Lentivirus-mediated silencing of MPHOSPH8 inhibits MTC proliferation and enhances apoptosis

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Abstract. Thyroid carcinoma (TC) is the most common malignancy of the endocrine organs, and its incidence rate has steadily increased over the last decade. For medullary thyroid cancer (MTC), a type of TC, a high mortality rate has been reported. In previous studies, M-phase phosphoprotein 8 (MPHOSPH8) displayed an elevated expression in various human carcinoma cells. Thus, MPHOSPH8 may be a sensitive biomarker that could be used for the diagnosis and follow-up of MTC. In the present study, plasmids of RNA interference targeting the MPHOSPH8 gene were constructed. Once these lentiviruses targeting MPHOSPH8 were transfected into the MTC cell line TT, cell viability and proliferation were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Flow cytometry was used to assess the cell cycle distribution and apoptosis. The expression levels of MPHOSPH8 were detected by reverse transcription quantitative-polymerase chain reaction and western blot analyses. Depletion of MPHOSPH8 significantly inhibited cell proliferation. Furthermore, knockdown of MPHOSPH8 in TT cells led to G0/G1 phase cell cycle arrest and apoptosis. The results of the present study suggest that MPHOSPH8 promotes cell proliferation and may be a potential target for anticancer therapy of MTC.

Introduction

Thyroid cancer (TC) is one of the most common endocrine malignancies (1), and its incidence has significantly increased in recent years (2). Thyroid tumors are usually classified into four subtypes [papillary TC, follicular TC, anaplastic TC and medullary TC (MTC) (3)], according to their histopathological characteristics, and treatments are selected depending on the subtype and stage of the tumor (4). MTC is a malignancy of the parafollicular cells (also called C cells), which accounts for ≤10% of all thyroid tumors (5). The majority of MTCs are sporadic (80%), while ~20% of cases are inherited as a germline mutation in the rearranged during transfection proto-oncogene (6-8). Metastases occur in ~70% of patients with MTC who have a palpable thyroid nodule (>1.0-cm diameter) (9). MTCs may present as an aggressive malignancy with metastases to the liver, lungs, bones and mediastinum (8,10,11). At that stage of the disease, patients cannot undergo surgical resection, and do not receive radioactive iodine. In consequence, biochemical cure rates drop to ≤30% (12,13). Surgical resection results in a recurrence rate of almost 50% (6). Therefore, it is important to develop novel therapies for the treatment of MTC.

Carcinogenesis is a progression of events resulting from the accumulation of genetic alterations and the disruption of epigenetic modifications, including epigenetic silencing of tumor suppressor genes, which is a common event during carcinogenesis and often involves aberrant DNA methylation and histone modifications (14) M-phase phosphoprotein 8 (MPHOSPH8 or MPP8), which is also known as hybrid-associated protein 3 with Ran-binding protein in the microtubule organizing center (RanBPM) and human source MPP8, was originally identified in the RanBPM complex (15). MPP8 was identified as a novel M phase phosphoprotein using expression and cloning by Matsumoto-Taniura *et al* (16) in 1996. MPP8 is capable of recognizing the methylated lysine 9 of histone H3, and it couples histone H3 K9 methylation with the promotion of DNA methylation for the silencing of tumor suppressor genes and induction of metastasis by recruiting DNA (cytosine-5)-methyltransferase 3A to target CpG sites (17). MPP8 predominantly localizes at the heterochromatin region during the interphase, and is important in the organization of heterochromatin by regulating the interplay between DNA methylation and histone H3 methylation (18,19). In addition, MPP8 causes cells in the G2 phase of the cell cycle to enter the M phase (20). Recently, targeted therapies for TC have been developed (21,22), and several potential drugs are currently in preclinical testing or in clinical use (23). However, the lack of systematic studies regarding the underlying molecular mechanisms of TC may lead to a high risk for TC patients to suffer unexpected side effects. A recent study has suggested that MPP8 may participate in the progression of MTC, and may

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be considered a potential biomarker (24). However, the role of MPP8 in MTC remains unclear. The present study conducted several experiments to investigate the role of MPP8 in MTC cells using an RNAi-based knockdown method. The depletion of MPP8 significantly inhibited the proliferation of TT cells and arrested the cell cycle at the G0/G1 phase. These findings may provide a novel insight into the treatment of MTC.

Materials and methods

Cell lines and cell culture. Human MTC TT cells and human embryonic kidney 293T cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). TT cells were cultured in F-12K medium (catalogue no. 21127022; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 20% fetal bovine serum (FBS; catalogue no. 10099-14; Gibco; Thermo Fisher Scientific, Inc.). 293T cells were maintained in Dulbecco's modified Eagle's medium (catalogue no. SH30243.01B+; HyClone; GE Healthcare Life Sciences, Chalfont, UK) with 10% FBS (catalogue no. 04-001-1a-1351574; Biological Industries, Cromwell, CT, USA). Both cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Construction of MPHOSPH8 small hairpin (sh)RNA lentivirus vectors and virus packaging. The following stem-loop-stem oligos were designed (Genechem, Shanghai, China) and cloned into the lentiviral expression vector pGP (ShanghaiBio China, Shanghai, China), which was digested with *Eco*RI and *Bam*HI (Takara Biotechnology, Dalian, China): MPHOSPH8 small hairpin (sh)RNA, S1 5'-GCTGTTTATCTTCCATGC AAACTCGAGTTTGCATGGAAGATAAACAGCTTT TT-3' and S2 5'-CAGTGTCCAGACTGCGTATTTCTCGAG AAATACGCAGTCTGGACACTGTTTTT-3'; and scramble shRNA 5'-GCGGAGGGTTTGAAAGAATATCTCGAG ATATTCTTTCAAACCCTCCGCTTTTTT-3', which was used as control. The above plasmids Lv-sh MPHOSPH8 (S1 and S2) and Lv-sh control were transformed into competent cells (*Escherichia coli* strain DH5α; Solarbio, Beijing, China), and extracted with a plasmid purification kit (Qiagen, Inc., Valencia, CA, USA). Successful ligation was determined by polymerase chain reaction (PCR) and sequencing analyses. The recombinant expression shRNA vectors and packaging helper plasmids pVSVG-I and pCMVΔR8.92 (ShanghaiBio China) were next co-transfected into 293T cells. Culture supernatants were harvested at 96 h post-transfection to purify lentiviruses expressing MPHOSPH8 shRNA or control shRNA. The lentiviruses were purified via ultracentrifugation at 400 x g for 10 min (MIKRO 200/200R; Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany), and their titer was measured by end point dilution through counting the numbers of infected green fluorescent protein (GFP)-positive cells at x100 magnification under a fluorescence microscope (Olympus, Tokyo, Japan). Titer (IU/ml) = (the numbers of green fluorescent cells) x (dilution factor) / (volume of virus solution). TT cells were infected with concentrated viruses at a multiplicity of infection of 60, and mock-infected cells were used as negative control. The infection efficiency was determined by observing GFP-positive cells under fluorescence microscope 96 h after infection. The efficiency of knocking down MPHOSPH8 was subsequently evaluated by reverse transcription-quantitative PCR (RT-qPCR) and western blot analyses.

RT-qPCR. TT cells were harvested following 7 days of lentivirus infection, and total RNA extraction was performed using TRIzol reagent (catalogue no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The purity and integrity of the RNA was assessed by spectrophotometry using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Inc.) and 1% agarose gel electrophoresis, respectively. The agarose gel electrophoresis was run in an electrophoresis tank with MOPS buffer (Dojindo Laboratories, Shanghai, China) at 100 V for 30min, and the results were observed under a ultraviolet lamp. First-strand complementary DNA was synthesized from 2 μg total RNA and the following PCR primers (Kangbeibio, Zhejiang, China): MPHOSPH8, forward 5'-AGTTATTGCTCGGCT CTGTG-3' and reverse 5'-CAGTCCCTTCTGTTTGGTCA-3'; and β-actin, forward 5'-GTGGACATCCGCAAAGAC-3' and reverse 5'-AAAGGGTGTAACGCAACTA-3'. RT-qPCR was performed in the linear range using SYBR® Green PCR Core Reagents (Applied Biosystems; Thermo Fisher Scientific, Inc.) on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR cycling conditions were as follows: Initial denaturation at 95°C for 60 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing and extension at 60°C for 20 sec. Data analysis was performed using the $2^{-\Delta\Delta Cq}$ method (25).

Western blotting. TT cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS) following 7 days of lentivirus infection. Then, cells were lysed in 2X sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl pH 6.8, 10 mM ethylenediaminetetraacetic acid, 4% SDS and 10% glycine). The total protein concentration in the cell lysate was quantified by BCA Protein Assay Kit (catalogue no. 23235; Pierce; Thermo Fisher Scientific, Inc.). A total of 30 μg cellular protein per lane was resolved on 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (catalogue no. 162-0177; Bio-Rad Laboratories, Inc.). The blots were probed overnight at 4°C with primary rabbit anti-MPHOSPH8 (1:500; catalogue no. HPA039701; Sigma-Aldrich, St. Louis, MO, USA) and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:100,000; catalogue no. 10494-1-AP; ProteinTech Group, Inc., Chicago, IL, USA) antibodies, and successively incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:5,000; catalogue no. SC-2054; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) secondary antibody for 2 h at room temperature. GAPDH served as internal standard. Signals were detected using the ECL Plus™ kit (catalogue no. RPN2132; GE Healthcare Life Sciences). Images were captured of the results (37X-V; Shanghai 5th optical factory, Shanghai, China).

Growth curve determination by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The effect of MPHOSPH8 on cell viability was determined based on growth curves of TT cells obtained by MTT assay. TT cells were seeded at a density of 10,000 cells/well in 96-well plates at 96 h post-lentivirus infection. Cell growth was examined

by MTT assay once a day for 5 days. For that purpose, 20 μl MTT solution (5 mg/ml in PBS; Sigma-Aldrich) was added to each well, followed by incubation for 4 h at 37˚C. Then, 100 μl stop buffer (0.01 M HCl, 10% SDS and 5% isopropanol; Sigma-Aldrich) was added to each well, which was then gently agitated for 10 min, prior to be analyzed on an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 595 nm.

Cell cycle analysis. Cell cycle analysis was performed with propidium iodide (PI) staining (Sigma-Aldrich), following the manufacturer's protocol. TT cells were seeded in 6-well plates at a density of $3x10^5$ cells/well subsequent to 4 days of lentivirus infection. The cell density was 50% after 72 h of culture, cells were washed and resuspended in PBS containing 50 μg/ml RNase A (Sigma-Aldrich), and next in cell cycle dying solution (50 μg/ml PI and 50 μg/ml RNase A) at room temperature in the dark for 1 h. Analysis of the cell cycle phase distribution was conducted on a FACScan™ system (BD Biosciences, Franklin Lakes, NJ, USA) using ModFit LT 3.2 software (Verity Software House, Topsham, ME, USA).

Apoptosis analysis by Annexin V staining. To identify apoptotic cells, the Annexin V-APC/7-AAD Apoptosis Detection Kit (catalogue no. KGA1026; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was used . TT cells were seeded in 6-well plates at a density of $3x10^5$ cells/well following 4 days of lentivirus infection. Upon 48 h of culture, cells were harvested and stained according to the manufacturer's protocol. The cells were analyzed on a FACSCalibur™ (BD Biosciences) using CellQuest Pro 5.1 software (BD Biosciences). The percentage of cells in each quadrant was calculated.

Statistical analysis. The results were expressed as the mean ± standard deviation. Differences between two groups were assessed using a two-tailed *t*-test. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed with SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Lv-sh MPHOSPH8 strongly suppressed MPHOSPH8 expression in TT cells. To explore the role of MPHOSPH8 in human MTC, lentivirus-mediated shRNA was used to silence the expression of MPHOSPH8 in TT cells. GFP was as used as a reporter gene. Lv-sh MPHOSPH8 (S1 and S2) was successfully infected into TT cells, since >61.2 and 69.4% cells, respectively, were GFP-positive under fluorescence microscopy at 96 h post-infection (Fig. 1A). RT-qPCR revealed that the lentiviruses containing S1 and S2 led to notable suppression of MPHOSPH8 expression (P<0.01), compared with the Lv-sh control group (Fig. 1B). In addition, Lv-sh MPHOSPH8 (S1 and S2) was efficiently transduced into TT cells and strongly reduced the expression of MPHOSPH8 protein, compared with the Lv-sh control group (Fig. 1C). Furthermore, the efficacy of S2 in knocking down MPHOSPH8 protein expression was higher than that of S1. These results indicated that Lv-sh MPHOSPH8 exerted successful knockdown effects on MPHOSPH8 expression in TT cells.

Lv-sh MPHOSPH8 suppressed the viability and proliferation of TT cells. To assess the inhibitory effect of silencing MPHOSPH8 on cell proliferation, a continuous 5-day MTT assay was performed. Both S1 and S2 lentiviruses exhibited a remarkable inhibition of proliferation in TT cells, compared with Lv-sh control (Fig. 2). Compared with cells infected with Lv-sh control, TT cell proliferation was markedly reduced from day 2 to day 5 (P<0.001). These data indicated that MPHOSPH8 depletion significantly decreased the proliferation of TT cells.

Lv-sh MPHOSPH8 affected the cell cycle progression in TT cells. To explore the underlying mechanism of inhibition of cell growth, cell cycle progression was evaluated by flow cytometry. As indicated in Fig. 3, the percentage of cells in different phases of the cell cycle (G0/G1, S and G2/M phases) was significantly different in the three groups (P<0.01 and P<0.001). Thus, Lv-sh MPHOSPH8 (S2)-infected TT cells exhibited a significant increase in the fraction of cells in the G0/G1 phase, and a reduction in the fraction of cells in the G2/M and S phases, compared with the Lv-sh control group. In addition, TT cells infected with Lv-sh MPHOSPH8 (S2) displayed a remarkable increase in the number of cells in the sub-G1 phase, suggesting that knockdown of MPHOSPH8 could induce cell apoptosis.

Lv-sh MPHOSPH8 promoted cell apoptosis in TT cells. Whether knockdown of MPHOSPH8 could enhance apoptosis in TT cells was next examined. As represented in Fig. 4, the apoptosis rate was significantly higher in Lv-sh MPHOSPH8 (S2)-transfected TT cells than in Lv-sh control-transfected TT cells (P<0.001). Following transfection, the apoptosis rate (early and late apoptotic cells) of TT cells was 20.45% for the Lv-sh MPHOSPH8 group, which was significantly higher than that of the Lv-sh control group (8.58%) (P<0.001). Therefore, the ratio of apoptotic TT cells was markedly increased following knockdown of MPHOSPH8, compared with that in the Lv-sh control group.

Discussion

TC is one of the most common malignancies in the world, and the mortality of MTC is the second highest of all thyroid tumors. Surgical resection results in a recurrence rate of almost 50% (6). Therefore, the identification of novel therapeutic targets and the development of novel therapeutic regimens able to more effectively regulate the cellular function of the target genes compared with traditional treatments is important.

Recently, MPHOSPH8 was identified in various human carcinoma cells, whereby it displayed an elevated expression (18). However, MPHOSPH8 as a potential target in human MTC has not been reported to date. RNA interference-mediated gene silencing is currently being tested in clinical trials as a potential therapy for a number of diseases (26). Thus, in order to investigate the role of MPHOSPH8 in MTC, TT cells were employed and infected with MPHOSPH8 lentivirus and control lentivirus to knockdown MPHOSPH8 expression in the present study. The selected shRNA-containing vector efficiently suppressed MPHOSPH8 expression at
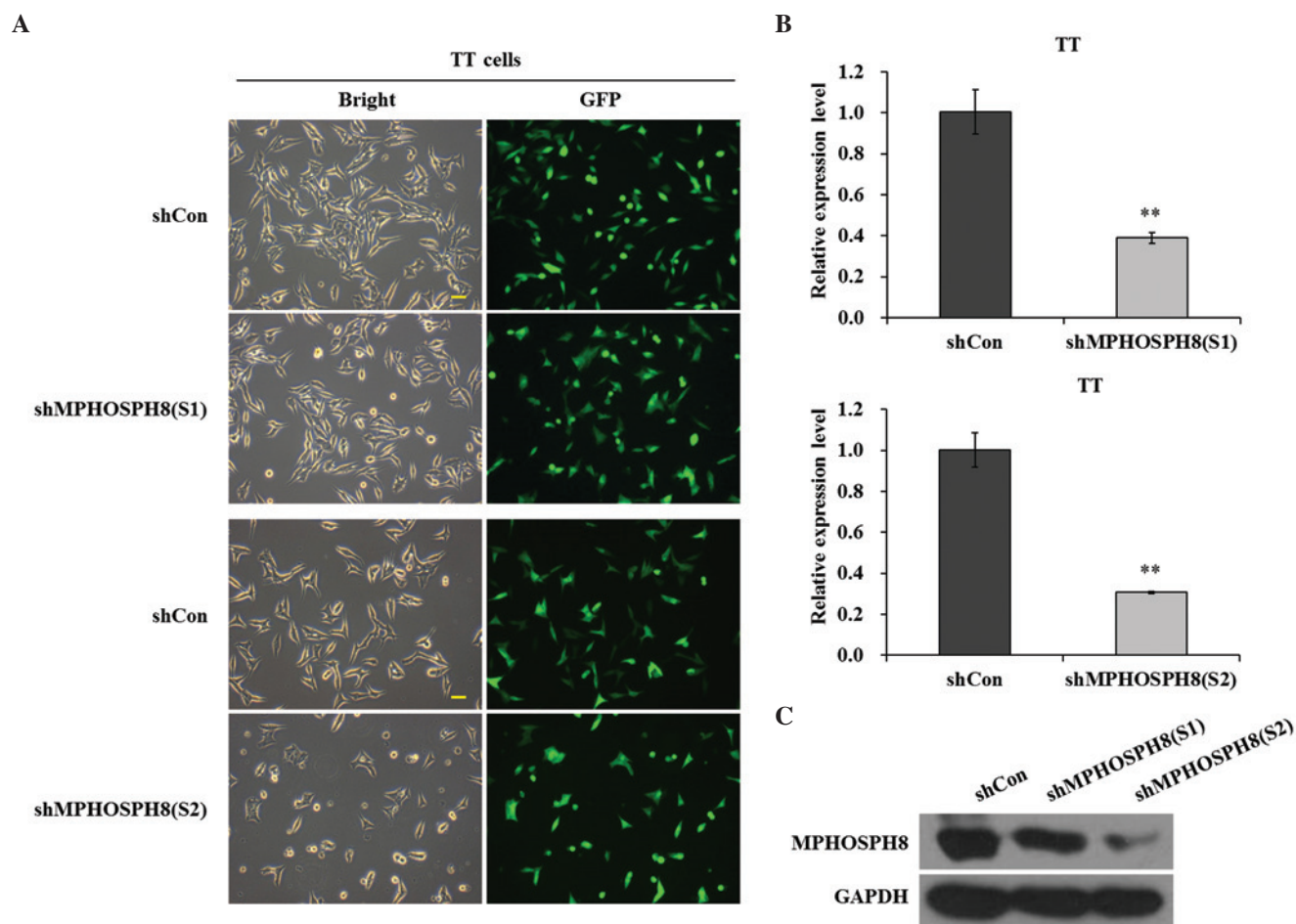
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Figure 1. Inhibition of MPHOSPH8 expression in TT cells by Lv-sh MPHOSPH8. (A) Representative micrographs of TT cells under a x100 magnification microscope and under an inverted fluorescent microscope at 96 h following infection with Lv-sh MPHOSPH8 (S1 and S2) . (B) Reverse transcription-quantitative polymerase chain reaction analysis revealed that in the Lv-sh MPHOSPH8 S1 and S2 groups, the messenger RNA levels of MPHOSPH8 decreased significantly, compared with those in Lv-sh-control TT cells. Data are represented as the mean ± standard deviation. (C) Western blotting demonstrated that the protein levels of MPHOSPH8 in Lv-sh MPHOSPH8 (S1 and S2) TT cells were significantly decreased, compared with those in Lv-sh control TT cells. **P<0.01 vs. control. Con, control; sh, small hairpin; MPHOSPH8, M-phase phosphoprotein 8; Lv, lentivirus; GAPDH, 3-phosphate dehydrogenase; GFP, green fluorescent protein.
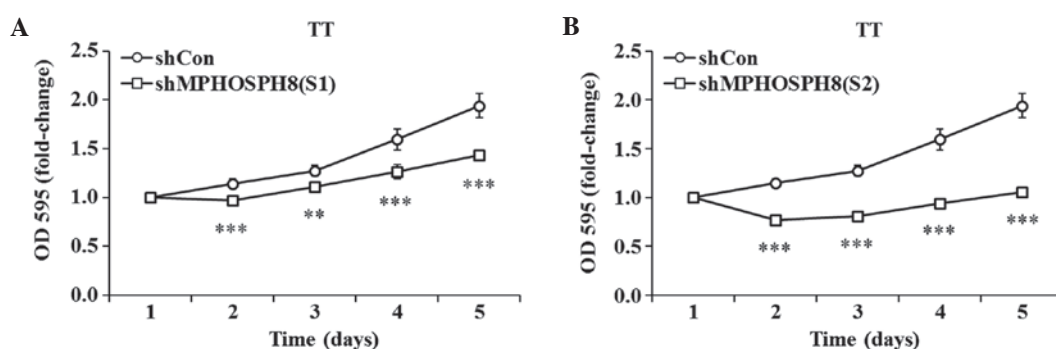
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Figure 2. Lv-sh MPHOSPH8 inhibited the viability and proliferation of TT cells, as indicated by the growth curve of TT cells infected with Lv-sh MPHOSPH8 (A) S1 and (B) S2, which was lower than that of TT cells infected with Lv-sh control. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01, ***P<0.001 vs. control. OD, optical density; Con, control; sh, small hairpin; MPHOSPH8, M-phase phosphoprotein 8; Lv, lentivirus.

both messenger RNA and protein levels. Next, the effect of MPHOSPH8 knockdown on the cellular functions of TT cells was explored. The results of MTT assay revealed that TT cells exhibited a reduced proliferation ability following infection with MPHOSPH8-targeted shRNA. In addition to cell growth and differentiation, the effect of MPHOSPH8 knockdown

on the cell cycle was also studied. The results indicated that suppressed MPHOSPH8 expression in TT cells led to G1 phase cell cycle arrest and decreased percentage of cells in S and G2/M phases, while flow cytometry analysis revealed an increase in apoptosis in Lv-sh MPHOSPH8 (S2)-treated cells. These results strongly suggest that MPHOSPH8 may play a
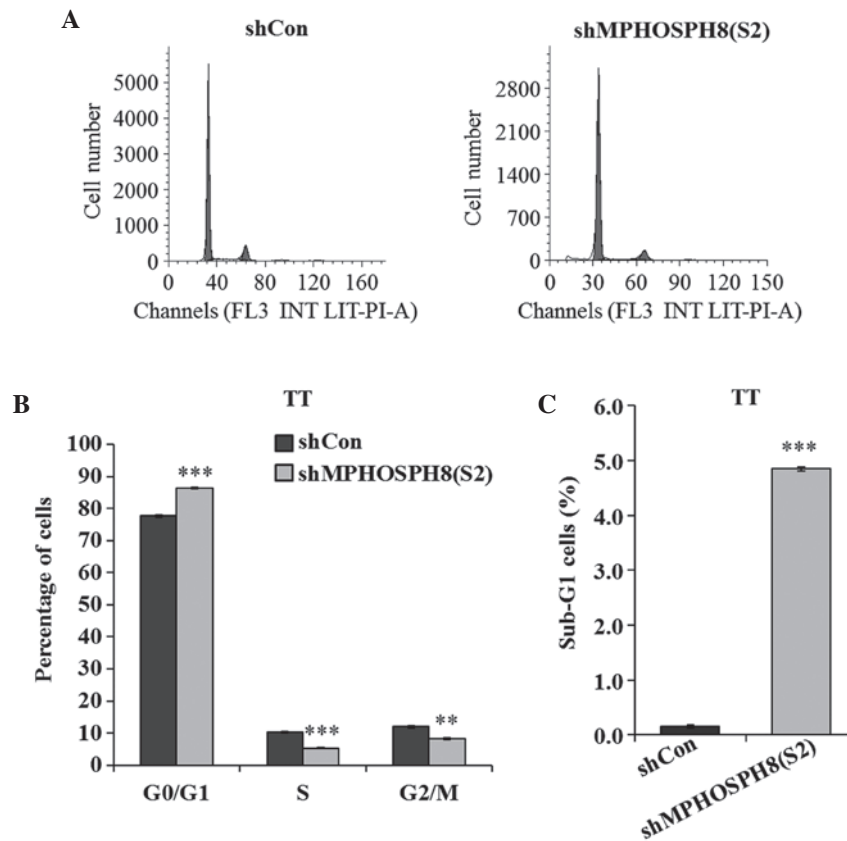
Figure 3. Lv-sh MPHOSPH8 inhibited the cell cycle progression of TT cells. (A) Representative data of fluorescence-activated cell sorting analysis of the cell cycle distribution in TT cells. A hypodiploid peak representing the apoptotic cells was observed prior to the G1 peak in TT cells infected with Lv-sh MPHOSPH8 (S2). (B) Inhibition of MPHOSPH8 expression in TT cells led to an increase in the number of cells at the G0/G1 phase and a concomitant decrease in the number of cells at the G2/M phase. (C) Inhibition of MPHOSPH8 expression in TT cells led to an increase in the number of cells at the sub-G1 phase. Data represents the mean ± standard deviation of three independent experiments. **P<0.01, ***P<0.001 vs. control. FL3 INT LIT-PI-A indicates the fluorescence signal intensity from different channels. Con, control; sh, small hairpin; MPHOSPH8, M-phase phosphoprotein 8; Lv, lentivirus; PI, propidium iodide.
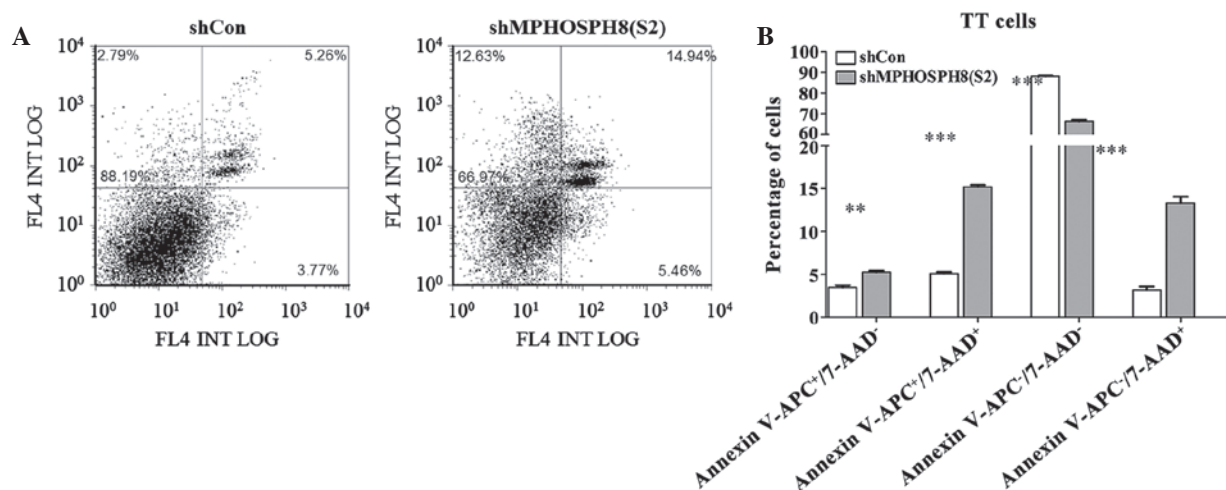

Figure 4. Detection of apoptosis using FACS. TT cells were assayed for apoptosis using Annexin V-APC/7-AAD staining in combination with FACS. (A) Scatter plots representing the FACS determination of the number of viable (APC$^-$/7-AAD$^-$), necrotic (APC$^-$/7-AAD$^+$), early apoptotic (APC$^+$/7-AAD$^-$) and late apoptotic (APC$^+$/7-AAD$^+$) TT cells following transfection with Lv-sh MPHOSPH8 (S2) for 4 days. (B) Proportion of viable, necrotic, early apoptotic and late apoptotic cells. Values are presented as the mean ± standard deviation. Representative results of three independent experiments are shown. **P<0.01; ***P<0.001 vs. control. FL4 INT LOG indicates the fluorescence signal intensity from different channels. Con, control; sh, small hairpin; MPHOSPH8, M-phase phosphoprotein 8; Lv, lentivirus; APC, allophycocyanin; 7-AAD, 7-amino actinomycin D; FACS, fluorescence-activated cell sorting.

central role in MTC. Further understanding of the molecular roles of MPHOSPH8 in human MTC may aid to clarify its pathophysiology and to develop novel therapeutic strategies.

In conclusion, the present study demonstrated that Lv-sh MPHOSPH8 successfully knocked down MPHOSPH8 expression in TT cells, which exerted an anti-proliferative effect,

caused cell cycle arrest in the G0/G1 phase and induced cell apoptosis. Although further studies are required, the present results suggest that MPHOSPH8 knockdown may constitute a potential therapeutic approach for the treatment of MTC, and may aid to improve the understanding of MTC progression.

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