anti-human CTPS antibodies (1:100; cat. no. abs138045; Absin Bioscience Inc.) at 4˚C for 12 h. The cells were washed with PBST three times and incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies (1:500; cat. no. 111-545-003 Jackson ImmunoResearch Laboratories, Inc.) for 2 h at room temperature. The cells were washed with PBST and slides were counterstained with DAPI to visualize the nuclei for 5 min at room temperature. All samples were imaged using the 63x objective of a laser-scanning confocal microscope (Leica TCS SP8 Confocal Microscope; Leica Microsystems GmbH). The proportion of the cells which contained CTPS cytoophidia was calculated from a minimum of five randomly chosen fields from three individual experiments using ImageJ 1.43 software (National Institutes of Health).

Cell viability analysis. MKN45 cells were seeded in 96-well plates at a density of $1x10^4$ cells/well. The cells were treated with different concentrations of TSN (0, 60, 80 and 120 nM) for 24, 48 and 72 h at 37˚C, then incubated with 0.5 μg/μl MTT at 37˚C for 4 h. Subsequently, the supernatant was removed and 150 μl DMSO was added to each well. The absorbance at 570 nm was quantified using a multi-well plate reader (Spark 10M, Tecan Group, Ltd.).

Cell cycle analysis. Following transfection with plasmids or treatment with TSN as aforementioned, cells were washed twice with PBS and detached from the plate surface by digestion using trypsin. Cells were centrifuged at 300 x g at 4˚C for 10 min, the pellet was resuspended in PBS, centrifuged again at 300 x g at 4˚C for 10 min and resuspended in ice-cold 70% ethanol and stored at 4˚C for 18 h. Samples were washed once in PBS and resuspended in DNA staining solution (propidium iodide, 5 μg/ml; RNase A, 0.5 mg/ml; PBS) and incubated at 37˚C in the dark for 30 min. All samples were assessed using a Cytomics FC500 Flow Cytometer (Beckman Coulter, Inc.) and analyzed using CXP Software version 2.3 (Beckman Coulter, Inc.).

Early apoptosis assay. Following transfection with plasmids or treatment with TSN as aforementioned, the cells were detached from the plate surface, digested by trypsin and centrifuged at 300 x g at 4˚C for 10 min, washed twice with cold PBS, then 500 μl Annexin V-FITC binding buffer (No. C1062M, Beyotime Institute of Biotechnology) was added to each sample. The cells were incubated with 5 μl FITC-annexin V and 5 μl PI for 15 min at 25˚C in the dark. After washing, aliquots of $2x10^4$ cells/sample were examined using a Cytomics FC500 Flow Cytometer and analyzed with CXP Software ver.2.3 (Beckman Coulter).

EdU proliferation assay. Cells were seeded in 96-well plates at $1x10^4$ cells/well and placed in a humidified incubator at 37˚C with 5% CO_2 for 12 h following treatment with TSN or transfection with plasmids for 48 h as aforementioned. Cell proliferation was assessed using the EdU Cell Proliferation Assay kit (Guangzhou RiboBio Co., Ltd.) as described by Wang *et al* (28). The percentage of EdU-positive cells was calculated from five random fields using ImageJ (National Institutes of Health).

JC-1 staining for mitochondrial membrane potential. To determine the mitochondrial membrane potential, cells were seeded in 6-well plates at a density of $1x10^4$ cells/well and transfection with plasmids or treatment with 80 nM TSN for 48 h. JC-1 staining was performed as described by Sabarwal *et al* (29).

Acridine orange nuclear staining. Cells were cultured on glass cover slides and transfected with plasmids or treatment with 80 nM TSN for 48 h. Cells were rinsed twice with PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Subsequently, the cells were stained with 0.1 mg/ml acridine orange (Solarbio Life Science Co., Ltd. Beijing) for 1-2 min. The images were captured using a Leica TCS SP8 confocal laser-scanning microscope (Leica Microsystems GmbH) at 488 nm excitation and 515 nm emission wavelengths (magnification, x63).

RT-quantitative (q)PCR. Following transfection with plasmids or treatment with 80 nM TSN for 48 h, mRNA expression levels of cyclin D1 (CCND1), Bax and Bcl-2 were assessed using RT-qPCR. The extraction of RNA and synthesis of first-strand cDNA were performed as described by Zhang *et al* (30) using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Life Science). TB Green® Premix Ex Taq™ (Tli RNase H Plus; cat. no. RR420L, Takara Bio Inc. Beijing) was used for qPCR. The thermocycling conditions were as follows: 95˚C for 2 min, followed by 40 cycles of 95˚C for 15 sec, and 60˚C for 30 sec. β-actin was used as an endogenous control for data normalization. Experiments were performed in triplicate. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (31). Sequences of the primers used are presented in Table I.

Western blotting. Whole-cell lysate was extracted using RIPA lysis buffer (cat. no. P0013B, Beyotime Institute of Biotechnology). The lysate was centrifuged at 12,470 x g for 15 min at 4˚C. The supernatant was collected and the protein concentration was determined using the BCA Protein Assay kit (Beyotime Institute of Biotechnology). Protein (12 μg/lane) was loaded and separated by 10% SDS-PAGE, followed by transfer to polyvinylidene fluoride membranes (EMD Millipore). Membranes were blocked with 5% (w/v) fat-free milk in Tris-buffered saline containing 0.05% Tween-20 at 20-25˚C for 1 h and probed with primary antibodies at 4˚C overnight, primary antibodies as follows: CTPS (1:300; cat. no. abs138045; Absin Bioscience Inc.), Bcl-2 (1:300; cat. no. ab196495; Abcam,), Bax (1:300; cat. no. ab53154; Abcam), CCND1 (1:5,000; cat. no. 60186-1-Ig; ProteinTech Group, Inc.) and β-actin (1:10,000; cat. no. ab8227; Abcam,).

Table I. Primers used for reverse transcription-quantitative PCR.

Gene	Sequence, 5'→3'
CCND1	F: tattgcgctgctaccgttga
	R: ccaatagcagcaaacaatgtgaaa
Bcl-2	F: atgtgtgtggagagcgtcaac
	R: agacagccaggagaaatcaaac
Bax	F: aagctgagcgagtgtctcaag
	R: caaagtagaaaagggcgacaac
β-actin	F: agcgagcatcccccaaagtt
	R: gggcacgaaggctcatcatt

CCND1, cyclin D1; F, forward; R, reverse.

The membranes were then incubated with IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody (1:15,000; code: 926-32211, LI-COR Biosciences) antibodies or IRDye 800CW Goat anti-Mouse IgG Secondary Antibody (1:15,000; code: 926-32210, LI-COR Biosciences) for 90 min at 20-25˚C and washed with PBS. The immunoblots were visualized using an Odyssey IR Imaging System (LI-COR Biosciences). Image Studio version 4.0 (LI-COR Biosciences) was used to analyze the bands and each band was normalized to β-actin.

Statistical analysis. All data are presented as the mean ± SEM, and data were obtained from three replications. Representative bands of western blotting were selected from independent experiments. All statistical tests were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc.). One-way ANOVA was used to compare independent groups with Dunnett's multiple comparisons test for comparisons against a single control and Tukey's multiple comparisons test when ≥3 groups were analyzed.

Results

TSN induces CTPS cytoophidia formation in MKN45 cells. Cell survival rate markedly decreased as the TSN concentration and treatment duration increased (Fig. 1A). CTPS cytoophidia were observed in MKN45 cells treated with different concentrations (0, 60, 80 and 120 nM) of TSN for 72 h (Fig. 1B). Compared with the control (0 nM TSN), cytoophidia were detected in 36.4% of MKN45 cells treated with 60 nM TSN and 46.65% of MKN45 cells treated with 80 nM TSN; this showed that CTPS cytoophidia formation was significantly increased compared with the control. However, the percentage decreased to 16.01% in MKN45 cells treated with 120 nM TSN (Fig. 1C). These results indicated that TSN decreased cell viability while induced CTPS cytoophidia formation in MKN45 cells.

CTPS cytoophidia formation inhibits proliferation of MKN45 cells. To determine the effect of CTPS cytoophidia on the proliferation rate of gastric cancer MKN45 cells, OE-CTPS and R294D-CTPS mutant (OE-$CTPS^{R294D}$) vector were generated (Fig. 2A). The formation of CTPS cytoophidia

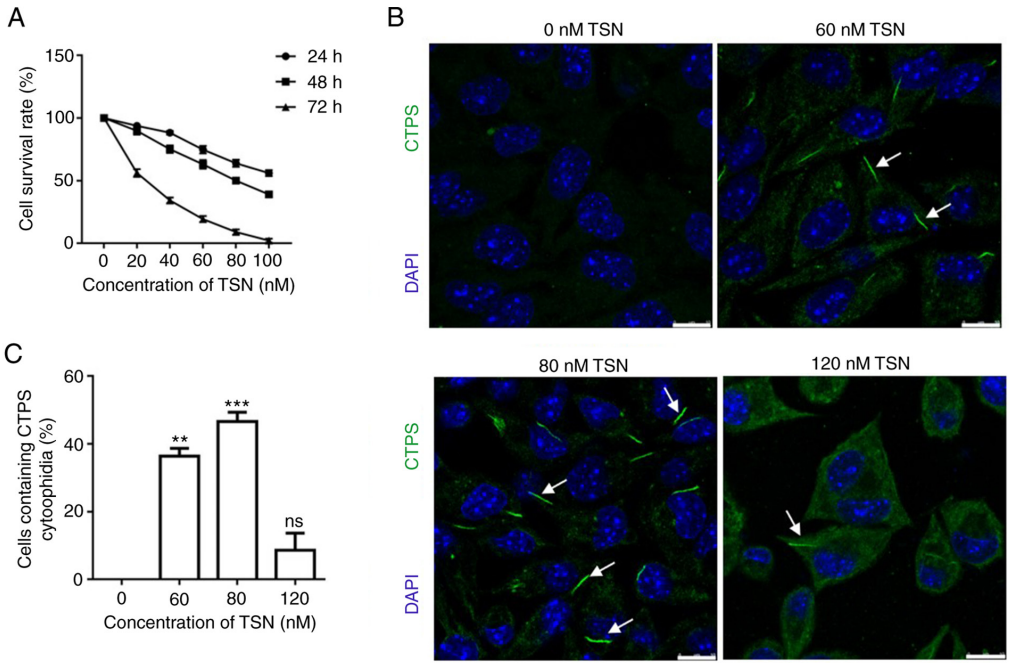

Figure 1. CTPS cytoophidia are induced by TSN in MKN45 cells. (A) Effect of TSN on survival of MKN45 cells. (B) Immunofluorescence analysis of CTPS cytoophidia in MKN45 cells following treatment with 0, 60, 80 and 120 nM TSN for 72 h. White arrows indicate CTPS cytoophidia. (C) Percentage of cells with CTPS cytoophidia. **P<0.01 and ***P<0.001 vs. 0. Scale bar, 10 μm. CTPS, cytidine triphosphate synthase; TSN, toosendanin; ns, not significant.

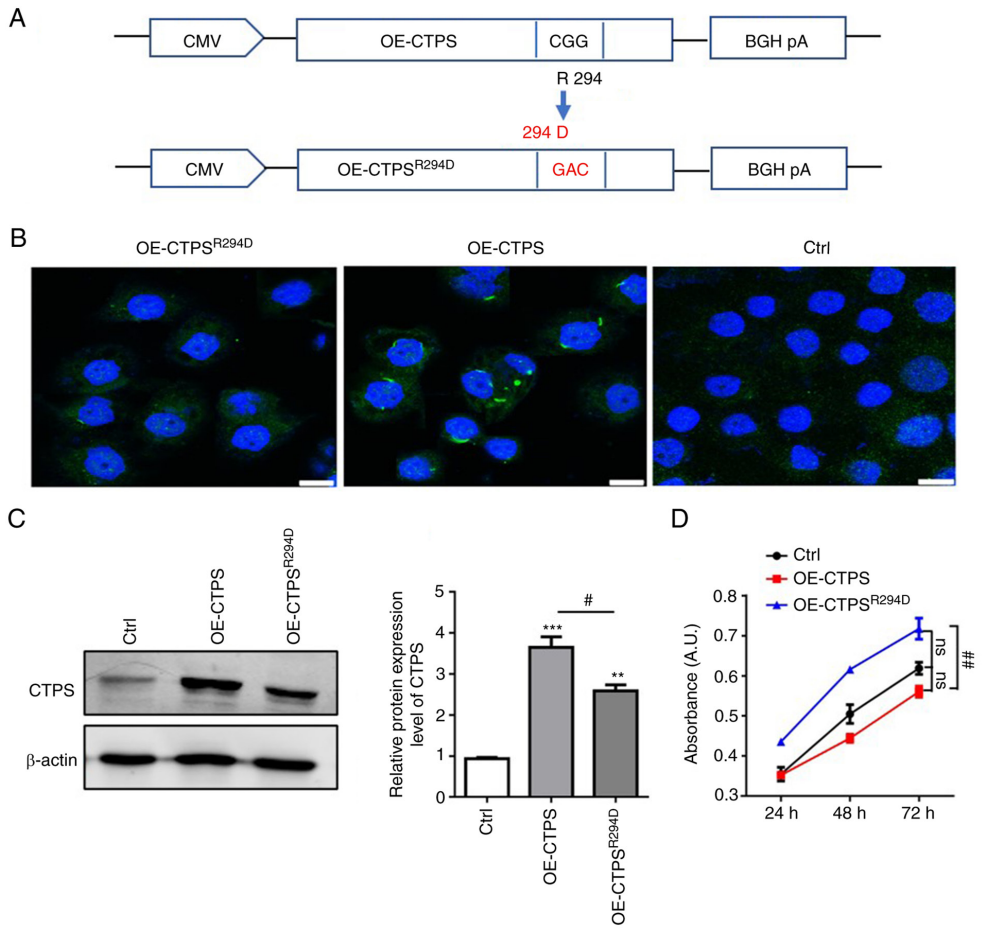

Figure 2. Effect of OE-CTPS and R294D-CTPS mutant on formation of CTPS cytoophidia in MKN45 cells. (A) Schematic diagram of OE-CTPS and OE-CTPS^{R294D} mutant vectors. (B) Formation of CTPS cytoophidia (green; blue, cell nuclei) and (C) protein expression levels of CTPS in MKN45 cells following transfection with OE-CTPS or OE-CTPS^{R294D} vectors. (D) Growth curves of Ctrl, OE-CTPS and OE-CTPS^{R294D} cells. **P<0.01 and ***P<0.001 vs. Ctrl; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. OE-CTPS. Scale bar, 10 μm. CTPS, cytidine triphosphate synthase; OE, overexpression; ns, not significant; Ctrl, control; CMV, cytomegalovirus; BGH pA, bovine growth hormone polyadenylation.

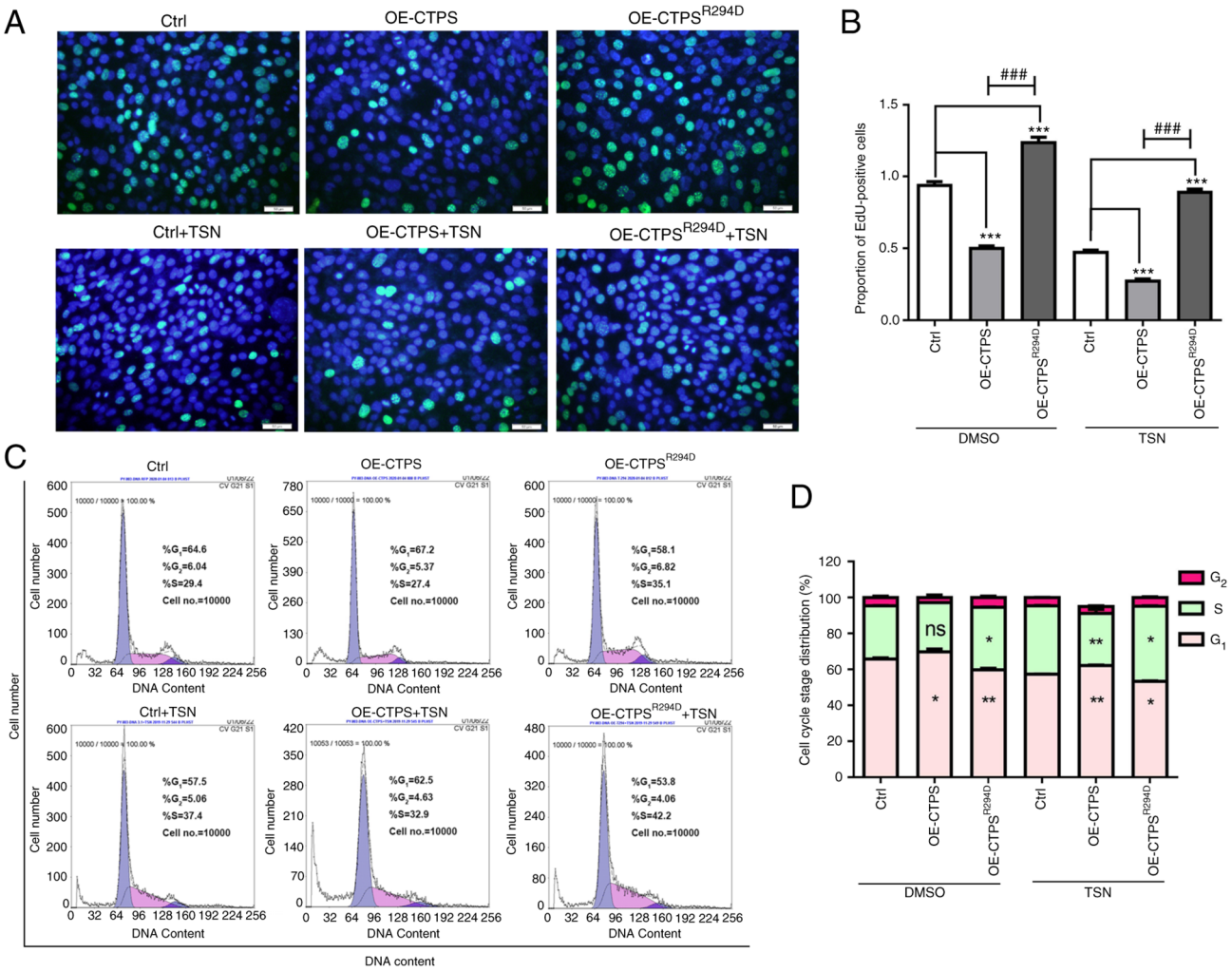

Figure 3. Effect of CTPS cytoophidia on proliferation of MKN45 cells. MKN45 cells were transfected with OE-CTPS or OE-CTPS^{R294D} vector for 48 h and treated with 80 nM TSN for 48 h. (A) Proliferation of MKN45 cells labeled with EdU (green; blue, cell nuclei). (B) Percentage of EdU-positive cells (n=6). (C) Cell cycle distribution of MKN45 cells assessed using flow cytometry. (D) Percentage of cells in G_1/G_0 and S phase (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. Ctrl; $^{\#\#\#}$P<0.001 vs. OE-CTPS. Scale bar, 50 μm. CTPS, cytidine triphosphate synthase; OE, overexpression; ns, not significant; Ctrl, control; TSN, toosendanin.

and CTPS protein expression levels were assessed. CTPS assembled into cytoophidia in OE-CTPS cells; but did not assemble into cytoophidia in OE-CTPS^{R294D} cells (Fig. 2B). CTPS protein expression levels in OE-CTPS^{R294D} cells were significantly lower compared with those in OE-CTPS cells (Fig. 2C). MKN45 cell viability decreased after being transfected with OE-CTPS compared with control; however, cell viability increased following transfection with OE-CTPS^{R294D} (Fig. 2D). These results indicated that the formation of CTPS cytoophidia decreased cell viability in MKN45 cells.

The proliferation rate of gastric cancer MKN45 cells was also assessed. Compared with the control, the percentage of EdU-positive cells significantly decreased in OE-CTPS cells (Fig. 3A and B). Furthermore, the percentage of G_1/G_0-phase cells significantly increased and the percentage of S-phase cells markedly decreased in OE-CTPS cells compared with the control (Fig. 3C and D). Following treatment with 80 nmol/l TSN, compared with group of control+TSN, the EdU-positive rate decreased, the percentage of G_1/G_0-phase cells increased and S-phase cells decreased in OE-CTPS +TSN group. The EdU-positive rate increased, percentage of G_1/G_0-phase cells decreased and the percentage of S-phase cells increased in OE-CTPS^{R294D} cells compared with the control. The same changes were observed in group of OE-CTPS^{R294D}+TSN compared with control+TSN group.

Furthermore, mRNA and protein expression levels of CCND1 markedly decreased in OE-CTPS cells compared with the control but significantly increased in OE-CTPS^{R294D} cells compared with both the control and OE-CTPS (Fig. 4A and B). The same changes of CCND1 mRNA and protein expression levels were observed following treatment with 80 nmol/l TSN in OE-CTPS cells and OE-CTPS^{R294D} cells compared with control. (Fig. 4C and D). These results indicated that CTPS cytoophidia formation could inhibit MKN45 cells proliferation by affecting cell cycle progression.

CTPS cytoophidia formation promotes apoptosis of MKN45 cells. To assess the effect of CTPS cytoophidia on apoptosis of gastric cancer MKN45 cells, cells were transfected with OE-CTPS or OE-CTPS^{R294} vectors. Subsequently, morphological changes and the presence of early apoptotic cells were evaluated. Compared with the control, chromosomes

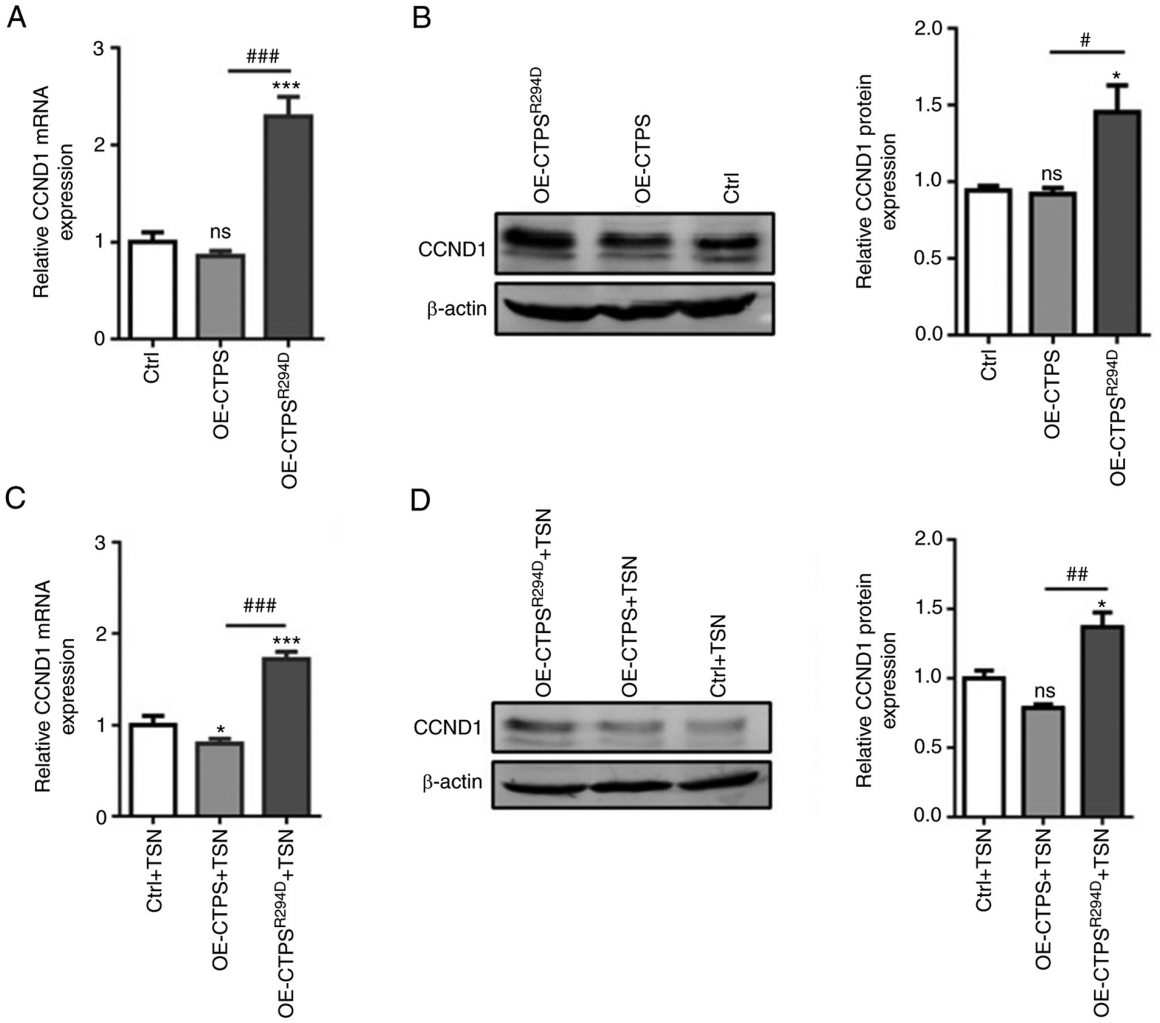

Figure 4. Expression of CCND1. (A) the mRNA expression levels of CCND1 were assessed using RT-qPCR after cells transfected with OE-CTPS and OE-CTPSR294D vectors. (B) the protein expression levels of CCND1 were assessed using western blotting after cells transfected with OE-CTPS and OE-CTPSR294D vectors. (C) the mRNA expression levels of CCND1 were assessed using RT-qPCR after treatment with 80 nmol/l TSN in OE-CTPS cells and OE-CTPSR294D cells (D) the protein expression levels of CCND1 were assessed using western blotting treatment with 80 nmol/l TSN in OE-CTPS and OE-CTPSR294D cells. (n=3) *P<0.05 and ***P<0.001 vs. Ctrl; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. OE-CTPS. CTPS, cytidine triphosphate synthase; OE, overexpression; ns, not significant; Ctrl, control; TSN, toosendanin; CCND1, cyclin D1.

were markedly more aggregated and marginalized in OE-CTPS cells; however, no notable morphological changes in OE-CTPS^{R294} cells were observed compared with the control (Fig. 5A). FITC-annexin-V/PI staining demonstrated that apoptosis rate was higher in OE-CTPS cells compared with the control and significantly lower in OE-CTPS^{R294} cells compared with both control and OE-CTPS (Fig. 5B and C). Following treatment of transfected cells with 80 nmol/l TSN, apoptotic bodies were prominent in OE-CTPS+TSN cells compared with in groups of control+TSN; however, in OE-CTPS^{R294}+TSN cells no apoptotic bodies was observed, chromosome aggregation or marginalization occurred only in some cells. Apoptosis rate was more pronouncedly increased in group of OE-CTPS +TSN cells compared with both control+TSN and OE-CTPSR294+TSN groups.

Mitochondrial membrane potential in OE-CTPS cells was markedly lower compared with control cells; however, the potential in OE-CTPS^{R294} cells was significantly higher compared with OE-CTPS cells without TSN treatment and markedly higher compared with OE-CTPS cells with TSN treatment (Fig. 6A and B). Furthermore, mRNA and protein expression levels of Bax and Bcl-2 were assessed by RT-qPCR and western blotting. The mRNA and protein expression levels of Bax increased significantly, whereas Bcl-2 mRNA expression levels markedly decreased and protein expression levels significantly decreased, in OE-CTPS cells compared with control cells. Furthermore, mRNA and protein expression levels of Bax significantly decreased, whereas mRNA and protein expression levels of Bcl-2 significantly increased in OE-CTPS^{R294} cells compared with OE-CTPS cells in the presence or absence of TSN treatment (Fig. 6C-H).

Discussion

TSN exhibits anticancer effects on numerous types of human cancer cell (32), such as suppresses hepatocellular carcinoma proliferative and metastasis (33), induces the apoptosis of human Ewing's sarcoma (34). In the present study, TSN significantly inhibited proliferation of MKN45 cells in a time- and dose-dependent manner. CTPS formed cytoophidia

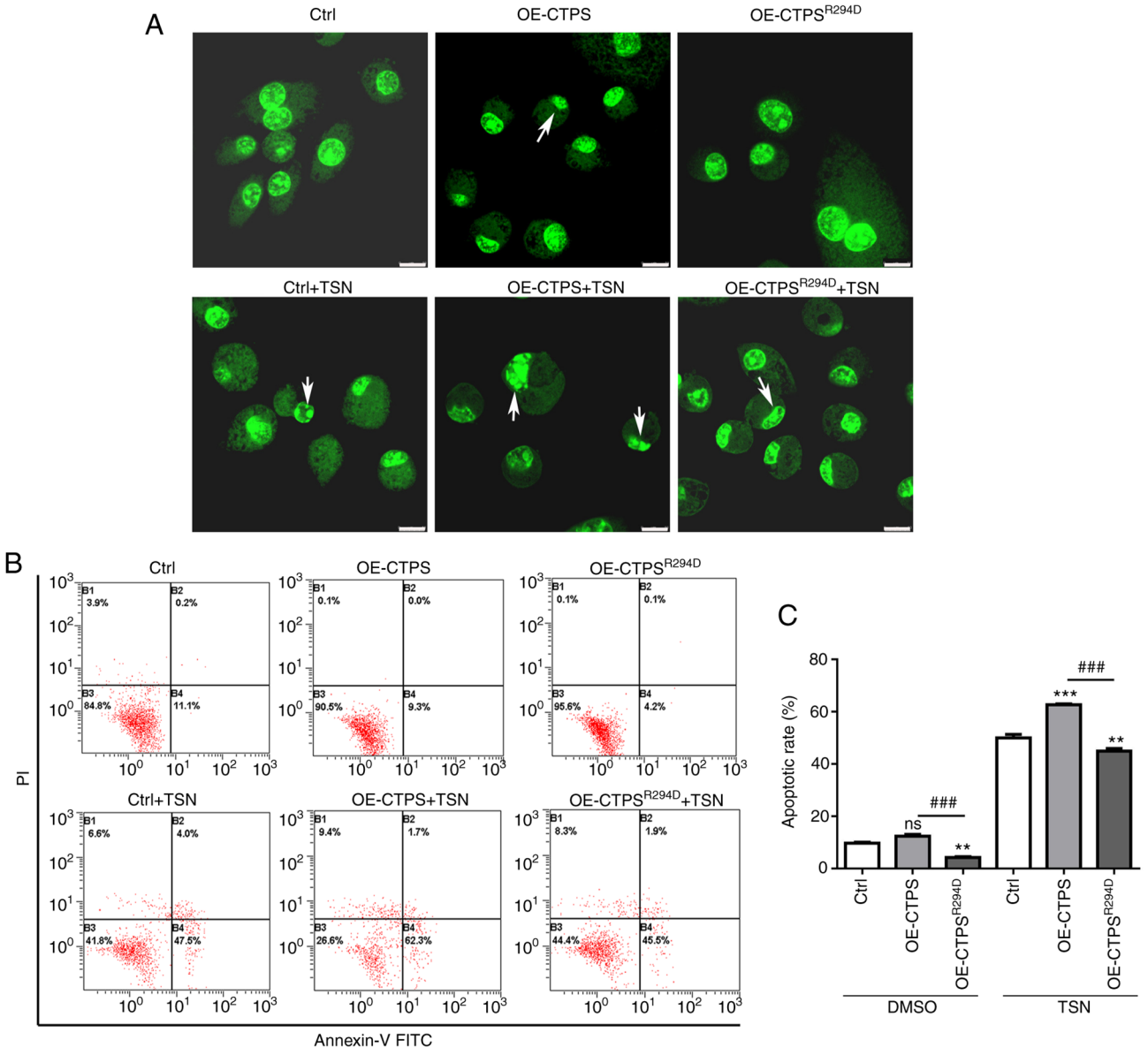

Figure 5. Effect of CTPS cytoophidia on apoptosis of MKN45 cells. MKN45 cells were transfected with OE-CTPS or OE-CTPS^{R294D} vectors for 48 h and treated with 80 nM TSN for 48 h. (A) Morphological changes in MKN45 cells observed following acridine orange staining, white arrows indicated the chromosomes aggregated and marginalized or apoptotic bodies. (B) Early apoptosis detected by flow cytometry (n=3). (C) Apoptotic rate. **P<0.01 and ***P<0.001 vs. Ctrl; $^{###}$P<0.001 vs. OE-CTPS. Scale bar, 10 μm. CTPS, cytidine triphosphate synthase; OE, overexpression; ns, not significant; Ctrl, control; TSN, toosendanin.

following TSN-induced inhibition of MKN45 cell proliferation; moreover, the number of CTPS cytoophidia increased with TSN dosage. However, high concentrations of TSN led to cell death and affected the formation of CTPS cytoophidia; fewer CTPS cytoophidia were observed when cells were treated with 120 nM TSN. These data suggested that cytoophidia formation may affect proliferation rate and apoptosis of cancer cells.

Cytoophidia are a type of intracellular compartment conserved across prokaryotes and eukaryotes and are involved in cell metabolism (35). The first reported component of the cytoophidia was CTPS (2-4). CTPS is a cytosol-associated glutamine amidotransferase enzyme that catalyzes *de novo* biosynthesis of CTP, a key nucleotide. Polymerization of CTPS into filamentous structures (cytoophidia) regulates its enzymatic activity (8,35). The formation of cytoophidia is reported to inhibit CTPS activity in *E. coli* and *Drosophila* (14,15). In the present study, R294D-CTPS mutants were generated and used to evaluate the effect of CTPS cytoophidia on TSN-induced proliferation and apoptosis. Although the R294D-CTPS mutant did not form cytoophidia, CTPS activity was not affected (10,36). As expected, significantly fewer EdU-positive cells were observed in the OE-CTPS group compared with the control in the present study. However, a significantly higher percentage of EdU-positive cells was observed in OE-CTPS^{R294D} compared with OE-CTPS cells. The decrease in EdU-positive cells demonstrated that formation of CTPS cytoophidia affected the proliferation rate of MKN45 cells.

Proliferating cells have been reported to demonstrate higher RNA and DNA synthesis rates during G_1- and S-phase (37).

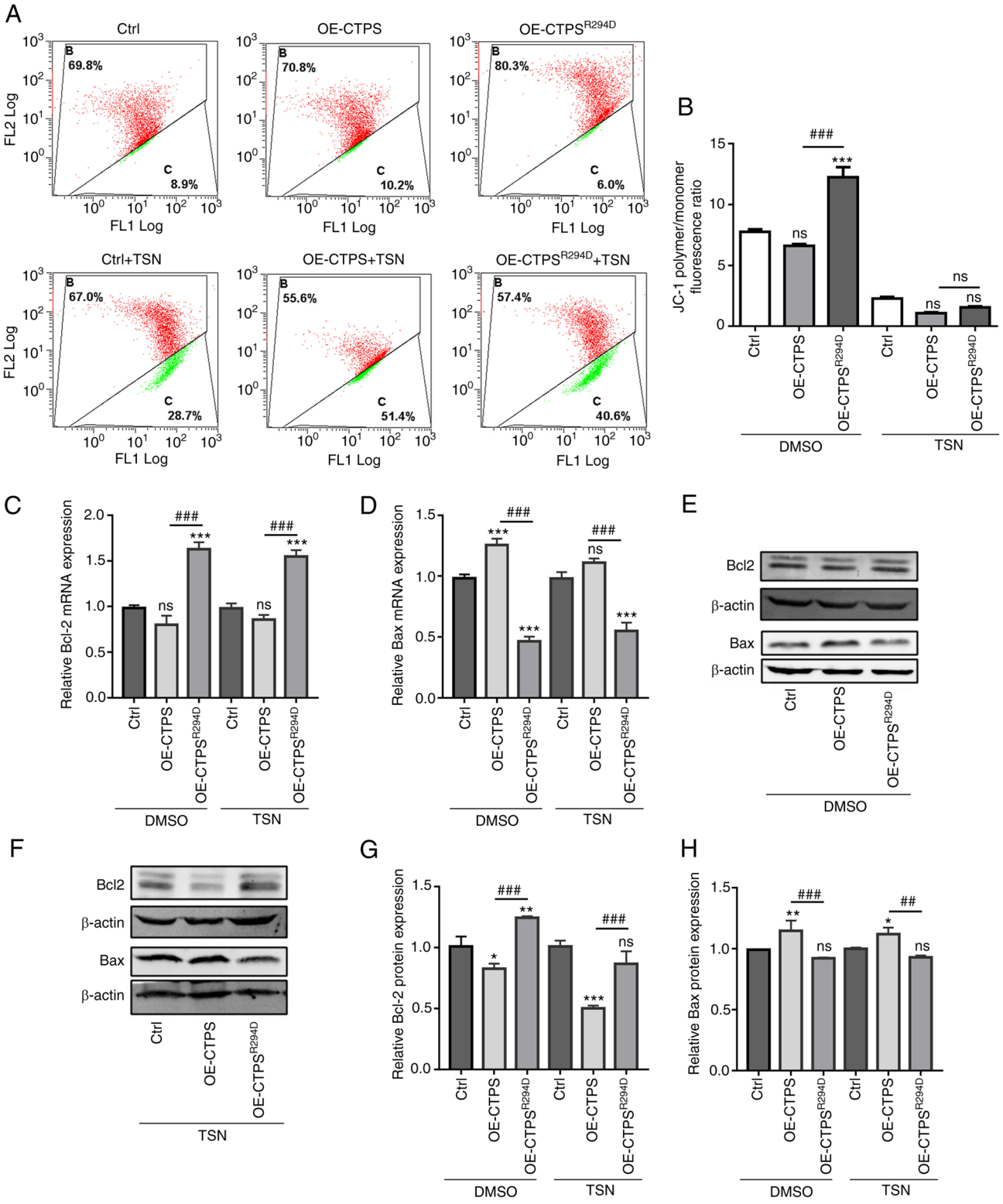

Figure 6. Effect of CTPS cytoophidia on mitochondrial membrane potential and mRNA and protein expression levels of Bax and Bcl-2. (A) Changes in mitochondrial membrane potential were assessed using JC-1 staining. (B) Fluorescence ratio of JC-1 polymer/monomer. mRNA expression levels of (C) Bcl-2 and (D) Bax in MKN45 cells quantified using reverse transcription-quantitative PCR. (E) Protein expression levels of Bax and Bcl-2 in MKN45 cells after transfected with OE-CTPS or OE-CTPS^{R294D} vectors semi-quantified using western blotting (n=3). (F) Protein expression levels of Bax and Bcl-2 in MKN45 cells after transfected with OE-CTPS or OE-CTPS^{R294D} vectors and following treatment with 80 nmol/l TSN semi-quantified using western blotting (n=3). (G) Statistical results of Bcl-2 protein expression. (H) Statistical results of Bax protein expression *P<0.05, **P<0.01 and ***P<0.001 vs. Ctrl; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. OE-CTPS. CTPS, cytidine triphosphate synthase; OE, overexpression; ns, not significant; Ctrl, control; TSN, toosendanin.

Therefore, proliferating cells synthesize increased amounts of ribonucleotides and deoxyribonucleotides. As CTPS is key for *de novo* synthesis of CTP, a precursor for RNA and DNA synthesis, it can be hypothesized that CTPS activity increases in G_1-phase of the cell cycle to support increased synthesis of nucleic acids. The present study demonstrated that the

percentage of G_1/G_0-phase cells significantly increased and the percentage of S-phase cells markedly decreased in OE-CTPS cells compared with the control. Moreover, the mRNA and protein expression levels of CCND1 markedly decreased in OE-CTPS cells compared with the control. OE-CTPS^{R294D} cells which demonstrated significantly decreased percentage of G1/G0-phase cells and increased the percentage of S-phase cells, meanwhile increased CCND1 mRNA and protein expression levels compared with both OE-CTPS and control cells. The aforementioned effects of OE-CTPS and R294D-CTPS mutation were greater following treatment with 80 nmol/l TSN, and subG1 peak was observed simultaneously, but the proportion of subG1 values were not calculated as the software cannot analyze it. The aforementioned results suggested that formation of CTPS cytoophidia affected RNA synthesis during the cell cycle, thereby inhibiting MKN45 cell proliferation induced by TSN.

TSN has been reported to suppress proliferation and induce apoptosis in numerous types of human cancer cell, such as hepatocellular carcinoma (26,33). The present study assessed the effect of CTPS cytoophidia on TSN-induced apoptosis in MKN45 cells. Following formation of TSN-induced CTPS cytoophidia, the number of apoptotic bodies and apoptotic rate increased markedly in OE-CTPS cells compared with the control. A decrease in mitochondrial membrane potential occurs during early cell apoptosis; the present study demonstrated that mitochondrial membrane potential markedly decreased following formation of TSN-induced CTPS cytoophidia in OE-CTPS cells. However, the mitochondrial membrane potential increased significantly when the formation of CTPS cytoophidia was prevented in OE-CTPS^{R294D} cells. Furthermore, mRNA and protein expression levels of Bcl-2 markedly decreased whereas those of proapoptotic Bax markedly increased in OE-CTPS compared with control; however, when formation of CTPS cytoophidia was prevented in OE-CTPS^{R294D} cells, increased Bcl-2 mRNA and protein expression levels and decreased Bax mRNA and protein expression levels were observed compared with both OE-CTPS and control cells.

In conclusion, the results of the present study suggested that CTPS promoted cell proliferation and inhibited apoptosis in MKN-45 cells. However, when CTPS formed cytoophidia after MKN45 cells were treated with TSN, CTPS activity was inhibited, which arrested the cell cycle in G_1 phase, inhibiting cell proliferation and promoting apoptosis. However, the mechanism by which TSN induces CTPS to form cytoophidia is still unclear and requires further study.

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Availability of data and materials

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

Authors' contributions

XPF analyzed data for the work and drafted the manuscript. WC and YP performed the experiments. CL performed data analysis. ZZZ performed the photography using the confocal laser microscope. SLS and WWZ designed the experiments and revised the manuscript. WWZ obtained funding. All authors have read and approved the final manuscript. YP and WWZ confirm the authenticity of all the raw data.

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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