Endocan silencing induces programmed cell death in hepatocarcinoma

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Abstract. Hepatocarcinoma is a type of high-grade malignant carcinoma identified worldwide. Its rapid development and late diagnosis prevents effective tumor resection in the majority of patients, and therefore recent studies have targeted metabolic signaling pathways and the tumor microenvironment for potential treatments. To investigate whether endocan may be a gene target for hepatocarcinoma treatment, the present study employed the following measures: MTT and Transwell assays, flow cytometry, western blotting and an mRFP-GFP-LC3 double fluorescence system. Following endocan gene silencing, cell proliferation was significantly inhibited and the number of invasive cells in the endocan siRNA-treated group was reduced compared with the control-siRNA treated-group. Furthermore, the apoptosis rate was 15% and autophagy was detected in the endocan short interfering (si)RNA-treated group compared with the control-siRNA treated-group. Using western blotting to detect NF-κB expression in the nucleus, the NF-κB expression was identified to be significantly reduced in the siRNA-treated group compared with the control groups. Endocan gene silencing inhibited hepatocarcinoma cell viability and invasion, whilst inducing apoptosis and autophagy. The results of the present study suggest that the effect of endocan gene silencing on cell survival was mediated via the NF-κB signaling pathway.

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Introduction

Hepatocarcinoma is a tumor with a high degree of malignancy. Hepatocarcinoma is the second most prevalent cause of cancer-associated mortality in males and the sixth leading cause in female patients (1). Typically, treatment for hepatocarcinoma is not administered in a timely manner due to late diagnosis. The majority of patients are diagnosed when effective surgery is not possible and in other patients, metastases are identified during surgery. Therefore patient prognosis is typically poor (2). Increasing attention has focused on identifying efficient therapeutic targets for hepatocarcinoma treatment.

Endocan expression has been reported in numerous types of tumor and therefore may represent a target for carcinoma treatment (3-8). In particular, endocan is overexpressed in tumor cells where it promotes tumor growth (9). In addition, the circulating levels of endocan increase over time and have been positively correlated with tumor size (9). Endocan was identified to be significantly overexpressed in endothelial cells isolated from hepatocellular carcinoma tissue compared with corresponding non-cancerous liver tissue (10). Endocan is also expressed at the periphery of tumor cells in hepatocellular carcinoma tissue (11). Furthermore, the mRNA expression of endocan in tissue samples has been associated with the tumor node metastasis stage, tumor vascular invasion and metastasis in patients with hepatocarcinoma (11). Further studies have demonstrated that the endocan expression in tumors is associated with their angiogenic and invasive properties (12,13). In vascular endothelial cells, certain factors promote the expression of endocan, including cultured hepatocyte growth factors, scatter factors, fibroblast growth factor-2 and vascular endothelial growth factor (14).

In the present study, endocan silencing experiments were performed using small interfering (si)RNA. In particular, cell proliferation and invasion were examined following endocan siRNA treatment in SK-HEP-1 cells, and cell survival was evaluated using flow cytometry and western blot analysis.

Materials and methods

Cell culture. Human SK-HEP-1 hepatocarcinoma cells were obtained from the American Type Culture Collection

(Manassas, VA, USA). Cells were cultured in minimal essential medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). All cells were incubated at 37˚C in an atmosphere containing 5% CO_2. The inoculum size was $3x10^3$ cells/well in a 96-multiwell plate, $2x10^4$ cells/well in a 24-multiwell plate or $3x10^5$ cells/well in a 6-multiwell plate (BD Biosciences, Franklin Lakes, NJ, USA). The medium was replaced every 2 days. The dose of pyrrolidine dithiocarbamate (PDTC) was 10 μM and the duration of treatment was 24 h at 37˚C. The controls used dimethyl sulfoxide (DMSO).

siRNA knockdown of endocan gene expression of human SK-HEP-1 hepatocarcinoma cells. In 6-well plates, 8 μl Lipofectamine 2000® (Thermo Fisher Scientific, Inc.). +2 μg pRNA-H1.1+2 $x10^6$ cells (70-80% confluence) were placed in every well. Endocan-targeting and control siRNAs (5'-TTC TCCGAACGTGTCACGT-3') were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). pRNA-H1.1 plasmids were constructed and used as the siRNA vector according to the protocol of the manufacturer (Jinsirui Science and Technology Biology Corp., Nanjing, China), and it was transfected into cells using Lipofectamine 2000®. The plasmid connection system contains 0.03 pmol pRNA-H1.1, 20 nmol Insert DNA, 2.5 μl 10xT4 DNA Ligase Buffer, 1 μl T4 DNA Ligase and ddH_2O up to 25 μl. The target sequence of endocan was 5'-GGTCTCCCGTAATGAGGAA-3'. A total of 2 μg siRNA vector was used to transfect into SK-HEP-1 cells with Lipofectamine 2000 kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Western blot analysis. Western blotting was performed 48 h following transfection. SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was conducted using 10 and 14% gels, according to the manufacturer's protocol. Protein extraction was performed with 2 ml radioimmunoprecipitation lysate (Beyotime Institute of Biotechnology, Haimen, China) on ice for 5 min. Centrifugation was performed at 24,000 x g and 4˚C for 10 min. The protein quantification method used was BCA, 200 μl of BCA at 37˚C for 20 min. A total of 40 μg protein was loaded per lane. Proteins were transferred onto polyvinylidene difluoride membranes. The membrane was blocked in a solution of TBS-Tween 20 containing 5% non-fat dry milk for 1 h at 25˚C with constant agitation. Proteins were probed with a number of primary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The primary antibodies were as follows: Endocan antibody (sc-515304; Santa Cruz Biotechnology, Inc.; 1:200) at 4˚C overnight; LC3 antibody (AL221; Beyotime Institute of Biotechnology; 1:500) at 4˚C overnight; ATG5 antibody (PA2260; Boster Biological Technology, Pleasanton, CA, USA; 1:400,) at 4˚C overnight; ATG7 antibody (PB9479; Boster Biological Technology; 1:400) at 4˚C overnight; DRAM antibody (bs-4233R, BIOSS, Beijing, China; 1:500) at 4˚C overnight; Beclin-1 antibody (AB123; Beyotime Institute of Biotechnology; 1:1,000) at 4˚C overnight and NF-κB p65 antibody (AF0246; Beyotime Institute of Biotechnology; 1:1,000) at 4˚C overnight. The membranes were incubated with secondary horseradish peroxidase-conjugated anti-Immunoglobulin G antibody (A0181; Beyotime Institute of Biotechnology; 1:5,000) at 37˚C for 45 min. β-actin was used as a loading control. Immunolabeled proteins were detected following incubation with ECL substrate (Beyotime Institute of Biotechnology) at room temperature for 50 sec, followed by exposure of the membrane to autoradiographic film. Density analysis was performed using a Gel Doc system (Gel Doc XR+; Bio-Rad Laboratories, Inc.). The nucleus protein and cell plasma protein extraction kit (P0028, contain reagent A and reagent B) was purchased from Beyotime Institute of Biotechnology and used to separate the cytoplasm and nuclear fractions. Reagent A digested the cell membrane and Reagent B digested the cell nucleus.

Cell viability measured using an MTT assay. Following varying durations following transfection (24, 48, 72 or 96 h), the culture medium was replaced with a medium containing 5 mg/ml MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and the cells were incubated for 24 h at 37˚C. The supernatant was discarded and DMSO was used to dissolve the purple crystals. The absorbance at a wavelength of 490 nm was measured using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Cell invasion assays. After 48-h of endocan siRNA treatment in Dulbecco's modified Eagle medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS, the SK-HEP-1 cells were harvested (24,000 x g for 5 min at room temperature) using trypsin. Then the cells were washed in DMEM with a soybean trypsin inhibitor and without serum. The cells were suspended in serum-free DMEM at a density of $1x10^5$ cells/ml. Matrigel (BD Biosciences) was put into Transwell. The cells ($2x10^4$/well) were allowed to migrate towards DMEM containing 20% FBS in the bottom chamber of the Transwell. The 24-well plates were incubated for 24 h at 37˚C. The invaded cells on the bottom surface of the membrane were stained with crystal violet at room temperature for 5 min. The number of migrated cells was counted in 5 randomly selected fields using a light microscope at a magnification of x200. Data presented are representative of the average of 3 individual wells.

Apoptosis detection using flow cytometry. The SK-HEP-1 cells were cultured for 48 h following treatment with endocan siRNA in DMEM containing 10% FBS. The cells were harvested (24,000 x g for 5 min at room temperature) by trypsinization and washed in DMEM with a soybean trypsin inhibitor and without serum. The cells were suspended in 500 μl of binding buffer (556547; BD Biosciences), and 5 μl Annexin V-FITC was added, together with 5 μl propidium iodide. The cells ware incubated for 15 min. The flow cytometer and software (WinMDI v2.8) used was FACSCalibur (BD Biosciences).

mRFP-GFP-LC3 fluorescence system assays. Following the appropriate cell culture period (density 80%), adenovirus infection was performed according to the manufacturer's protocol (mRFP-GFP-LC3; Hanbio, Shanghai, China; adenovirus number: cell number, 15:1). The plates were incubated for 4 h at 37˚C with 5% CO_2 in a humidified atmosphere. Stationary infected cells, or those cells that were able to stably present fluorescent signals, were incubated in 4% paraformaldehyde

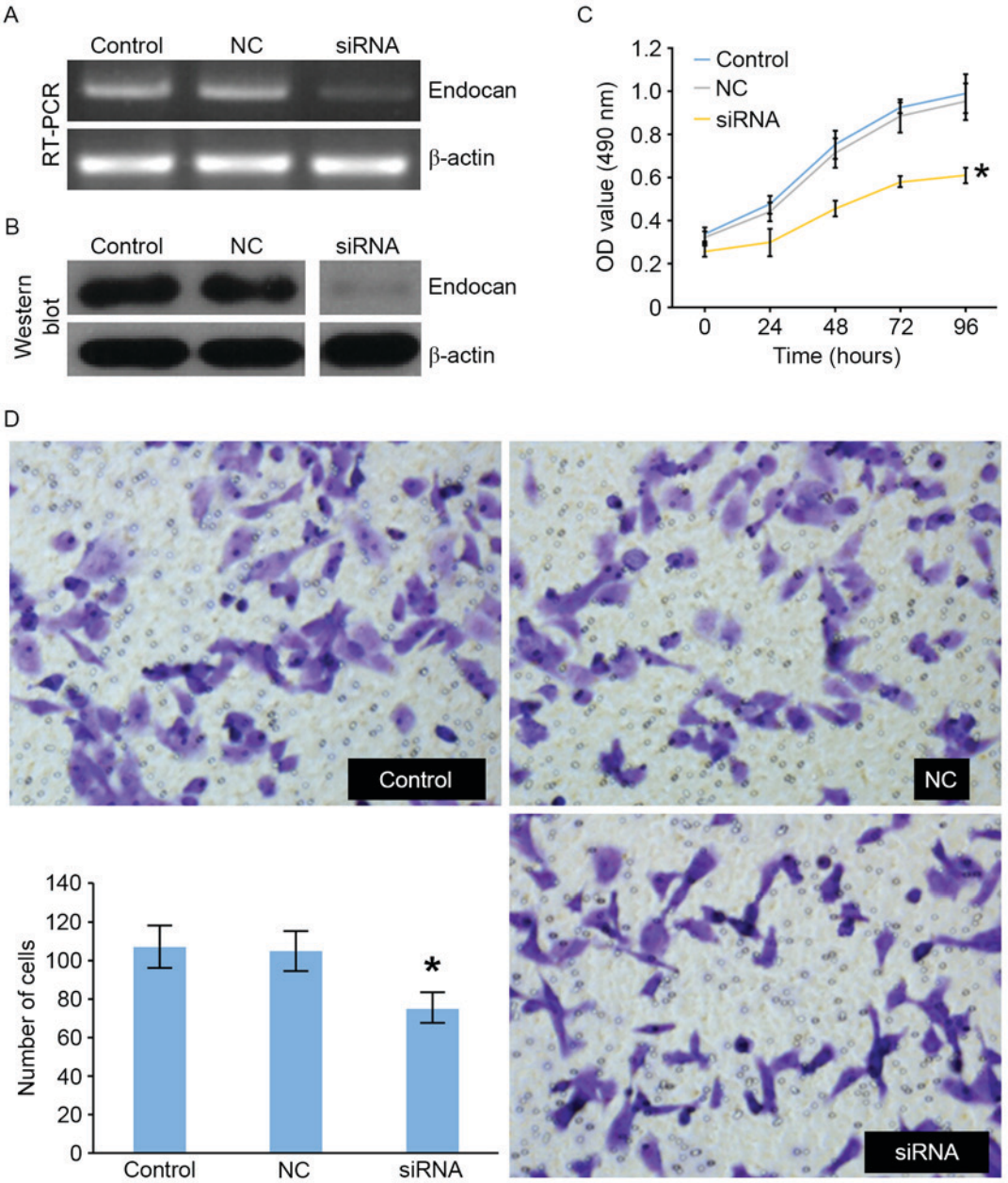

Figure 1. Endocan siRNA and NC siRNA constructs were transfected in SK-HEP-1 cells. siRNA against endocan suppressed its expression, evaluated using (A) RT-PCR and (B) western blotting (normalized to β-actin). (C) SK-HEP-1 cells cultured in 96-well plates were transfected with endocan siRNA, and cells were analyzed 0, 24, 48, 72 and 96 h following transfection. The cell proliferation was determined with an MTT assay. Data were presented as the mean ± standard deviation (n=3). The average OD value from each sample was obtained from 5 replicates. (D) Transwell assays were used to investigate the migratory and invasive abilities of the control, NC and siRNA groups. Invading cells were stained with crystal violet and counted in ≥5 fields using a light microscope (magnification, x200). *P<0.05 in siRNA groups vs. NC groups. OD, optical density; NC, negative control; siRNA, small interfering RNA; RT-PCR, reverse transcription polymerase chain reaction.

for 0.5 h at 4˚C. The level of fluorescence in the cells was observed using a fluorescence microscope. Cells in every 5 fields of view were counted.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA extraction buffer was purchased from BioTek Instruments, Inc. (RP5611). TIANScript RT kit (KR104) was supplied by Tiangen Biotech Co., Ltd. (Beijing, China). The primer sequences were as follows: Endocan forward, 5'-CTG GGAAACATGAAGAGCG-3' and reverse, 5'-GCCTGAGAC TGTGCGGTAG-3' and β-actin forward, 5'-CTTAGTTGC GTTACACCCTTTCTTG-3' and reverse, 5'-CTGTCACCT TCACCGTTCCAGTTT-3'. The fluorophore used Gold View (Beijing Solarbio Science and Technology, Co., Ltd, Beijing, China). The RT reaction was performed with 1 μl oligo (dT)15, 1 μl random primer, 2 μl dNTP (2.5 mM each), 10.5 μl ddH_2O (2), and the thermocycler conditions were as follows: 70˚C for 5 min, cooling on ice for 2 min, and then 4 μl 5X First-Strand Buffer, 0.5 μl RNasin, 1 μl (200 U) TIANScript M-MLV were placed in each tube at 42˚C for 50 min, then at 95˚C for 5 min. The thermocycler conditions were: 95˚C for 5 min, 95˚C for 20 sec, 52˚C for 20 sec, 72˚C for 30 sec for 40 cycles. The experiment was repeated 3 times. The method of quantification was gel electrophoresis, using 1.5% sepharose

gel. The gel mixture was heated in the microwave oven until boiling. When cooled to 50-60˚C, Gold View (Beyotime Institute of Biotechnology, Shanghai, China) dye (0.01%) was blended. The DNA ladder was purchased from Beyotime Institute of Biotechnology.

NF-κB inhibitor treatment. Cell cultures were performed as aforementioned. The four groups prepared as follows: the NC + DMSO group, siRNA + DMSO group, NC + PDTC group, siRNA + PDTC group. DMSO and 10 μM NF-κB pathway inhibitor ammonium PDTC (Beyotime Institute of Biotechnology) were added to the culture for 24 h at room temperature.

Statistical analysis. Data were assessed using the Student's t-test or one-way analysis of variance, as appropriate, followed by the Turkey's post-hoc test. All statistics were calculated using SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference. Data are presented as the mean ± standard deviation. The number of replications was three.

Results

Gene silencing of endocan decreases cell proliferation and cell invasion. In the present study, SK-HEP-1 cells treated with endocan siRNA were monitored for cell proliferation and invasion using an MTT and a Transwell assay, respectively. The following 3 groups were established: Untreated control; negative control (NC) siRNA; and endocan siRNA groups. To determine siRNA efficiency, the endocan mRNA transcript and protein level were evaluated using RT-PCR and western blotting, respectively (Fig. 1A and B). The level of endocan expression was decreased in the endocan siRNA compared with the NC and control groups (Fig. 1A and B). Cell proliferation was evaluated using the MTT assay and was significantly reduced in siRNA-treated cells compared with the two control groups (Fig. 1C). In the Transwell assay, the invasive ability of endocan siRNA-treated cells was significantly reduced compared with the NC and control groups (Fig. 1D).

Gene silencing of endocan enhances apoptosis in SK-HEP-1 cells. The apoptosis assay demonstrated that endocan silencing resulted in a marked increase in the apoptosis rate (Fig. 2). Overall, these results suggest that endocan silencing promotes cell apoptosis.

Gene silencing of endocan enhances autophagy in SK-HEP-1 cells. To determine whether the gene silencing of endocan is able to induce autophagy, autophagy-associated protein expression levels were evaluated using western blotting (Fig. 3A). The expression levels of microtubule associated protein 1 light chain 3 α (LC3), autophagy related (ATG)5, ATG7, DNA damage regulated autophagy modulator 1 (DRAM) and Beclin-1 protein following transfection with endocan siRNA were significantly increased compared with those of the NC, and control groups (Fig. 3B). These results suggest that endocan silencing promotes autophagy in hepatocarcinoma cells. In Fig. 4A, no increased level red fluorescence was observed compared with in Fig. 4B or C. In Fig. 4B, an increased level red fluorescence was observed compared with in Fig. 4A or C, this revealed that there was an increased level of autophagy in Fig. 4B. In Fig. 4C, no increased level red fluorescence was observed compared with in Fig. 4A or B.

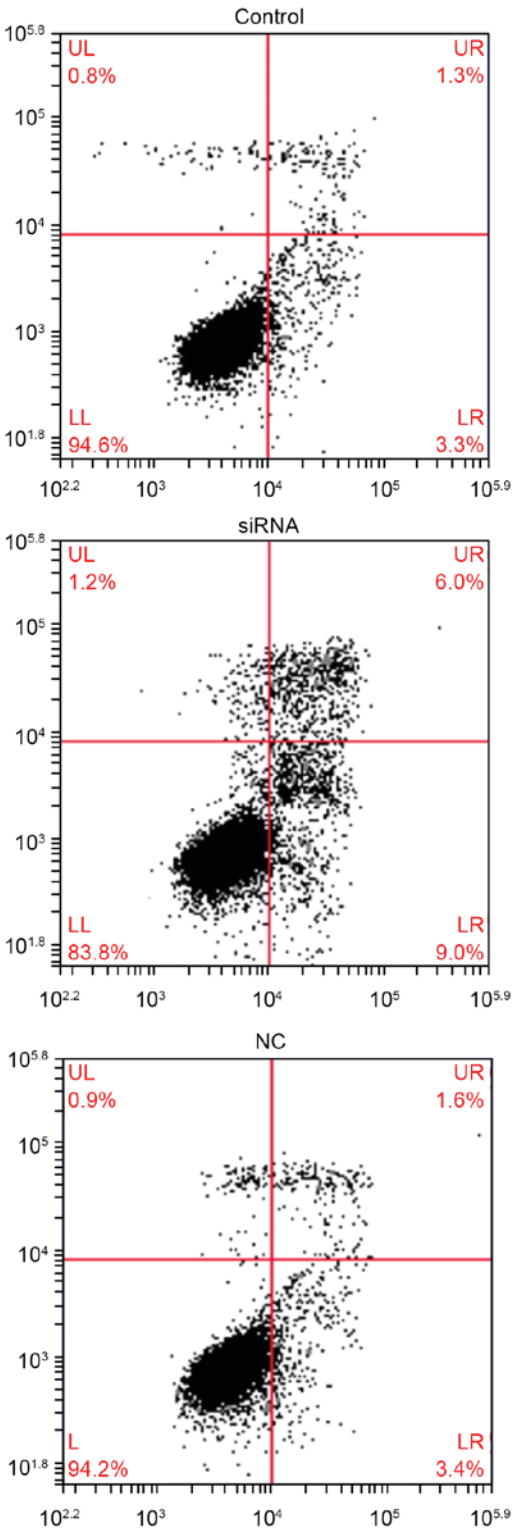

Figure 2. Endocan silencing increases the apoptosis rate compared with NC siRNA treated and control cells. UR+LR quadrants represent apoptotic cells and were included in the apoptosis rate. The horizontal axes represent positive Annexin V- fluorescein isothiocyanate. The vertical axis represents positive propidium iodide. UL, upper left; UR, upper right; LL, lower left; LR, lower right; NC, negative control; siRNA, small interfering RNA.

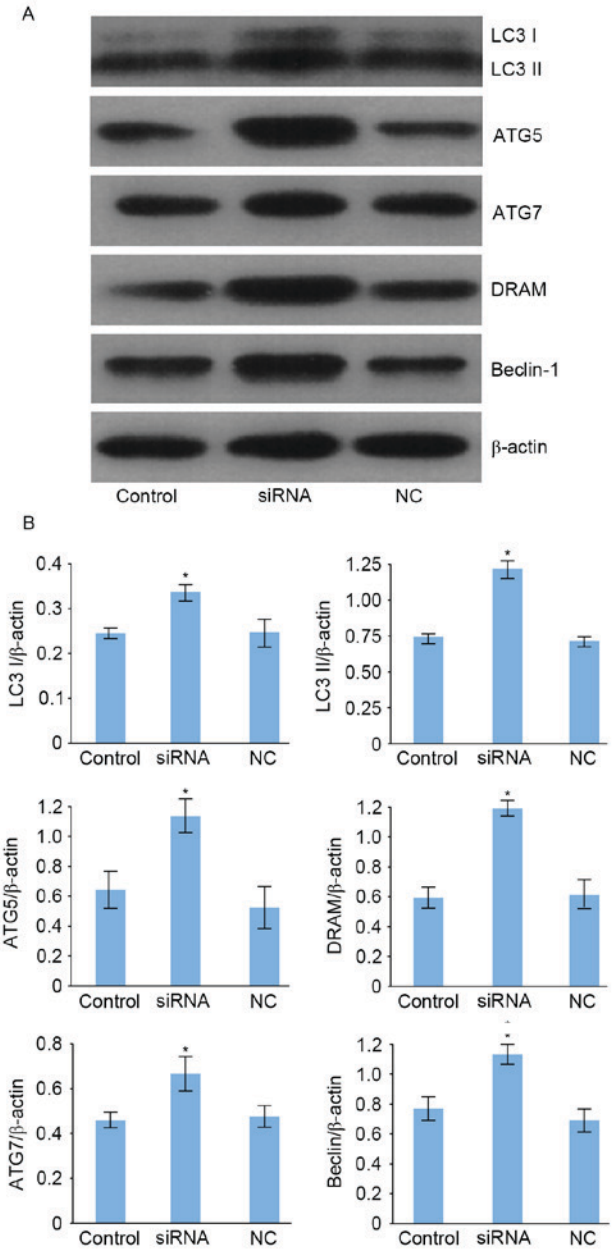

Figure 3. The levels of LC3, ATG5, ATG7, DRAM and Beclin-1 protein expression were measured following transfection with endocan siRNA and normalized to β-actin. (A) Representative blot and (B) quantification of autophagy-associated protein expression. The expression levels of all the autophagy-associated proteins were significantly increased in endocan siRNA-treated cells compared with those in the NC and control groups. *P<0.05 in siRNA groups vs. NC groups. NC, negative control; siRNA, small interfering RNA; LC3, microtubule associated protein 1 light chain 3 α; ATG, autophagy related; DRAM, DNA damage regulated autophagy modulator 1.

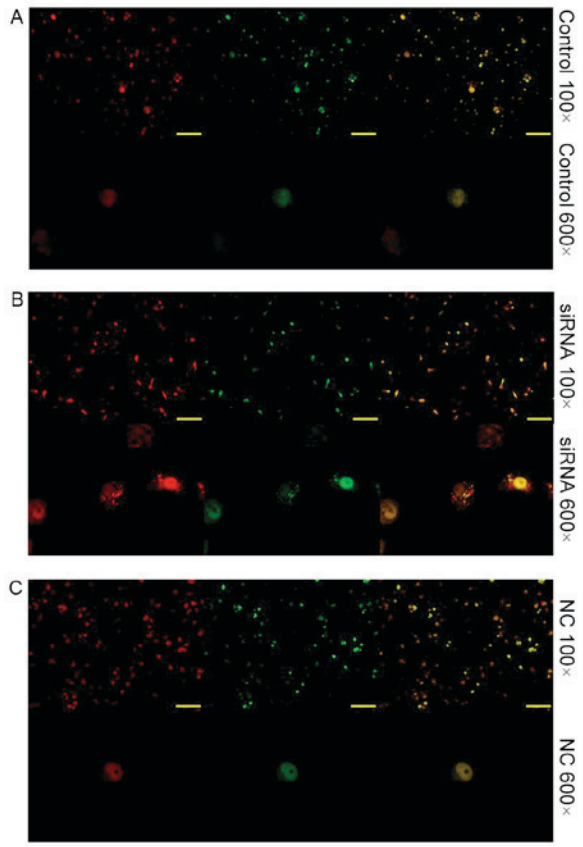

Figure 4. Autophagy was detected using the mRFP-GFP-LC3 system. Red fluorescence, lysosomes; green fluorescence, autophagosome. mRFP-GFP-LC3 was transfected into cells of the (A) control, (B) endocan-targeting siRNA and (C) NC siRNA groups, and after 48 h expression was analyzed using fluorescence microscopy (magnification, x100 or x600). The mRFP-GFP-LC3 microtubule associated protein 1 light chain 3 α foci were monitored. Yellow fluorescence is the result of merging the red and green fluorescence images. Green fluorescent foci are observed to reduce alongside the formation of the autophagic lysosome. NC, negative control; siRNA, small interfering RNA. Scale bar=10 μm.

Association between endocan gene expression and the nuclear factor (NF)-κB signaling pathway. To determine whether the gene silencing of endocan is able to influence the NF-κB signaling pathway, NF-κB p65 protein expression was evaluated using western blotting (Fig. 5A). The level of NF-κB p65 protein within the nucleus following transfection with endocan siRNA was significantly reduced compared with that of the cells in the NC and control groups (Fig. 5A and B). Furthermore, the level of endocan protein was significantly reduced following treatment with NF-κB pathway inhibitor ammonium PDTC (Fig. 5C and D) compared with DMSO. These results demonstrated the synergistic effects of endocan gene expression on the NF-κB signaling pathway. The expression level of the endocan protein were as follows: NC+DMSO group>siRNA+DMSO group; siRNA+DMSO group> siRNA+PDTC group; NC+PDTC group> siRNA+PDTC group.

Discussion

Effective tumor treatment remains a challenge for clinicians and researchers. There are currently 2 types of non-surgical therapy for hepatocarcinoma; One therapy targets the tumor and the other targets the tumor microenvironment. Previous studies have demonstrated that endocan may represent a target for tumor-targeted therapy (3-8). Although there is insufficient data regarding whether endocan may be an effective tumor marker similar to α-fetoprotein, a number of previous studies have reported that endocan is associated with tumor cell survival (9,10,12,14). Indeed, endocan gene silencing was utilized to inhibit tumor cell survival in human colon carcinoma (14), concordant with the results of the present study. Using MTT and Transwell assays, the results of the current

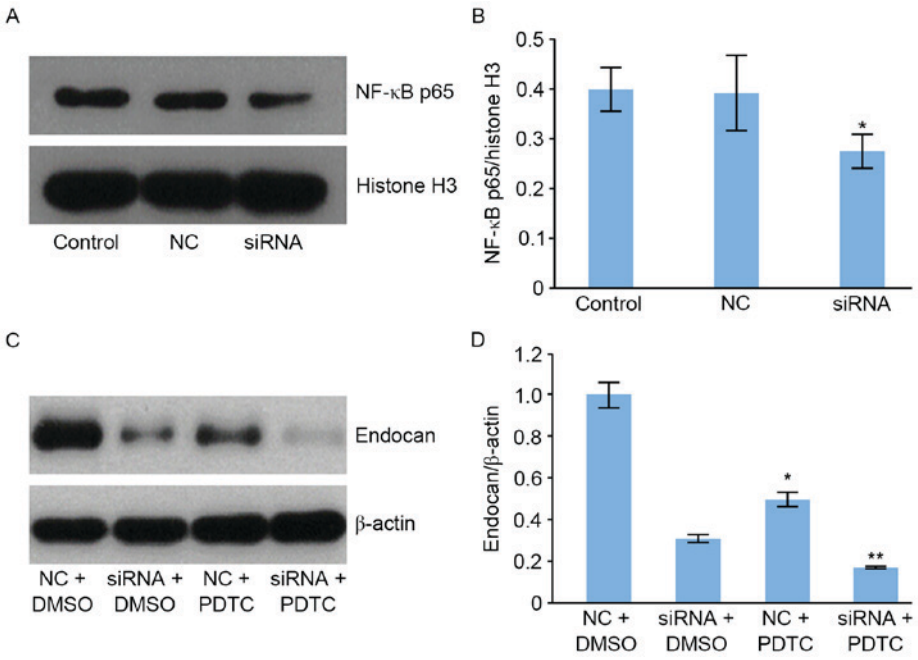

Figure 5. The expression of NF-κB p65 protein within the nucleus was measured using western blotting following transfection with endocan siRNA. (A) Representative blot and (B) quantification of NF-κB p65 expression. The expression was significantly reduced in endocan siRNA-treated cells compared with cells in the NC and control groups. *P<0.05 on siRNA groups vs. NC groups. (C) The cells were incubated with 10 μM PDTC and an equal volume of DMSO, and then the level of endocan protein was determined. (D) Quantification of endocan protein expression. *P<0.05, NC+PDTC vs. NC+DMSO; **P<0.05, siRNA+PDTC vs. NC+DMSO or siRNA+DMSO. NC, negative control; siRNA, small interfering RNA; PDTC, ammonium pyrrolidine dithiocarbamate; NF-κB, nuclear factor-κB.

study further demonstrate that endocan silencing inhibits SK-HEP-1 cell proliferation and invasion, which indicates that endocan may serve as a treatment target in hepatocarcinoma.

The induction of programmed cell death (PCD) is the preferred result for tumor treatment. Specifically, tumor-cell PCD may be targeted without injuring healthy cells. Apoptosis represents type I PCD and involves a series of morphological and biochemical processes, including alterations in the mitochondrial membrane potential, opening of mitochondrial pores, phosphoserine shifts and the gathering of nuclear chromatin (15). An Annexin V-FITC kit was used with flow cytometry analysis to determine alterations in apoptotic rates, and the results identified that apoptosis was induced in siRNA-treated SK-HEP-1 cells. The low apoptotic cell ratio may be due to the short observation time; therefore, if the observation time had been extended, the ratio may have increased. Autophagy serves an important role in cell survival and death, and a certain degree of autophagy occurs during normal cellular metabolism. The purpose of autophagy is to digest metabolic waste or remove misfolded proteins. Furthermore, autophagy leads to cell death, specifically type II PCD (16). Through autophagy-associated protein determination, evidence of autophagy was observed in endocan siRNA-treated cells. The autophagy-associated proteins investigated include LC3 (17,18), ATG5 (19), ATG7, Beclin-1 (20) and DRAM (21). The significant increase in the expression of these proteins in siRNA-treated cells compared with NC and control cells indicate the increased level of autophagy in the endocan siRNA-treated cells. Furthermore, red and green fluorescent dyes were used for the detection of LC3, as observed through the lack of green fluorescence due to the formation of autophagic lysosomes, it is suggested that endocan silencing induces autophagy.

NF-κB serves an important role in cell survival and this transcription factor may be phosphorylated to regulate gene expression in the nucleus (22,23). NF-κB is also an important regulator of gene promoters. In the process of cell apoptosis, NF-κB may regulate the function of apoptotic proteins (24,25), anti-apoptotic proteins (26-28) and tumor-suppressor proteins (29-31). To investigate the underlying mechanism of endocan gene silencing in hepatocarcinoma cell apoptosis and autophagy, the NF-κB signaling pathway was used as an indicator. In the current study, it was demonstrated that following endocan gene silencing, NF-κB expression decreased significantly in the cell nucleus compared with NC and control cells that were not treated with endocan siRNA, which may have contributed to the impaired survival of hepatocarcinoma cells. In addition, the influence of the NF-κB signaling pathway on endocan expression was demonstrated as following inhibition of the NF-κB signaling pathway through treatment of cells with PDTC, endocan expression was significantly decreased compared with untreated cells.

In conclusion, the results of the present study demonstrated that endocan silencing induced PCD in SK-HEP-1 cells. Thus, endocan may be a treatment target for hepatocarcinoma. Furthermore, these results identified an association between endocan gene expression and the NF-κB signaling pathway, suggesting that combined treatments may improve the efficiency of inhibiting hepatocarcinoma progression.

Acknowledgements

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