# Lanosterol synthase loss of function decreases the malignant phenotypes of HepG2 cells by deactivating the Src/MAPK signaling pathway

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**Abstract.** Cholesterol is critical for tumor cells to maintain their membrane components, cell morphology and activity functions. The inhibition of the cholesterol pathway may be an efficient strategy with which to limit tumor growth and the metastatic process. In the present study, lanosterol synthase (LSS) was knocked down by transfecting LSS short hairpin RNA into HepG2 cells, and cell growth, apoptosis and migratory potential were then detected by Cell Counting Kit-8 cell proliferation assay, flow cytometric analysis and wound healing assay, respectively. In addition, proteins associated with the regulation of the aforementioned cell biological behaviors were analyzed by western blot analysis. The activity of the Src/MAPK signaling pathway was measured by western blotting to elucidate the possible signal transduction mechanisms. LSS knockdown in the HepG2 liver cancer cell line inhibited cell proliferation, with cell cycle arrest at the S phase; it also decreased cell migratory ability and increased apoptosis. The expression proteins involved in the regulation of cell cycle, cell apoptosis and migration was altered by LSS knockdown in HepG2 cells. Furthermore, a decreased Src/MAPK activity was observed in the HepG2 cells subjected to LSS knockdown. LSS loss of function decreased the malignant phenotypes of HepG2 cells by deactivating the Src/MAPK signaling pathway and regulating expression of genes involved in cell cycle regulation, cell apoptosis and migration.

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## Introduction

In cancer cells, aberrant lipid metabolism, particularly cholesterol, disrupts normal cell signaling and motility, as cholesterol plays key roles in these processes (1). The inhibition of the cholesterol pathway may thus be an efficient strategy which can be used to limit tumor growth and the metastatic process (2,3). The *do novo* synthesis of cholesterol is a complex multi-step process catalyzed by enzymes (4). The antitumor efficacy may differ when targeting different nodes within the cholesterol metabolic pathway due to the nature of feedback responses elicited, and whether synthesis and transport processes are simultaneously inhibited (5).

Some researchers have reported that an increased flux through lanosterol, rather than merely the upregulation of the cholesterol biosynthesis pathway, leads to more malignant phenotypes of cancer cells (6). In the process of the cholesterol anabolic pathway, lanosterol synthase (LSS) catalyzes the formation of the first cyclized product, lanosterol, and also the shunt pathway to 24(S),25-epoxycholesterol. Increased 24(S),25-epoxycholesterolby the partial inhibition of LSS reduces the effects of compensatory mechanisms to maintain cholesterol by tumor cells due to a higher affinity for diepoxysqualene than epoxysqualene (7). The inhibition of LSS as a therapeutic target is potentially advantageous and represents an attractive target in this regard. RO 48-8071, a specific inhibitor of LSS, has been shown to inhibit the proliferation of various tumor types (8,9).

The present study aimed to investigate the effects of the knockdown of LSS on liver cancer progression and the possible molecular mechanisms in HepG2 cells.

## Materials and methods

*Cells and cell culture.* The human liver cancer cell line, HepG2, was stored cultured in a humidified incubator at 37°C with 5% $CO_2$ in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% heat-inactivated FBS (cat. no FB15011; Clark Bioscience) supplemented with penicillin and streptomycin.

*Construction of LSS short hairpin (sh)RNA.* In total, three shRNAs were designed targeting human LSS gene sequences GGACTGCGCTCAACTATGT. The sequences were digested with restriction endonuclease *Bam*HI at the 5' end and *Hin*dIII at the 3' end and inserted into the pRNAT-U6.1/Neo plasmid. The recombinant plasmids were then transformed into *E. coli* DH5α and screened using medium containing ampicillin. The positive clones were selected with 50 $\mu$g/ml ampicillin and expanded to obtain the recombinant DNA for sequencing. The primers for each target sequence are presented in Table I.

*LSS knockdown by shRNA in HepG2 cells.* The confirmed shLSS- pRNAT-U6.1/Neo and pRNAT-U6.1/Neo vector plasmids were transfected into HepG2 cells using Lipofectamine 2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) following the protocol provided by the manufacturer. A total of 500 ng plasmid DNA diluted in 250 $\mu$l Opti-MEM® medium was mixed together with 5 $\mu$l Lipofectamine® in 50 $\mu$l Opti-MEM® and incubated for 5 min at room temperature. The DNA-lipid complex was added to HepG2 cells at ~70% confluency, cultured in 24-well plates. Following a 24-h incubation at 37°C, the cells were selected with G418 at 500 $\mu$l/ml. Viable cells transfected with the plasmids exhibit green fluorescence under a fluorescence microscope. Thus, the transfection efficiency was detected using a fluorescence microscope. The stably transfected HepG2 cell clones were harvested and LSS expression was detected using western blot analysis to verify the efficiency of RNA interference by shRNAs targeting different human LSS gene sequences.

*Cell Counting Kit-8 (CCK-8) cell proliferation assay.* Cell proliferation was detected using CCK-8 assay according to the manufacturer's instructions (cat. no. K1018; APeXBIO Technology LLC). The stably transfected HepG2 cells, at a density of 2,000, 3,000 or 6,000 cells in 100 $\mu$l 10% FBS/DMEM, were seeded in 96-well plates and cultured for 48 h. This was followed by the addition of 10 $\mu$l CCK-8 to each well and incubation for 4 h at 37°C. The absorbance at 450 nm was measured using a microplate reader (ELx800; BioTek Instruments, Inc.) following gentle mixing on a shaker.

*Cell cycle analysis.* Cells were seeded in six-well plates and cultured routinely to ~80% confluency. The cells were then trypsinized, washed and fixed in ice-cold 70% ethanol at 4°C for 30 min followed by treatment with 50 $\mu$g/ml propidium iodide (PI, Beijing Biosea Biotechnology Co., Ltd.) in staining buffer for 30 min at 4°C away from light. Cell cycle distribution was then analyzed on a BD FACSV flow cytometer (BD Biosciences). The data obtained from flow cytometric analyses were examined using ModFit software (v3.1; Verity Software House, Inc.).

*Wound healing assay.* Cells were seeded in 24-well plates and cultured to 100% confluency. Scratches were then made in the middle of the confluent monolayer cells using sterile pipette tips. The debris was removed by washing with PBS before obtaining images using a light microscope (Nikon TS2-FL; Nikon Corporation). The cells were incubated with fresh DMEM with 2% FBS for 48 h and images were captured (10). The wound area was measured using Quantity One software (v4.6.6; Bio-Rad Laboratories, Inc.) and the wound area changes at 48 h were calculated by measuring the area of the wound at 0 h minus the wound area at 48 h.

*Western blot analysis.* The cells were harvested, lysed in RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) on ice and centrifuged at 4°C to yield total cellular protein. The protein concentration was determined using BCA assay; Beyotime Institute of Biotechnology). Subsequently, ~30 $\mu$g lysate were loaded on each lane of 12% SDS-PAGE and transferred onto a PVDF membrane. Following a 2-h blocking in 5% non-fat milk in PBST (0,05% Tween-20 in PBS) at room temperature, the membrane was incubated with primary antibody (LSS, cat. no. sc-514507, 1:500; cyclin B1, cat. no. sc-245, 1:500, both Santa Cruz; cyclin E, cat. no. sc-377100, 1:500, Santa Cruz, CA; GRP78, cat. no. ab21685, 1:500, Abcam, Cambridge, UK; CLPX 1, cat. no. PA5-79052, 1:500, Thermo Fisher Scientific, Inc., Waltham, MA; Bcl-2, cat. no. 9662S, 1:400, Cell Signaling Technology, Danvers, MA; NF-κB, cat. no. ab32536, 1:500, Abcam, Cambridge, UK; endocan, cat. no. sc-515304, 1:300, Santa Cruz, CA; pSrc, cat. no. 12432S, 1:500, Cell Signaling Technology, Danvers, MA; Src, cat. no. 2109S, 1:500, Cell Signaling Technology, Danvers, MA; pAKT, cat. no. sc-514032, 1:500, Santa Cruz, CA; AKT, cat. no. sc-5298, 1:500, Santa Cruz, CA; pERK, cat. no. sc-81492, 1:500, Santa Cruz, CA; ERK1/2, cat. no. sc-514302, 1:500, Santa Cruz, CA; β-actin, cat. no. sc-47778, 1:1,000, Santa Cruz, CA.) at 4°Covernight and HRP-conjugated secondary antibody (1:10,000, goat anti-rabbit IgG with cat. no. AP132P and goat anti-mouse IgG with cat. no. AP130, Sigma Aldrich, Merck KGaA) for 2 h at room temperature. The SuperSignal™ West Femto Trial kit (Thermo Fisher Scientific, Inc.) and Chemi Scope (Clinx Science Instrument Co., Ltd.) were used to visualize the signals and obtain images of the bands. The specific bands were quantified using Quantity One software (v4.6.6; Bio-Rad Laboratories, Inc.).

*Statistical analysis.* SPSS version 15.0 software (SPSS, Inc.) was used to analyze all the data in the present study. Significant differences between groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Knockdown of LSS in HepG2 cells is successful and effective.* The shRNA sequences targeting LSS were constructed into the pRNAT-U6.1/Neo plasmid and the recombinant plasmids were sequenced. The sequences of each recombinant plasmids containing LSS shRNA are presented in Table II. The confirmed plasmids were transfected into HepG2 cells and selected using G418 to obtain stable-transfection cell clones.

Table I. Primers used for shRNA construction.

| Primer name | Sequence (5'→3') |
| --- | --- |
| shLSS | Forward: GATCCCGGACTGCGCTCAACTATGTTTGATATCCGACATAGTTGAGCGCAGTCCTTTTTTCCAAA<br>Reverse: AGCTTTTGGAAAAAAGGACTGCGCTCAACTATGTCGGATATCAAACATAGTTGAGCGCAGTCCGG |

sh-, short hairpin; LSS, lanosterol synthase.

Table II. Sequences of recombinant plasmids containing LSS shRNA.

| Target sequences | DNA sequencing of cloned fragments (5'→3') |
| --- | --- |
| h-LSS-shRNA (GGACTGCGCTCAACTATGT) | AAAATTCTTGGGTAGTTTGCAGTTTTAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGGTTTATATATCTTGTGGAAAGGACGCGGG<u>ATCCC</u>**GGACTGCGCTCAACTATGTTTGATATCCGACATAGTTGAGCGCAGTCCTTTTTTC**<u>C</u>AAAAGCTTAAGTTTAAACCGCTGATCAGCCTCGACTGTGCCTTCTAAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGC |

The underlined section indicates the cloned target sequence, and the two sides are the sequences on the vector skeleton. The sequence shown in bold font is the target sequence and complementary sequence. sh-, short hairpin; LSS, lanosterol synthase.

Cells stably transfected with LSS shRNA exhibited an evident decrease in LSS expression. Together with labeled green fluorescent protein observed under a fluorescence microscope, it could be concluded that LSS knockdown cell line was successful and that LSS expression was effectively suppressed by shRNA targeting LSS (Fig. 1).

*LSS knockdown inhibits the proliferation and migration but induces the apoptosis of HepG2 cells.* Fundamentally, the disruption of cell growth and proliferation are basic characteristics of malignant tumors. CCK-8 cell proliferation assay revealed a cytostatic effect of LSS knockdown on HepG2 cells. The viability of the HepG2 cells was significantly reduced following LSS knockdown when compared with the vector control-transfected cells, with initial seeding densities at 2,000, 3,000 or 6,000 cells per well (Fig. 2C).

Cell cycle analysis using flow cytometry revealed that the proportion of cells in the G1 phase was significantly lower than that of the vector control, whereas the proportion of cells in the S phase was higher. The cell proportion in the G2/M phase was slightly decreased in the cells following LSS knockdown, although no significant difference was detected between groups (Fig. 2A and B). This indicated that cells subjected to LSS knockdown were blocked in the S phase. Although there was genomic DNA replication, the cells could not enter the division phase and thus, an inhibitory effect on cell proliferation was observed.

Moreover, flow cytometry was performed to analyze the extent of apoptosis. As shown in Fig. 3A and B, there were higher proportions of apoptotic cells in the cells subjected to LSS knockdown than in the cells transfected with the control vector, whether in the early stages or in metaphase. The migratory potential is another characteristic of cancer cells. Wound healing assay demonstrated a reduced migratory ability of HepG2 cells following stable transfection with LSS shRNA (Fig. 4A and B).

*Multiple proteins associated with proliferation, cell cycle regulation, apoptosis and migration are altered by LSS knockdown in HepG2 cells.* The aforementioned results demonstrated that HepG2 cells subjected to LSS knockdown exhibited a decreased proliferative activity and migratory potential, as well as an increased apoptotic rate with cell cycle arrest at S phase. These findings suggested that LSS may be closely related to liver cancer and may play a role in the malignant biological behavior of liver cancer cells such as a high proliferative and invasive ability. LSS loss of function exerted inhibitory effects on the development and progression of liver cancer. The expression of proteins associated with proliferation, apoptosis and migration was examined by western blot analysis to reveal the possible molecules involved in the inhibitory effects of LSS knockdown on HepG2 cells.

The analysis of cell cycle distribution revealed that the cells subjected to LSS knockdown were blocked in the S phase (Fig. 2A and B), which may suggest that although there was genomic DNA replication, the cells could not enter the division phase and thus, an inhibitory effect on proliferation was observed. As HepG2 cells subjected to LSS knockdown were arrested in the S phase, as detected using flow cytometry, the expression of cyclin B1 and cyclin E was then
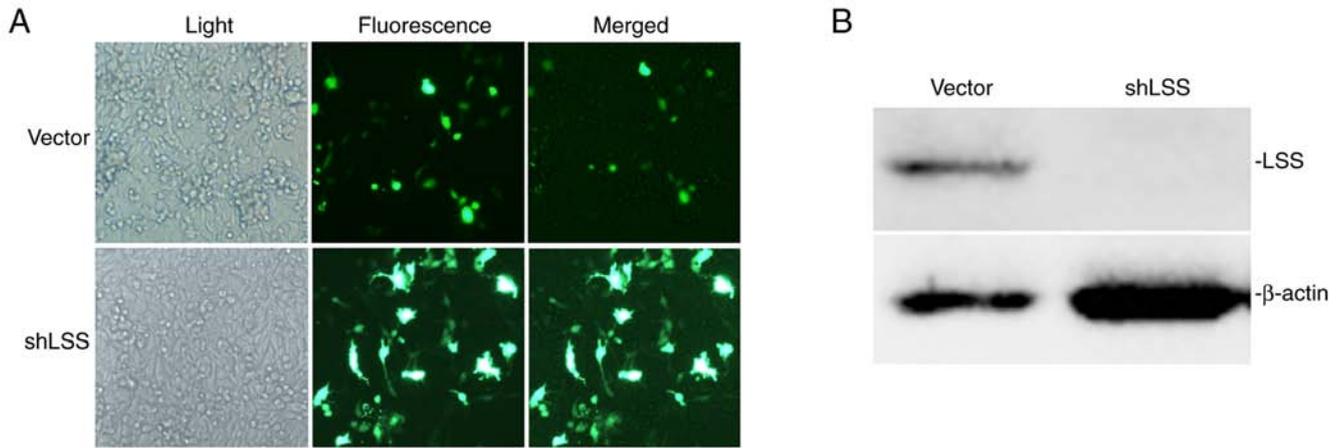
Figure 1. LSS shRNA transfection efficiency detected using GFP reporter and LSS knockdown efficiency detected using western blot analysis. (A) LSS shRNA transfection efficiency. Left, cell morphology examined under a bright light; middle panel, the corresponding fluorescent images of the left penal; right panel, the merged image corresponding to the left panel. 20x magnification. (B) The LSS expression level was successfully knocked down using shRNA. LSS, lanosterol synthase; shRNA, short hairpin RNA.
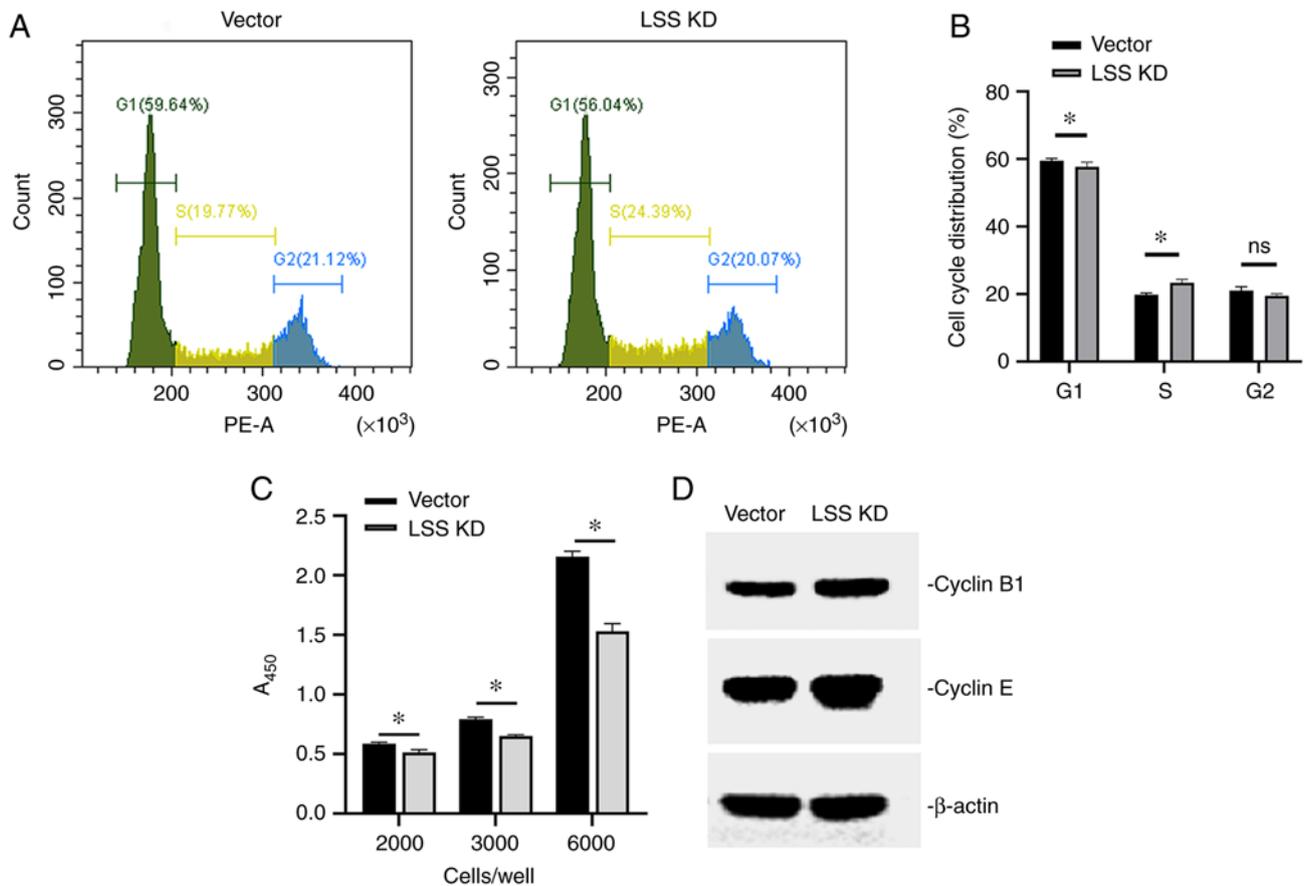


Figure 2. Effects of LSS knockdown on cell cycle, cell viability and cell cyclin expression. (A) Representative cell cycle distribution of transfected HepG2 cells using flow cytometric analyses, and (B) quantification of the results. *P<0.05, LSS knockdown vs. vector control. (C) Cell Counting Kit-8 assay was used to examine the effects of LSS knockdown on HepG2 cell viability, with initial seeding densities at 2,000, 3,000 or 6,000 cells per well. *P<0.05. (D) Western blot analysis of cyclin levels in transfected HepG2 cells. Vector, HepG2 cells with vector control; LSS KD, HepG2 cells subjected to LSS knockdown using shRNA; LSS, lanosterol synthase; shRNA, short hairpin RNA.

detected. Cyclin B1 is known to be highly expressed in cells in the S phase and cyclin E has been proven to regulate the G2/M phase transition. An increased expression of cyclin B1 and cyclin E was found in the HepG2 cells following LSS knockdown (Fig. 2D), suggesting that cyclin B1 and cyclin E may participate in the S phase cell cycle arrest following LSS knockdown.

The mitochondrial apoptotic pathway is one of the most critical pathways mediating cell apoptosis. In HepG2 cells subjected to LSS knockdown, a higher apoptotic ratio
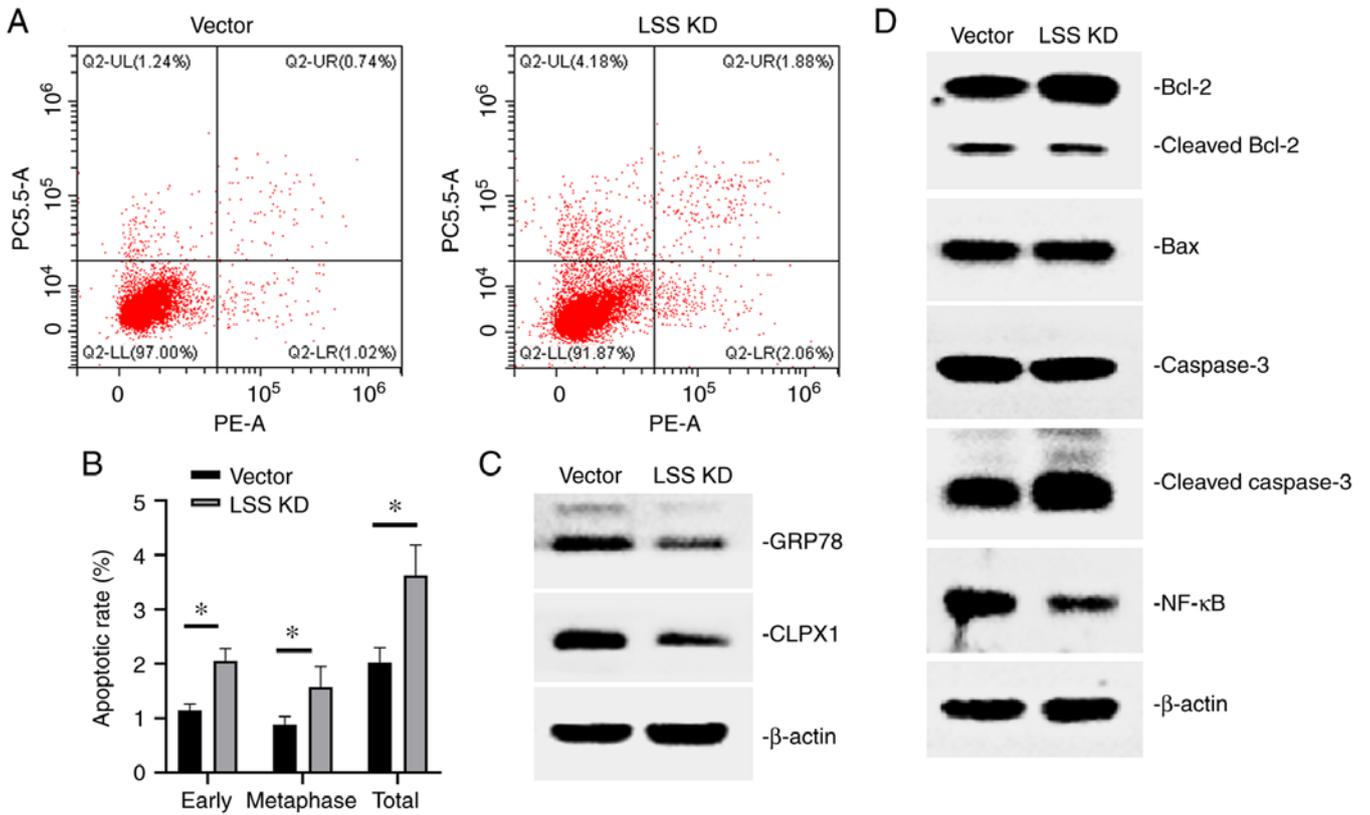
Figure 3. Effects of LSS knockdown on cell apoptosis and the expression of proteins related to apoptosis. (A) Representative results of apoptosis detection of transfected HepG2 cells using flow cytometry, and (B) quantification of the results. *P<0.05, LSS KD vs. vector control. (C) Western blot analysis of proteins involved in endoplasmic reticulum-mediated apoptosis. (D) Western blot analysis of proteins involved in the mitochondrial apoptotic pathway. Vector, HepG2 cells with vector control; LSS KD, HepG2 cells subjected to LSS knockdown using shRNA; LSS, lanosterol synthase; shRNA, short hairpin RNA.
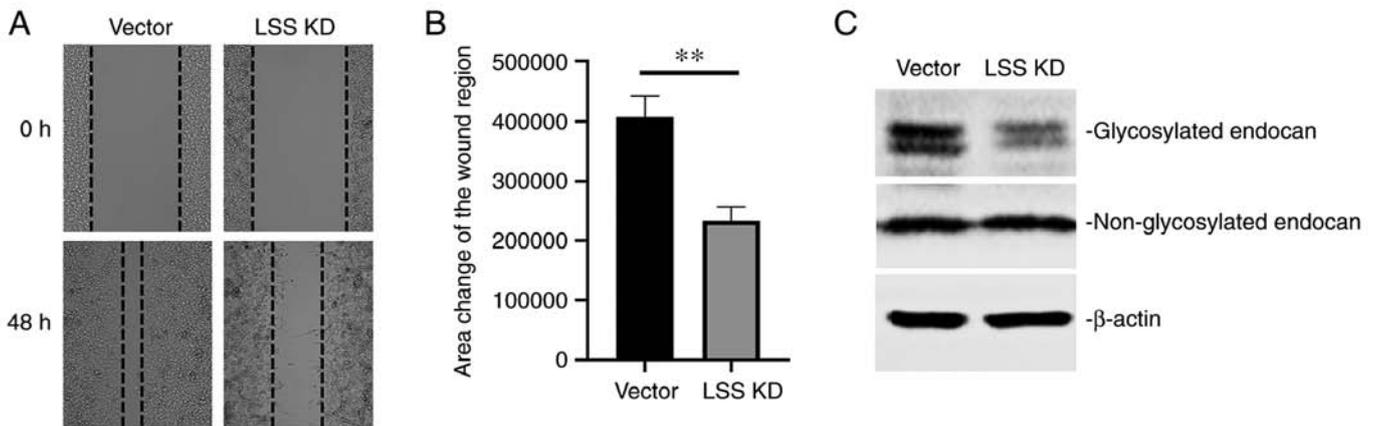


Figure 4. Effects of LSS knockdown on HepG2 cell migration and the expression of endocan. (A) Cell migration was detected using wound healing assay. (B) Quantification of the area and changes in the wound region. **P<0.01, LSS KD vs. vector control. (C) Western blot analysis of two alternative endocan forms. Vector, HepG2 cells with vector control; LSS KD, HepG2 cells subjected to LSS knockdown using shRNA; LSS, lanosterol synthase; shRNA, short hairpin RNA.

was observed. Molecular mechanistic analysis revealed an activated mitochondrial apoptotic pathway, as evidenced by an altered Bcl-2, Bax, caspase and NF-κB expression (Fig. 3D). The expression of GRP78 and CLPX1, endoplasmic reticulum and mitochondrial unfolded protein reaction-related proteins, was decreased in cells subjected to LSS knockdown, mediating cell apoptosis (Fig. 3C).

Furthermore, the endocan level was investigated. This has been proven to play differential roles in various malignancies. Western blot analysis revealed two specific bands corresponding to the glycosylated endocan at 50 kDa and non-glycosylated endocan at 20 kDa, respectively. In cells subjected to LSS knockdown, there was a decreased glycosylated endocan level when compared with the vector control-transfected cells, with no change of non-glycosylated

endocan (Fig. 4C). This suggested LSS loss of function inhibited HepG2 cell migration by decreasing the ratio of glycosylated and non-glycosylated endocan.

*Src/MAPK is one of the possible signaling pathway by which LSS KD plays its roles in HepG2 cells.* The aforementioned results demonstrated that several proteins are involved in the inhibitory effects on HepG2 liver cancer cells mediated by LSS knockdown. The exploration of the related mechanisms of signal transduction in HepG2 cells (Fig. 5) demonstrated that pSrc was downregulated by LSS knockdown, while no difference was observed in the level of total Src compared with the vector control-transfected cells. No marked differences were detected in p-AKT and p-ERK levels between the cells transfected with the vector control or those subjected to LSS knockdown. However, there was a decreased ERK level in the HepG2 cells with LSS knockdown when compared with the vector control-transfected cells. These results suggest a possible signaling mechanism, which involves the deactivation of the Src/MAPK pathway by LSS knockdown in HepG2 cells.

## Discussion

As a rapidly growing tissue, the tumor requires large quantities of cholesterol for its membrane components, and for the maintenance of cell morphology and functions. The derivation of cholesterol in tumor cells may thus be an efficient strategy with which to limit tumor growth and the metastatic process. Enzymes involved in cholesterol synthesis efflux have been found to be increasingly required for cancer cell proliferation, in addition to their role in maintaining the homeostasis of lipid metabolism (11).

Although studies have suggested that inhibiting the cholesterol pathway can be used as a target for tumor therapy, the antitumor efficacy may differ when targeting different nodes within the cholesterol metabolic pathway due to the nature of feedback responses elicited, and whether synthesis and transport processes are simultaneously inhibited. Some researchers have noted that not the upregulation of the cholesterol biosynthesis pathway, but rather an increased flux through lanosterol translates into a more malignant phenotype of cancer cells (6); this suggests that lanosterol may be a pro-survival factor for cancer cells. Thus, the present study focused on biosynthetic intermediate lanosterol for hepatic carcinoma progression.

A high LSS activity or expression has been reported to be positively associated with tumor metastasis and to be predictors of a poor prognosis of patients with cancer (12). As a therapeutic target, the inhibition of LSS is potentially advantageous as function of LSS is not limited to cholesterol synthesis, but also catalyzes an alternate flux through the shunt pathway to 24(S),25-epoxycholesterol. When the conversion of epoxysqualene to lanosterol is inhibited, part of the accumulated epoxysqualene can be converted into diepoxysqualene and 24(S),25-epoxycholesterolis ultimately catalyzed by LSS (13). Oxysterols act as ligands of certain receptors such as LXRα and LXRβ, or regulate several cellular signaling pathways acting on cellular receptors to regulate the transcription of target genes which are involved in the modulation of TGF-β1, Hedgehog, Wnt or MAPK signaling pathways to regulate cell proliferation and apoptosis (14). Increased 24, 25-
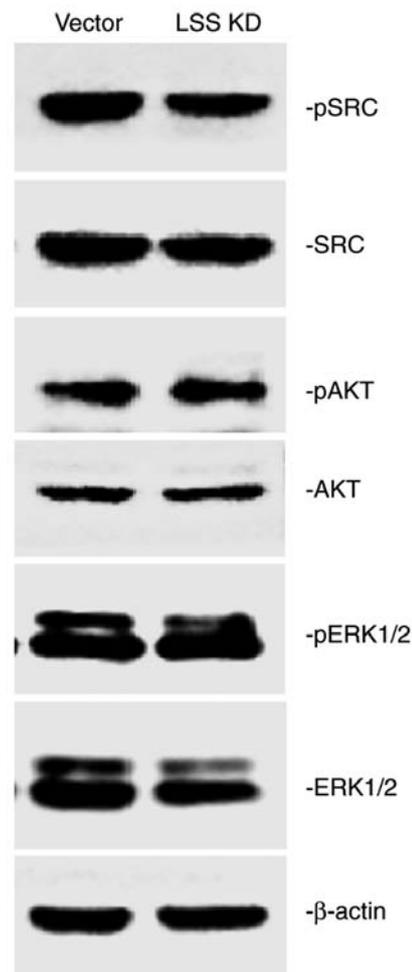


Figure 5. Western blot analysis of the changes in the activity of possible signaling pathways mediated by LSS knockdown in HepG2 cells. Vector, HepG2 cells with vector control; LSS KD, HepG2 cells subjected to LSS knockdown using shRNA; LSS, lanosterol synthase.

epoxycholesterol, due to the combined effect of LSS partial inhibition, and due to a higher affinity for diepoxysqualene than epoxysqualene, activates cholesterol export mediated by LXR, while concurrently reducing post-oxidosqualene cholesterol synthesis (7,15). This 'double hit' cholesterol depletion mechanism may further reduce the effect of compensatory mechanisms to maintain cholesterol by tumor cells.

It has been reported that the specific inhibitor of LSS, RO 48-8071, decreased cell growth in various tumor types including pancreatic ductal adenocarcinoma, glioblastoma, breast cancer and colon carcinoma, and these inhibitors may thus be potential anticancer drugs (8,16-19). The present study investigated the functional importance of LSS in regulating the proliferation, apoptosis and migration of human liver cancer cells *in vitro*, although with limitations that the effects of RO48-8071 on HepG2 cells have not been tested yet and the lack of *in vivo* studies. Inhibition of LSS activity by shRNA led to a decreased proliferation and led to cell cycle arrest. Cell cycle dysregulation may be due to the altered expression of cyclins and may thus result in tumorigenesis and cancer development. In the present study, it was found that cyclin B1 and cyclin E expression levels were upregulated in HepG2 cells following LSS knockdown. It is known that cyclin E promotes

the transition from the G1 phase to the S phase, and that cyclin B1 undergoes dynamic changes throughout the cell cycle; a marked increase in the levels of these markers is observed when the cells enter the S phase (20-22). In the present study, following LSS loss of function in HepG2 cells, the expression of cyclin B1 and cyclin E increased, regulating the cell cycle in S phase, and leading to S phase arrest and the inhibition of cell proliferation.

Moreover, the knockdown of LSS expression markedly altered the cell apoptotic ratio. Mitochondria are dependent on accumulation of cholesterol in mitochondrial membranes and also mitochondrial cholesterol metabolism into oxysterols. The function of cholesterol in mitochondria is not limited to synthesis of steroid hormones in steroidogenic tissues or bile acids in the liver. In mitochondria, cholesterol is metabolized into oxysterols which regulate multiple pathways such as ERK, Hedgehog, Wnt and TGF-β1 signaling pathways to regulate cell proliferation and apoptosis, not limited on synthesis of steroid hormones in steroidogenic tissues or bile acids in the liver. Thus, mitochondria are dependent on cholesterol components although lower cholesterol level in mitochondrial membrane compared with other bilayers (23). The activated mitochondrial apoptotic pathway, which is characterized by an altered Bcl-2, Bax, caspase and NF-κB expression, was observed in HepG2 cells following LSS knockdown; this may be responsible for the higher apoptotic ratio in the cells compared with the vector control-transfected cells. Bcl-2 is known as an apoptosis-antagonizing protein (24). The present study found that in HepG2 cells subjected to LSS knockdown, Bcl-2 expression was markedly higher than that in the control cells; thus, more apoptotic cells were detected in the HepG2 cells subjected to LSS knockdown. Further analysis revealed a smaller molecule binding to Bcl-2 antibody, which is shown as cleaved Bcl-2. It has been reported that Bcl-2/Bax and caspase 3 are interrelated and are mutually restricted in the process of apoptosis transmission (25). Bcl-2 and Bax can not only act as the upstream regulatory mechanism of caspase-3 and participate in the regulation of caspase-3 activity but can also act as the direct substrate of caspase-3, downstream of caspase-3, to form a positive apoptotic feedback pathway. Bcl-2 protein can be cleaved by caspase-3 into fragments with Bax; this pro-apoptotic activity accelerates the process of apoptosis (26). The results of the present study are inconsistent with this mechanism, which involves a higher level of total Bcl-2, and a lower level of cleaved Bcl-2, with no change in the Bax level. However, a higher level of cleaved caspase-3 and lower intracytoplasmic NF-κB were both detected in cells in which LSS was knocked down. NF-κB, known as a transcription factor, is translocated into the nucleus to activate multiple target genes involved in cell survival and apoptosis (27). This suggested that the mitochondrial apoptotic pathway participated in the promotion of apoptosis following LSS knockdown; however, the inconsistent changes in the level of Bcl-2/Bax warrant further investigation. Moreover, lower levels of GRP78 (28) and CLPX1 (29) were found in the cells subjected to LSS knockdown when compared with the vector control-transfected cells; this suggested that the endoplasmic reticulum and mitochondrial stress response were also involved in the apoptotic effects induced by LSS knockdown, and not merely the classic mitochondrial apoptotic pathway.

Endocan is a type of protein which has been demonstrated to play opposite roles in different types of tumors (30-32). The roles of promoting or inhibiting tumor development by endocan may be dependent on the ratio between glycosylated and non-glycosylated endocan; glycosylated endocan, but not non-glycosylated form, plays roles as a stimulus in tumor progression, by promoting the activity of HGF/SF, VEGF and EGFR and inducing tumor growth and angiogenesis (33-36). A decreased glycosylated endocan level, but not non-glycosylated endocan, in HepG2 cells subjected to LSS knockdown revealed that the glycosylation of endocan was also regulated by LSS loss of function to inhibit HepG2 cell migration and malignant behaviors.

As a consequence of LSS knockdown in the HepG2 liver cancer cell line, the ability of migration and the rate of cell proliferation were decreased, while the percentage of apoptotic cells was significantly increased. This phenomenon may be related to LSS loss of function, at least in part.

Moreover, multiple cellular signaling pathways are involved in the suppressive effects of inhibitors of the cholesterol synthesis pathway, as well as in the occurrence and progression of liver cancer, such as Src, AKT and MAPKs (37,38). The present study also examined the effects of LSS knockdown by shRNA on tumor progression. A decreased level of p-Src level and a lower level of total ERK were observed following LSS knockdown; however, this was not observed for pERK. In addition, no change was observed in the levels of p-AKT or AKT in the cells subjected to LSS knockdown. This suggested that the activities of Src and the ERK signaling pathways were inhibited by LSS knockdown to regulate the expression of numerous genes, thus reducing the malignant potential of HepG2 cells.

In conclusion, the findings of the present study suggest that LSS loss of function decreases the expression of genes associated with HepG2 proliferation, apoptosis and migration via the deactivation of the Src/MAPK signaling pathway. These findings establish the molecular basis of LSS loss of function in the inhibition of tumorigenesis and development, and provide a rationale for targeting LSS and other enzymes catalyzing cholesterol biosynthesis, as a promising approach for cancer treatment.

## 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

SuZ and ShZ designed the study. XS, JZ and HL performed the experiments. ShZ analyzed data and revised the manuscript. SuZ drafted the manuscript. ML, LL, ZY, WH, HB, JXu, JXi, ZX, AM, ZG, YB, QZ and YW collected, analyzed and interpreted data. All authors have read and approved the final manuscript. SuZ and ShZ 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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