

Inhibitory Effects of LOXL2 Knockdown On Cellular Functions of Liver Cancer Stem Cells

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Research Article

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Abstract

Background and aim: Lysyl oxidase-like 2 (LOXL2) plays a role in tumor microenvironment formation and metastasis of hepatocellular carcinoma (HCC), which has a high mortality burden. Liver cancer stem cells (LCSCs) are related with the major malignant phenotypes of HCC. The function of LOXL2 in regulation of LCSCs remains unknown.

Methods: CD133⁺HepG2 and CD133⁺Hep3B cells were sorted by fluorescence-activated cell sorting (FACS) from two human hepatoblastoma cell lines. Spheroid formation, apoptosis, cell cycle, as well as transwell assays were performed upon *LOXL2* knock down in CD133⁺HepG2 and CD133⁺Hep3B cells. Protein and mRNA levels were quantified by Western blotting, Immunofluorescence and real-time PCR.

Results: Knockdown of *LOXL2* decreased spheroid formation, migration and invasion ($p < 0.05$), also induced apoptosis ($p < 0.05$) and cell cycle arrest ($p < 0.05$) in CD133⁺HepG2 and CD133⁺Hep3B cells. Knockdown of *LOXL2* effectively inhibited expression of the anti-apoptosis proteins baculoviral IAP repeat-containing 3 (BIRC3) and murine double minute 2 (MDM2) ($p < 0.01$), as well as autophagy marker microtubule-associated protein 1 light chain 3 B (LC3) and autophagy gene ATG5 in CD133⁺HepG2 and CD133⁺Hep3B cells ($p < 0.01$).

Conclusions: The results revealed that LOXL2 inhibition could reduce the proliferation and expansion of LCSCs, making LOXL2 inhibitors an attractive and novel therapeutic strategy of HCC.

Introduction

Hepatocellular carcinoma (HCC) is very common and has a high mortality burden [1, 2]. Hepatitis B and C, metabolic dysfunction associated fatty liver disease and alcohol intake are the main risk factors of HCC [2, 3]. Hepatocarcinogenesis is accompanied by resistance to cellular death signals, metabolic changes, and a failure to clear damaged precancerous hepatocytes by the immune system [4]. The poor prognosis and response to therapy of HCC is in part due to the presence of CSCs, an immune-privileged cell population that can evade immune surveillance more effectively than non-CSCs [5, 6]. Liver cancer stem cells (LCSCs) are a subset of hepatocarcinoma cells with pluripotent and self-renew properties related to the major malignant phenotypes of HCC [3, 7, 8]. The expression of CSC-related genes such as NANOG, SOX2 and OCT4 can be promoted by oncogenes and/or overexpression of oncoproteins such as the HBV envelope protein PreS1, thereby driving the malignant biological behaviors of LCSCs [9]. LCSCs can be characterized through cell surface markers, such as CD133 [10, 11]. CD133 is a transmembrane cell surface single chain glycoprotein, which is the original marker of hematopoietic stem cells, neural stem cells and liver stem / progenitor cells. It is an important indicator of malignant progression, patient survival and recurrence rate. The expression of CD133 in tumor cells is related to the stem cell-like characteristics [12~14].

Lysyl oxidase-like 2 (LOXL2) belongs to the lysyl oxidase (LOX) family, members of which are implicated in diverse pathophysiology including developmental regulation, cell motility, cellular senescence, and tumor suppression or promotion [15~17]. The roles played by LOXL2 in fibrosis, tumorigenesis, and metastasis have been extensively reported, and it has also been recognized as a therapeutic target [16, 18, 19]. Overexpression of LOXL2 is related with poor prognosis of patients with malignant tumors, such as colon cancer, esophageal cancer, and squamous cell carcinoma [15, 20, 21]. LOXL2 can induce epithelial to mesenchymal transition (EMT), its overexpression negatively affects the clinicopathological features of different tumor types [22]. In HCC, LOXL2 is upregulated and plays a key role in HCC metastasis by mediating EMT and extracellular matrix remodeling, subsequently enhancing HCC invasion [22, 23]. LOXL2 is also crucial in tumor microenvironment formation and metastatic niche in HCC [23, 24]. However, the functions and regulatory mechanism of LOXL2 in LCSCs remains unknown.

In this study, we sorted the CD133⁺HepG2 and CD133⁺Hep3B cells from two human hepatoblastoma cell lines. The cellular function of the two cell lines, including proliferation, migration, invasion as well as apoptosis and cell cycle assay, were demonstrated upon LOXL2 gene knock down. To understand whether LOXL2 benefited LCSCs from escaping apoptotic cell death, we investigated the change of gene expression of anti-apoptosis proteins baculoviral IAP repeat-containing 3 (BIRC3, also known as cIAP2) and murine double minute 2 (MDM2) in CD133⁺HepG2 and CD133⁺Hep3B cells upon LOXL2 knockdown. Moreover, to explore whether LOXL2 was related with autophagic cell death, we investigated the expression change of autophagy marker microtubule-associated protein 1 light chain 3 (LC3) B and autophagy gene ATG5 in CD133⁺HepG2 and CD133⁺Hep3B cells upon LOXL2 gene knock down.

Materials And Methods

Cell lines

The human hepatoblastoma cell lines HepG2 and Hep3B were purchased from Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS; PAN, Germany) and 1% penicillin and streptomycin (Gibco) at 37.0°C in 5% CO₂.

Isolation of LCSCs through fluorescence activated cell sorting (FACS)

HepG2 or Hep3B cells were resuspended in phosphate-buffered saline (PBS), then incubated with FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) and phycoerythrin (PE)-conjugated CD133 antibodies (Miltenyi Biotec) at 4°C for 20 min before sorting by FACS on a BD Influx (BD Biosciences, Franklin Lakes, NJ, USA). About 60% of CD133⁺ cells were sorted based on strong positivity. Isotype-matched mouse IgG was used to exclude non-specifically-stained cells. CD133⁺HepG2 and CD133⁺Hep3B cells were cultured in DMEM/F12 (HyClone, Logan, UT, USA) with 20 ng/ml recombinant

epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ, USA), 20 ng/ml basic fibroblast growth factor (bFGF; PeproTech) and 2% B27 supplement (Gibco) at 37.0°C in 5% CO₂. The CD133 positive proportion of cells was detected by flow cytometry.

In vitro LOXL2 gene silencing

LOXL2 gene in CD133⁺HepG2 cells and CD133⁺Hep3B cells were stable knockout using siRNA lentiviral vector (LV; GeneChem, Shanghai, China), the control group were transfected with siRNA negative control lentiviral vector. The vectors were encoded with green fluorescent protein (GFP). Cells were transduced with 25 multiplicity of infection (MOI) lentiviral particles, and HiTransG P (GeneChem, Shanghai, China) was used to promote transfer efficiency. After 48 to 72 hours, the effect of cell infection was observed under fluorescence microscope to make sure that more than 80% of cells show GFP. Cells were transduced for 72 h before treated with 2 µg/mL puromycin for 2 weeks. Transfection efficiency was confirmed by RT-PCR and western blotting.

After stable knockout of LOXL2 by transfected with LOXL2 siRNA lentivirus, the CD133⁺Hep3B cell line presented with growth retardation, even died. CD133⁺Hep3B cell line was more susceptible to LOXL2 gene deletion. Thus, we adjusted to transient transfection of CD133⁺Hep3B cell line by siRNA in the follow-up study after spheroid formation assay. Pre-validated Silencer® select siRNAs against *LOXL2*, negative control siRNA, and *GAPDH* siRNA (Thermo Fisher Scientific) were transfected respectively into 5 × 10⁵ CD133⁺Hep3B cells at a dose of 25 pmol in six-well plates using the Lipofectamine RNAiMAX™ transfection reagent (Invitrogen, Waltham, MA, USA) for 24 h.

Quantitative real-time PCR

Total RNA was extracted from CD133⁺HepG2 cells and CD133⁺Hep3B cells. Then, cDNA was synthesized by reverse transcription with the Evo M-MLV RT Kit for qPCR (AG Bio, China). PCR was performed by Applied Biosystems 7500 PCR System (Thermo Fisher Scientific) with SYBR® Green Premix Pro Taq HS qPCR Kit (AG Bio). *GAPDH* was taken as the internal control. The amplification conditions were as follows: pre-denaturation (95°C, 30s), denaturation (95°C, 5s), annealing and extension (60°C, 30s), all for a total 40 cycles. The experimental Ct values were normalized against *GAPDH*. 2^{-ΔΔCT} method was used to analyze the data.

Western blotting

Total protein lysate was extracted on ice using radioimmunoprecipitation assay (RIPA) buffer, phenylmethanesulfonyl fluoride, phosphatase inhibitor, and protease inhibitor (KeyGEN, Nanjing, China). After separated in SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membrane. Then, the PVDF membrane was blocked with 5% buffer bovine serum albumin in TBST and exposed to primary antibody at 4°C overnight. Next, the PVDF membrane was washed in TBST (10 min × 3 times) and exposed to secondary antibody (1:5000) at room temperature for 1-1.5 h. The band was incubated in BeyoECL Plus (Beyotime China) and detected by chemiluminescence.

Spheroid formation

Cells were collected and washed with PBS. After ACCUTASE (Sigma-Aldrich, USA) digestion, single cells (1×10^4) were seeded in ultra-low adhesion 6-well plate (Crystalgen, Commack, NY, USA) containing serum-free culture (SFC) medium, which composed of DMEM/F12 (Hyclone), 20 ng/ml EGF (PeproTech), 20 ng/ml bFGF (PeproTech), and 2% B27 supplement (Life Technologies). Add 1 ml of fresh SFC into the cells twice a week. After culturing for 10-15 days, the number of tumor spheroids ($>50 \mu\text{m}$) were determined.

Flow cytometric apoptosis analysis

Cells in logarithmic growth phase were collected and digested into single ones by ACCUTASE (Sigma-Aldrich). $1 \times 10^5 \sim 1 \times 10^6$ single cells were washed with cold PBS and 1x binding buffer, respectively. Next, cells were resuspended in 100 μL binding buffer with 5 μL Annexin V-FITC and 10 μL PI staining solution, and incubated in the dark for 15 min at room temperature. Then, apoptosis was analyzed with a FACS Calibur instrument.

Flow cytometry cell cycle analysis

Cells were collected and digested into single ones by ACCUTASE (Sigma-Aldrich). Then, the cells were washed with cold PBS, and fixed with 70% ethanol at 4°C overnight. After being washed, cells were incubated with 2 mL staining solution (propidium iodide) in the dark for 30 min at room temperature. Then, cell cycle was analyzed with a FACSCalibur instrument (BD Biosciences).

Transwell assay for migration and invasion

Cells were starved for 24 hours, and suspended with RPMI 1640 medium. 5×10^4 cells in 200 μL RPMI 1640 medium were added into the upper chambers of 8 μm pore size transwells. 700 μL medium with 10% FBS were added into the lower ones. For the invasion assay, the bottom of the transwell chamber was laid with diluted Matrigel (BD Biosciences). The cells in transwell were cultured at 37°C in 5% CO_2 for 24 hours. Next, the transwell was treated with 4% paraformaldehyde for 30 min, and 0.1% crystal violet for 25 min. After PBS washing, cells in the upper chambers were wiped off, and cells at the bottom of the chamber were observed under the microscope, and five fields were randomly selected for counting ($\times 100$ objective).

Immunofluorescence assay

Cells were fixed in 4% paraformaldehyde for 30 min and permeabilized in 0.25% Triton X-100 for 15 min. After blocked by 5% goat serum at room temperature for 45 min, cells were exposed to primary antibody (1:500; Abcam, Cambridge, UK) at 4°C overnight. Next, cells were exposed to fluorescein (FITC)-conjugated goat anti-rabbit antibody (1:250; Abbkine, China) in the dark for 1 h at room temperature. Fluorescence images were acquired by a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed in Prism 8 software (GraphPad Software, La Jolla, CA). Data of continuous variables are shown as mean \pm standard deviation (S.D). The difference between two independent groups was analyzed with two-sided unpaired Student's *t*-test. *p*-value <0.05 is regarded as statistically significant.

Results

Knock down of LOXL2 impairs LCSCs spheroid formation

To understand the effect of LOXL2 knockdown on the cellular functions of LCSCs, LOXL2 or control siRNA lentiviral vectors were transferred into CD133⁺ HepG2 and CD133⁺ Hep3B cell lines. Results of Western blot and PCR showed that LOXL2 was effectively knocked down in the two cell lines (Fig. 1a, b). The ability of colony formation depends on the replication immortality of tumor cells, which is one of the hallmarks of tumor [25]. To explore whether knock down of LOXL2 gene could impair tumor spheroid formation of LCSCs, CD133⁺ HepG2 and CD133⁺ Hep3B cells were digested into single cells and cultured in SFC medium for 1-2 weeks. For CD133⁺ HepG2 cells, spheroid formation was significantly decreased upon silencing *LOXL2* (Fig. 1c). When switching to CD133⁺ Hep3B cells, a more significant inhibition effect was observed (Fig. 1d). CD133⁺Hep3B cell line was more susceptible to LOXL2 gene deletion. After stable knockout of LOXL2 by transfected with LOXL2 siRNA lentivirus, the growth of CD133⁺Hep3B cell line significantly slowed down, stagnated or even died. These results indicate that LOXL2 knockdown impairs LCSCs spheroid formation.

Knock down of LOXL2 inhibits LCSCs migration, and invasion

As LOXL2 gene plays a key role in HCC metastasis, subsequently enhancing HCC invasion, we asked whether silencing LOXL2 could inhibit LCSCs expansion. The transwell migration and invasion assays were performed in CD133⁺HepG2 (Fig. 2a, b) and CD133⁺Hep3B cells (Fig. 2c, d) which subjected to siRNA-mediated *LOXL2* knockdown. The migration and invasion effect of both two cell lines were decreased upon LOXL2 knockdown compared to the controls. It indicates that LOXL2 knockdown inhibits LCSCs migration, and invasion.

Knock down of LOXL2 induces apoptosis and cell cycle arrest in LCSCs

Anti-apoptosis is one of the signs of malignant tumors. We assessed apoptosis of CD133⁺HepG2 and CD133⁺Hep3B cells by flow cytometry using Annexin V-FITC and PI. The results showed that knock down of *LOXL2* significantly increased the proportion of cell apoptosis in both CD133⁺ HepG2 (Fig. 3a) and CD133⁺ Hep3B cells (Fig. 3b). Furthermore, we explored whether knock down of LOXL2 could also affect

cell cycle progression in the two cell lines. We found that the proportion of CD133⁺ HepG2 cell decreased in G0/G1 phase, increased in G2/M phase, and was unaltered in S phase upon *LOXL2* knockdown (Fig. 3c). Correspondingly, the proportion of CD133⁺Hep3B cells increased in G0/G1 phase, decreased in S phase, and did not change in G2/M phase (Fig. 3d). These results indicate that knock down of *LOXL2* makes CD133⁺ HepG2 cell cycle arrest in G2/M phase, and CD133⁺Hep3B cells arrest in G0/G1 phase. *LOXL2* knockdown induces the apoptotic phenotype and regulates the cell cycle of LCSCs.

Knock down of LOXL2 inhibits the expression of the anti-apoptosis proteins BIRC3 and MDM2 in LCSCs

The activation of anti-apoptosis proteins benefits tumor cells from escaping apoptotic cell death [4, 26]. To understand whether *LOXL2* favored the survival of LCSCs by anti-apoptosis, we explored the expression of BIRC3 and MDM2 in CD133⁺ HepG2 and CD133⁺ Hep3B cells upon *LOXL2* knockdown. The results showed that *LOXL2* knockdown significantly decreased protein and mRNA expression of BIRC3 and MDM2 in the two cell lines compared to controls (Fig. 4a-d). Immunofluorescence analysis of CD133⁺ HepG2 cells was consistent with the western blotting and RT-PCR results, with reduced level of BIRC3 and MDM2 upon *LOXL2* knockdown (Fig. 4e, f). These results indicate that one of the molecular mechanisms of apoptosis induced by *LOXL2* knockdown in LCSCs may lies in the downregulation of anti-apoptosis protein BIRC3 and MDM2.

Knock down of LOXL2 inhibits the expression of autophagy marker LC3B and autophagy gene ATG5 in LCSCs

As to CD133⁺Hep3B cells proliferated significantly slow or even died after stable knockout of *LOXL2*, we wondered whether *LOXL2* took a part in regulation of autophagic cell death of LCSCs besides anti-apoptosis. So, we further explored the expression of autophagy marker LC3B and autophagy gene ATG5 in CD133⁺HepG2 and CD133⁺Hep3B cells upon *LOXL2* gene knock down. The results showed that LC3B and ATG5 expression at protein and mRNA level were both significantly decreased in the two cell lines upon *LOXL2* knockdown (Fig. 5a-d). Also, it was found using immunofluorescence that inhibition of *LOXL2* reduced the expression of LC3B and ATG5 in CD133⁺HepG2 cells. (Fig. 5e, f). These results indicate that *LOXL2* knockdown inhibits the expression of autophagy marker LC3B and autophagy gene ATG5 in LCSCs.

Discussion

LCSCs, a subset of hepatocarcinoma cells with CSC-like properties, are an important therapeutic target in HCC [3, 27]. Here we show that knock down of *LOXL2* inhibits the proliferation, invasion and migration of CD133⁺ HepG2 and CD133⁺ Hep3B cells, and induces cell apoptosis and cell cycle arrest. This is consistent with the conclusions of Wong et al., they demonstrated that *LOXL2* facilitates HCC metastasis by inducing bone marrow-derived cells recruitment into the metastatic site [23]. These results indicated that *LOXL2* inhibition could reduce the proliferation and expansion of LCSCs. Targeting LCSCs by *LOXL2* inhibitors may become an attractive and novel therapeutic strategy in HCC.

We tried to explore the potential mechanisms of LOXL2 inhibiting LCSCs function. In the current study, the growth of CD133⁺ HepG2 and CD133⁺Hep3B cells significantly slowed down after stable knockout of LOXL2. The CD133⁺Hep3B cell was even stagnant or dead. Apparently, knock down of LOXL2 may lead to LCSCs death via some mechanism. Cell death is subdivided into apoptosis, autophagy death and necrosis [28]. The activation of anti-apoptosis proteins benefits tumor cells from escaping apoptotic cell death [4, 26]. We first investigated whether knock down of LOXL2 blunted the anti-apoptosis mechanism of LCSCs. We found that knock down of *LOXL2* effectively downregulated the anti-apoptosis proteins BIRC3 and MDM2 expression in CD133⁺ HepG2 and CD133⁺ Hep3B cells. This is consistent with our previous finding by microarray that BIRC3 and MDM2 are possible LOXL2 downstream genes in SMMC-7721 human hepatoblastoma cell lines [29]. BIRC3, one of the human inhibitor of apoptosis protein (IAP) family, is often overexpressed in HCC tissues and indicates a poor prognosis of HCC patients [30~32]. BIRC3 also induces EMT, proliferation, migration of hepatoblastoma cells in vitro [32]. Moreover, MDM2 is an up-regulator of XIAP, which also belongs to the IAP family. It favors resistance of neoplastic cells to irradiation-induced apoptosis [33, 34]. MDM2 is also a negative-regulatory factor of p53, which is a central player in apoptosis [33, 35~37]. We speculate that LOXL2 might benefit LCSCs from escaping apoptotic cell death by regulating BIRC3 and MDM2. Knockdown of LOXL2 could induce LCSCs apoptosis through downregulation of BIRC3 and MDM2, which may become a promising strategy of targeting LCSCs.

Another mechanisms of LOXL2 inhibiting LCSCs function is that LOXL2 might play a role in regulation of LCSCs autophagy. We investigated the expression change of autophagy marker LC3B and autophagy gene ATG5 upon LOXL2 gene knock down to understand whether LOXL2 was related with autophagic cell death. Our results show that knock down of *LOXL2* downregulated the expression of LC3B and ATG5 in CD133⁺HepG2 and CD133⁺Hep3B cells. This phenomenon could be explained by the previous reports that autophagy favors LCSCs resistance to hypoxia and nutrient deficiency in the tumor microenvironment of HCC [38~40]. Autophagy positively regulates LCSCs by suppressing p53, which would normally be upregulated by mitochondrial PINK1 to downregulate CSC-related NANOG [41]. Moreover, BIRC3 was upregulated in the active autophagy response and inhibition of autophagy suppressed the expression of BIRC2 and BIRC3 [42, 43]. Thus, we speculate that LOXL2 might take a part in regulation of LCSCs autophagy, favoring LCSCs survival under cellular stress and hypoxia.

However, the specific mechanism of LOXL2 in LCSCs is not well established. The relationship between LOXL2, apoptosis and autophagy need to be further explored. Moreover, BIRC3, MDM2 and other possible LOXL2 downstream genes need further investigations, such as p53 which is known associated with MDM2 and autophagy.

In summary, knock down of *LOXL2* inhibits the proliferation, invasion and migration of LCSCs, and induces cell apoptosis and cell cycle arrest. Knockdown of LOXL2 effectively inhibited the expression of anti-apoptosis proteins BIRC3 and MDM2, as well as autophagy marker LC3B and autophagy gene ATG5 in LCSCs. LOXL2 inhibition could reduce the proliferation and expansion of LCSCs, making LOXL2 inhibitors an attractive and novel therapeutic strategy of HCC.

Statements And Declarations

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Competing Interests: All authors have no conflicts of interest to declared.

Authors Contributions YZ conceived the work, and approved the final version of manuscript. Material preparation and experimental operation were performed by NL, HG, LL, XLZ and QLC. Data collection and analysis were performed by NL and HG. The manuscript was written by NL. All authors approved the final revision.

Ethics approval: The scheme of the current study was approved by the Institutional Review Board and Ethics Committee of the First Affiliated Hospital of Dalian Medical University. This study does not involve human or animal experiment.

Consent to participate: Not applicable.

Consent for publication: Not applicable.

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Figures

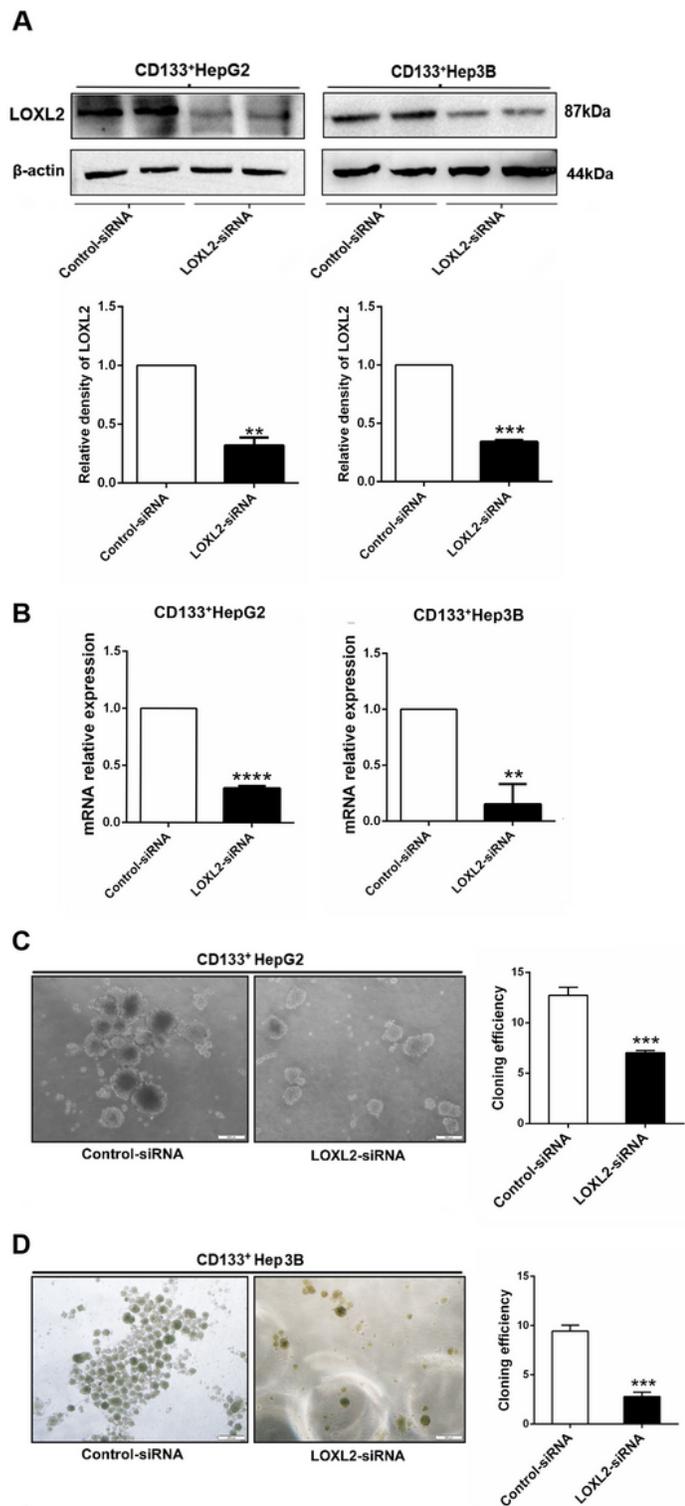


Figure 1

Knock down of lysyl oxidase-like 2 (LOXL2) impairs the spheroid formation of liver cancer stem cells (LCSCs) (A) Western blotting and (B) PCR were performed to determine transfection efficiency of LOXL2 gene in CD133+ HepG2 and CD133+ Hep3B cell lines 72~48h after LOXL2 or control siRNA lentiviral vectors were transferred. Relative intensity values for the proteins were obtained using Image J software. LOXL2 was effectively knocked down in CD133+ HepG2 and CD133+ Hep3B cell lines. Spheroid

formation assay were performed to determine the effect of LOXL2 knockdown on cell survival in (C) CD133+ HepG2 and (D) CD133+ Hep3B cells. Knock down of LOXL2 impairs the spheroid formation of LCSCs. The data were analyzed upon three independent experiments and shown as mean \pm S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

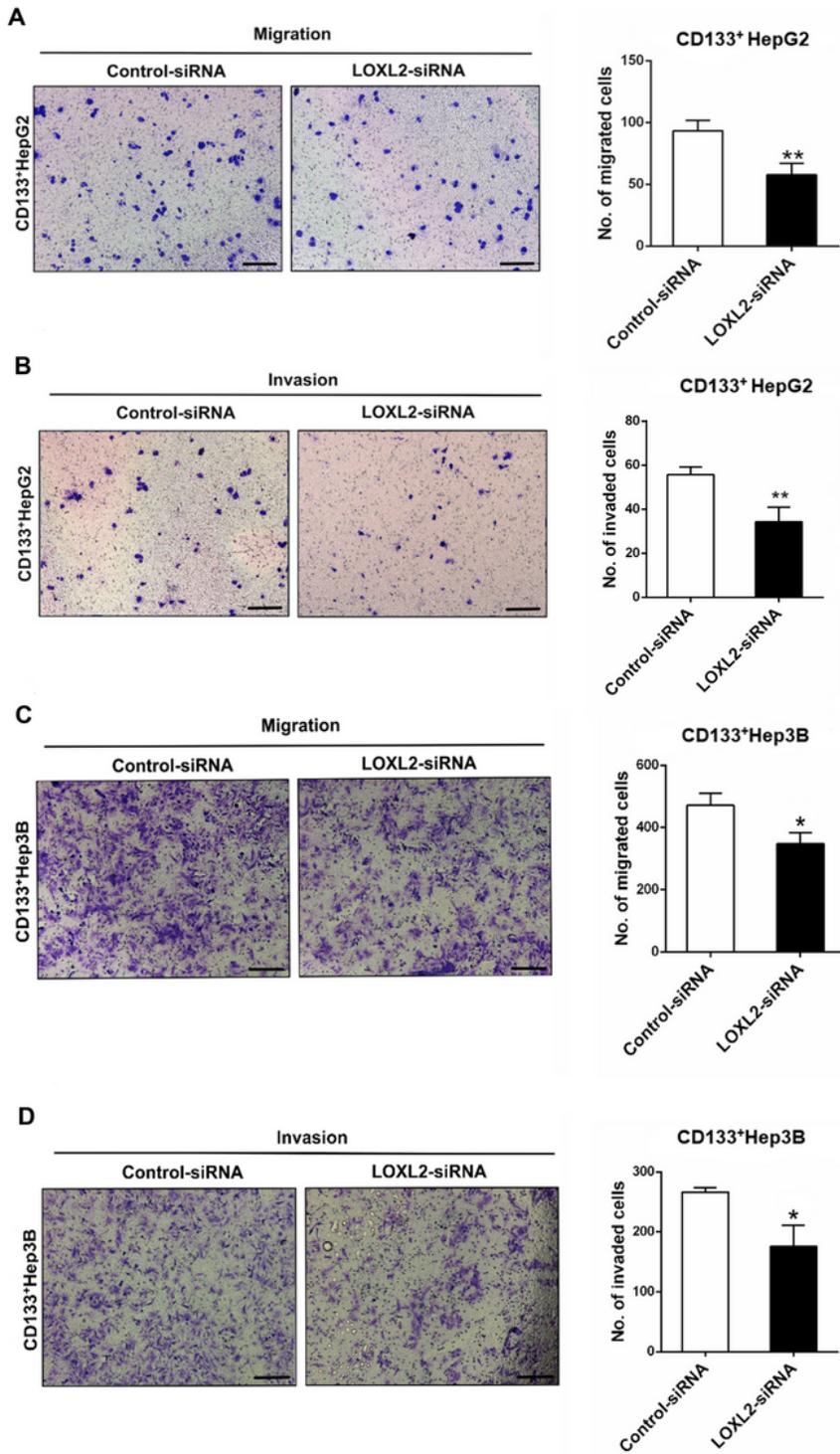


Figure 2

Knock down of lysyl oxidase-like 2 (LOXL2) inhibits LCSCs migration and invasion (A, C) The transwell migration assays were performed upon LOXL2 knockdown in CD133+HepG2 and CD133+Hep3B cells. The average number of migrating cells are showed in graphs. (Scale bar: 50 μ m). Knock down of LOXL2 inhibits LCSCs migration. (B, D) The transwell invasion assays were performed upon LOXL2 knockdown in CD133+HepG2 and CD133+Hep3B cells. The average number of invading cells are showed in graphs. (Scale bar: 50 μ m). Knock down of LOXL2 inhibits LCSCs invasion. The data were analyzed upon three independent experiments and shown as mean \pm S.D. * $p < 0.05$, ** $p < 0.01$.

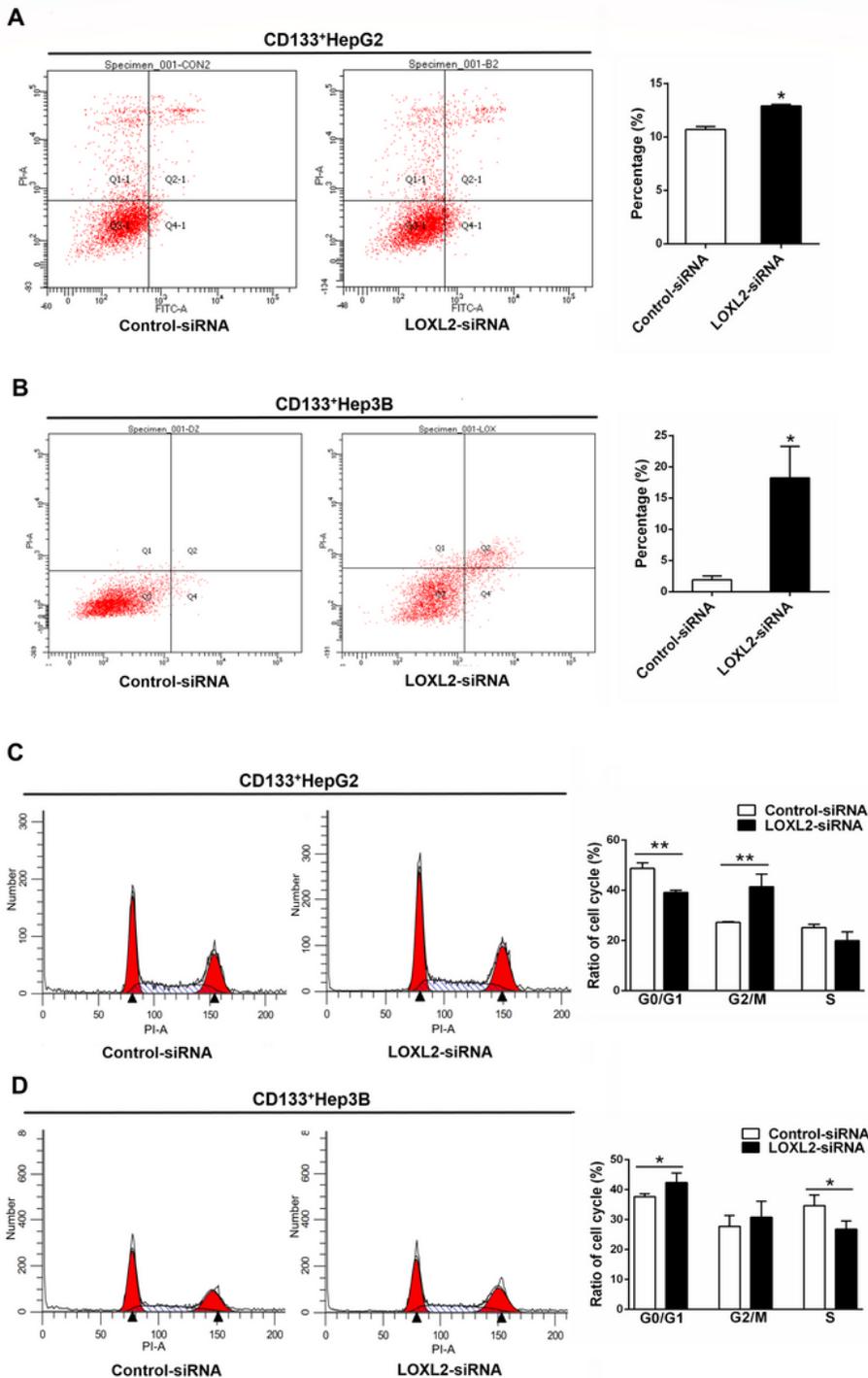


Figure 3

Knock down of lysyl oxidase-like 2 (LOXL2) induces apoptosis and cell cycle arrest in liver cancer stem cells (LCSCs) Fluorescence activated cell sorting (FACS) analysis showing apoptosis of CD133+ (A) HepG2 and (B) Hep3B cells transfected with control siRNA or LOXL2 siRNA. Cells were gated according to Annexin V-FITC and PI staining. Knock down of LOXL2 induces the apoptosis of LCSCs. Cell cycle assay of CD133+ (C) HepG2 and (D) Hep3B cells performed by flow cytometry upon LOXL2 knockout. Knock down of LOXL2 makes CD133+ HepG2 cell cycle arrest in G2/M phase, and CD133+Hep3B cells arrest in G0/G1 phase. Data represented as % of total cells. The data were analyzed upon three independent experiments and presented as mean \pm S.D. *p < 0.05, **p < 0.01.

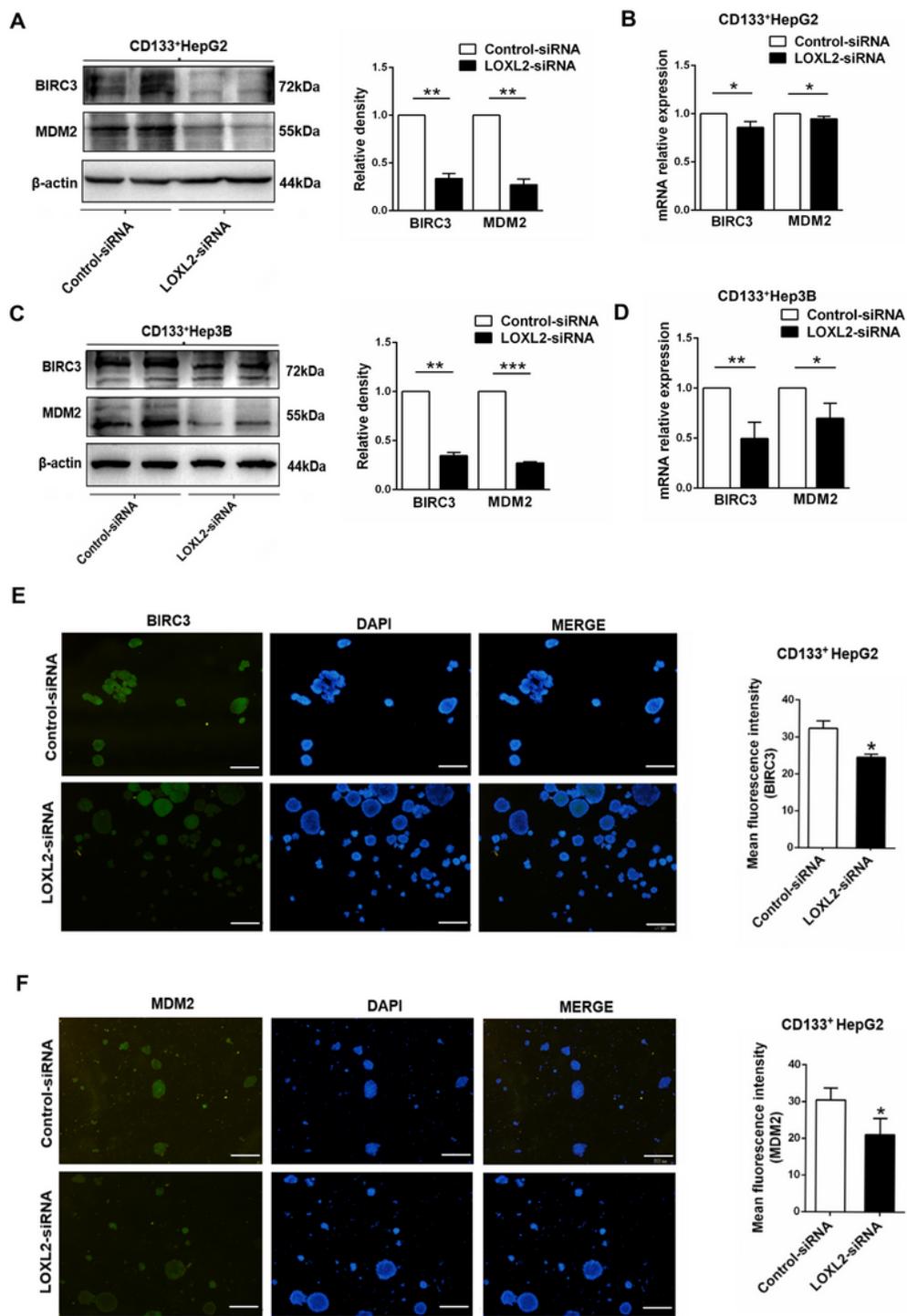


Figure 4

Knock down of Lysyl oxidase-like 2 (LOXL2) downregulates the anti-apoptosis proteins baculoviral IAP repeat-containing 3 (BIRC3) and murine double minute 2 (MDM2) in liver cancer stem cells (LCSCs). Western blotting and PCR were performed to determine the expression of BIRC3 and MDM2 in CD133+ (A, B) HepG2 and (C, D) Hep3B cells transfected with control siRNA or LOXL2 siRNA. Relative intensity values for the proteins were obtained using Image J software. (E, F) Immunofluorescence staining was

performed for BIRC3 and MDM2 in CD133+ HepG2 cells upon LOXL2 knock down. Scale bars 200 μ M. Mean fluorescence intensity values were obtained using Image J software. The data were analyzed upon three independent experiments. * $p < 0.05$, ** $p < 0.01$. *** $p < 0.001$.

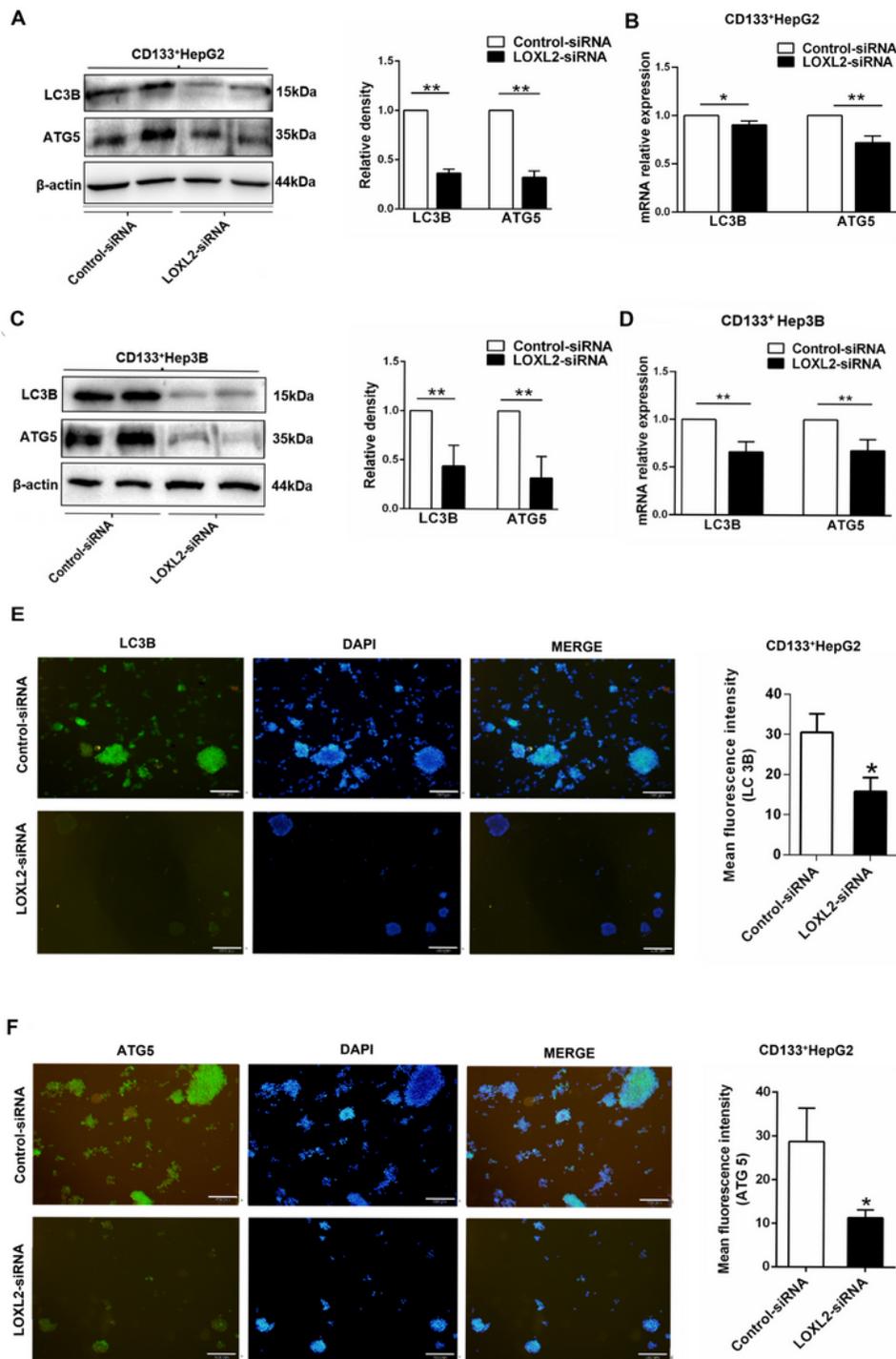


Figure 5

Knock down of Lysyl oxidase-like 2 (LOXL2) inhibits the expression of autophagy marker LC3B and autophagy gene ATG5 in liver cancer stem cells (LCSCs) Western blotting and PCR were performed to

determine the expression of the autophagy marker type B microtubule-associated protein 1 light chain 3 (LC3B) and autophagy gene ATG5 in CD133+ (A, B) HepG2 and (C, D) Hep3B cells transfected with control siRNA or LOXL2 siRNA. Relative intensity values for the proteins were obtained using Image J software. (E, F) Immunofluorescence staining was performed for LC3B and ATG5 in CD133+ HepG2 cells upon LOXL2 knock down. Scale bars 200 μ M. Mean fluorescence intensity values were obtained using Image J software. The data were analyzed upon three independent experiments. * $p < 0.05$, ** $p < 0.01$.