LOXL2 upregulates hypoxia-inducible factor-1α signaling through Snail-FBP1 axis in hepatocellular carcinoma cells

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Abstract. Lysyl oxidase-like 2 (LOXL2), a member of the lysyl oxidase gene family, is involved in the progression of hepatocellular carcinoma progression and metastasis. Increased expression of LOXL2 has been identified in several types of cancer, including hepatocellular carcinoma. Recently, LOXL2 has been reported to promote epithelial-mesenchymal transition by reducing E-cadherin expression via the upregulation of Snail expression. The present study provided evidence demonstrating that LOXL2 inhibited the expression of fructose-1, 6-biphosphatase (FBP1) and enhanced the glycolysis of Huh7 and Hep3B hepatocellular carcinoma cell lines in a Snail-dependent manner. Overexpression of the point-mutated form of LOXL2 [LOXL2(Y689F)], which lacks enzymatic activity, does not affect the expression of Snail1 or FBP1. Notably, targeting extracellular LOXL2 of Huh7 cells with a therapeutic antibody was unable to abolish its regulation on the expression of Snail and FBP1. Knockdown of LOXL2 also interrupted the angiogenesis of Huh7 and Hep3B cells, and this effect could be rescued by the overexpression of Snail. Furthermore, upregulation of hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF) expression was observed in Huh7 and Hep3B cells expressing wild-type LOXL2. Notably, the selective LOXL2 inhibitor LOXL2-IN-1 could upregulate the expression of FBP1 and inhibit the expression of Snail, HIF-1α and VEGF in HCC cells, but not in FBP1-knockdown cells. The results of the present study indicated that the intracellular activity of LOXL2 upregulated HIF-1α/VEGF signaling pathways via the Snail-FBP1 axis, and this phenomenon could be inhibited by LOXL2 inhibition. Collectively, these findings further support that LOXL2 exhibits an important role in the progression of hepatocellular carcinoma and implicates LOXL2 as a potential therapeutic agent for the treatment of this disease.

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Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver malignancy, which results in chronic inflammation in the liver (1,2). Typical HCC progression is a multistep process involving transformation, survival, proliferation, invasion, angiogenesis and metastasis (3,4). Although, a number of clinicopathological factors are important in the treatment of HCC, there is no marked and effective medical therapy for patients with advanced HCC (5-8). Therefore, it is necessary to develop more efficient therapeutic strategies and targets for patients with HCC.

Hypoxia-inducible factor-1 (HIF-1), a heterodimer composed of an oxygen-regulated HIF-1α subunit and a HIF-1β subunit, activates the transcription of genes involved in proliferation and angiogenesis (9-11). HIF-1β is stable under hypoxic or normoxic conditions. By contrast, HIF-1α is an oxygen sensitive subunit and its expression is induced under hypoxic conditions (12). The degradation of HIF-1α subunit is facilitated by ubiquitination following the hydroxylation of proline residues (13,14). The other post-transnational modifications, such as acetylation and phosphorylation reactions, can also affect the stability of HIF-1α (15,16). Activated HIF-1 serves a crucial role in the regulation of the expression of certain enzymes in the glycolytic pathway, such as glucose transporters, hexokinase, phosphofructokinase, pyruvate kinase and pyruvate dehydrogenase (17-19). Vascular endothelial growth factor (VEGF), one of the most important angiogenic factors in cancer progression, is a HIF-1α target gene. VEGF has also been revealed to regulate cancer cell proliferation and migration (20).

HIF-1α functions by binding hypoxia response elements (HREs) within its target metabolism-related genes (21). Fructose-1,6-biphosphatase (FBP1), which catalyzes the splitting of fructose-1,6-bisphosphate into fructose 6-phosphate and inorganic phosphate, can inhibit HIF-1α activity via direct interaction with the HIF-1α C-terminus (22). The epithelial-mesenchymal transition (EMT) promotes invasiveness and stem cell-like features in cancer cells (23,24). The EMT involves the downregulation of E-cadherin to reinforce

the destabilization of adherens junctions. EMT-related transcription factors, such as Snail, Slug and Twist, can suppress E-cadherin expression (25,26). Snail interacts with H3K9 methyltransferase G9a and DNA methyltransferase Dnmt1 to silence E-cadherin expression. Similarly, the Snail-G9a-Dnmt1 complex also suppresses FBP1 expression, and thus enhances aerobic glycolysis (27).

LOX-like protein 2 (LOXL2) is a member of the lysyl oxidase gene family, with both intracellular and extracellular functions (28,29). Extracellularly, LOXL2 catalyzes the covalent cross-linkages of collagen and elastin in the extracellular matrix. Intracellularly, LOXL2 modifies histone tails and reduces cell polarity, which increases the metastatic potential of tumors (30-32). Intracellular LOXL2 has also been revealed to stabilize Snail protein and promote EMT in certain breast cancer cell lines through interaction with Snail (33). Thus, small molecule LOXL2 inhibitors were considered as suitable drug candidates for treatment of numerous types of cancers. LOXL2-IN-1 hydrochloride (LOXL2-IN-1) is the first published small molecule inhibitor selective for LOXL2 with a strong inhibitory effect (32). The most established LOXL2 inhibitor is β-aminoproprionitrile (BAPN), which irreversibly inhibits the enzyme activity of LOXL2 (34). Recently, a subseries of LOXL2 specific inhibitors containing an aminomethiophene (AMT) scaffold were revealed to inhibit tumor growth (35-37).

The present study demonstrated that LOXL2 upregulated HIF-1α/VEGF signaling pathways via the Snail-FBP1 axis, and this phenomenon could be inhibited by LOXL2 inhibition. LOXL2 represents a potential therapeutic target for HCC. These findings further support the role of LOXL2 in the progression of HCC and implicates LOXL2 as a potential therapeutic agent for the treatment of HCC.

Materials and methods

Cell cultures. 293T cells and human HCC cell lines Huh7 and Hep3B were purchased from the American Type Culture Collection. Huh7, Hep3B and 293T cells were cultured in Dulbecco's modified Eagles medium (DMEM; HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37˚C. Human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell Academy and cultured in Endothelial Cell Growth Medium 2 (PromoCell Academy) with Supplement Mix according to the manufacturer's guidelines.

Reagents. LOXL2-IN-1 hydrochloride (LOXL2-IN-1) was purchased from MedChemExpress. Anti-human LOXL2 therapeutic antibody (Simtuzumab; cat. no. HPAB-0160-LSX) was obtained from Creative Biolabs. Antibodies against LOXL2 (cat. no. ab179810; 1:1,000), Snail (cat. no. ab53519; 1:1,000), FBP1 (cat. no. ab109020; 1:1,000), VEGF (cat. no. ab69479; 1:1,000), Transferrin (cat. no. ab88165; 1:2,000) and β-actin (cat. no. ab6276; 1:5,000) were all obtained from Abcam. Antibody against HIF-1α (cat. no. 14179; 1:500) and GAPDH (cat. no. 5174; dilution 1:2,000) were purchased from Cell Signaling Technology, Inc.

Quantification of glucose uptake and lactate generation. Glucose uptake was assessed in the cell lysates with Glucose Uptake Colorimetric assay kit (BioVision, Inc.). The extracellular lactate was assessed in the medium with a lactate assay kit (BioVision, Inc.). Both assays were performed according to the manufacturer's protocol.

Lentiviral vector construction. Wild-type LOXL2 and the point-mutated form of LOXL2 [LOXL2(Y689F)] in pBOBI lentiviral vector were purchased from Rosetta Stone Biotech Co., Ltd. Wild-type Snail in pBOBI lentiviral vector was purchased from Rosetta Stone Biotech Co., Ltd. The sequences of the lentiviral short hairpin RNAs (shRNAs) in pLKO.1-puro vectors were as follows: sh1LOXL2, 5'-GAAACCCTCCAGTCTATTATA-3'; sh2LOXL2, 5'-GGCAATGAGAAGTCCATTATA-3'; sh1FBP1, 5'-CCACCATCAAATGCTGTAGAA-3'; and sh2FBP1, 5'-CCACCATCAAATGCTGTAGAA-3'. The sequence of the scrambled hairpin lentiviral shRNA pLKO.1-puro control (PLK) was 5'-CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG-3'.

Viral production and infection. 293T cells ($2x10^6$/cell culture dish) were plated and transfected with different lentiviral vectors together with packaging plasmids using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h of transfection, culture supernatants were harvested and filtered with a Millipore Stericup filter (0.45-μm). Huh7 or Hep3B cells were infected with different lentivirus for 24 h. After 4 days, the effects on these cells were examined by western blotting.

RT-qPCR assay. Total RNA was isolated from Huh7 cells by TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). The purity and quantity of RNA were assessed with a NanoDrop 100 spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was synthesized with a reverse transcriptase kit (Thermo Fisher Scientific, Inc.). SYBR-Green (Takara Biotechnology Co., Ltd.) was added to quantify the expression of VEGF, according to the protocol provided. The sequences of the primers were: VEGF, 5'-TTGCAGATGTGACAAGCCGA-3' and 5'-GGCCGCGGTGTGTCTA-3'; GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' and 5'-TGGTGAAGACGCCAGTGGA-3'. PCR reaction conditions were performed as follows: 95˚C for 60 sec, and 40 cycles of 95˚C for 15 sec, 60˚C for 15 sec, and 72˚C for 45 sec. The relative expression levels were determined using the comparative ΔΔCq method (38).

Colony forming analysis. The different types of HCC cells were seeded at a density of 300 cells/well in 6-well plates and cultured for 14 days. The culture medium was replaced every 3 days. Colonies were fixed with 10% buffered formalin at 37˚C for 15 min and stained with 2% crystal violet at 37˚C for 30 min. Colonies were counted under a microscope (Micropublisher 3.3RTV; Olympus Corporation), and colony forming was determined as the colony formation rate (number of cell clones experimental group/cell clones in the control group x100%).

Western blot analysis. To analyze the expression of proteins, western blot analysis was performed. Total protein was isolated

from the cells using RIPA buffer containing a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc.). The lysate was centrifuged at 20,8000 x g for 15 min at 4˚C. The supernatants were collected and mixed with 5X loading buffer, followed by incubation in a boiling water bath for 5 min. Protein concentration was determined by Bradford protein assay. Western blot analysis was performed using 12% SDS-PAGE gels for different proteins (50 μg per lane). Following transfer to PVDF membranes, 5% non-fat milk in TBST was used for blocking for 1 h at room temperature, followed by incubation overnight at 4˚C with each of the indicated primary antibodies. Subsequently, the membranes were washed with PBST three times for 10 min. The membranes were then probed with HRP-conjugated secondary antibodies (cat. no. ab205719 and cat. no. ab205718; Abcam) in TBST for 1 h at room temperature, and washed with TBST three times. Following washing, enhanced chemiluminescence was used to incubate the membrane and bands were visualized using the ECL Plus Western Blotting Detection system™ (GE Healthcare Life Sciences). Signals were detected and documented with the densitometry system LAS-3000 (Fujifilm).

Tube formation assay. A tube formation assay was used to evaluate vascular activity of HCC cells on HUVECs tube formation. The upper chamber was prepared by plating HUVECs onto Matrigel basement membrane matrix (BD Biosciences) in Transwell filters (0.4-μm pore size; Costar) followed by incubation at 37˚C for 24 h. HUVECs and HCC cells were co-cultured at 37˚C for 48 h. Tubes were observed under an inverted light microscope.

Immunoprecipitation assays. Huh7 cells were seeded in a 10-cm dish at an initial concentration of $2x10^6$ cells. After 24 h, the medium was replaced with serum-free DMEM and cultured for a further 24 h. The medium was centrifuged at 20,8000 x g for 15 min at 4˚C and the supernatant was then filtered by ultrafiltration using Amicon Ultra-4 (5K; EMD Millipore, Merck KGaA). Concentrated protein (30 μl) was collected (per dish) and then added to 1 ml RIPA buffer (Sigma-Aldrich; Merck KGaA). Following incubation with simtuzumab antibody for 8 h at 4˚C, A/G-agarose beads were added with gentle rocking for 3 h at 4˚C. Following centrifugation at 1,008 x g for 30 sec at 4˚C, the pellets were washed with RIPA buffer three times and re-suspended in 2X SDS sample buffer. The samples were then subjected to 12% SDS gel electrophoresis.

Statistical analysis. All statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc.). The data were presented as the mean ± standard deviation (SD), and the experiments were performed in triplicate. For data analyses, a two-tailed Student's t-test was used to examine the differences between groups, and comparisons between multiple groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. In all comparisons, $P<0.05$ was considered to indicate a statistically significant difference.

Results

Intracellular LOXL2 inhibits the expression of FBP1 in HCC cell lines. LOXL2 has been reported to promote EMT by reducing E-cadherin expression via the upregulation of Snail expression (33). Snail interacts with the H3K9 methyltransferase G9a and the DNA methyltransferase Dnmt1 to induce E-cadherin gene repression. Similarly, the Snail-G9a-Dnmt1 complex also suppresses FBP1 expression, and thus enhances aerobic glycolysis (27). Therefore, the present study examined whether knockdown of LOXL2 can affect the expression level of FBP1 in HCC cells. As presented in Fig. 1A, LOXL2-knockdown markedly inhibited the expression of Snail. In addition, the expression of FBP1 was upregulated in LOXL2-knockdown Huh7 and Hep3B HCC cells (Fig. 1A). Furthermore, overexpression of wild-type LOXL2 increased

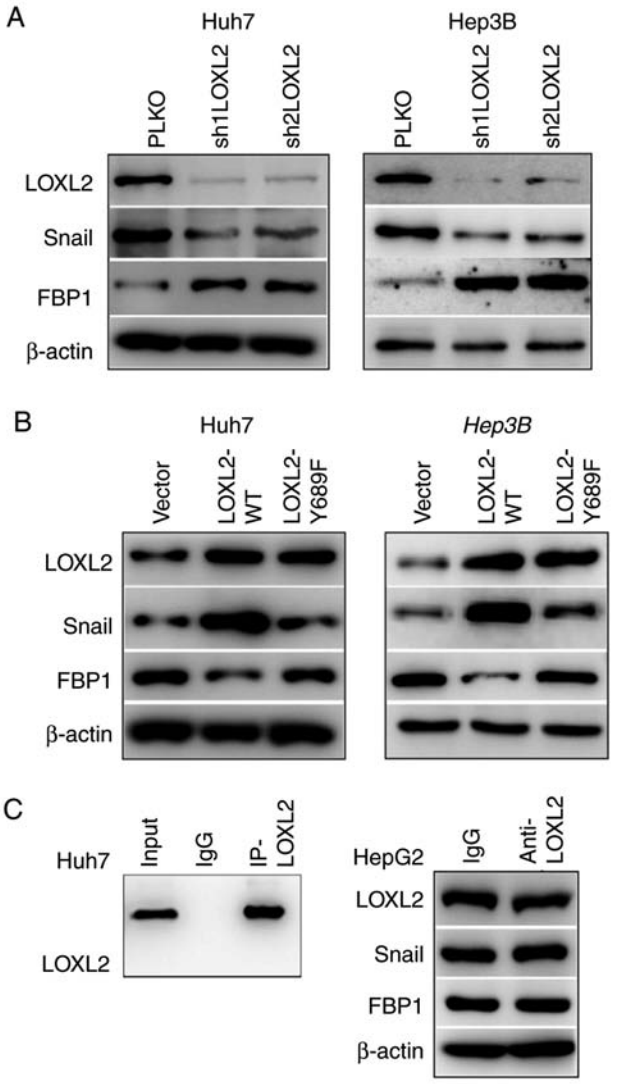

Figure 1. Intracellular LOXL2 inhibits the expression of FBP1. (A) Western blot detection of LOXL2, Snail and FBP1 in LOXL2-knockdown Huh7 or Hep3B cells. β-actin was used as a loading control. (B) Overexpression of wild-type or point-mutated form of LOXL2 [LOXL2(Y689F)] lacking enzymatic activity in Huh7 or Hep3B cells. The expression levels of LOXL2, Snail and FBP1 were detected using western blotting. β-actin was used as a loading control. (C) Left, the binding between simtuzumab and extracellular LOXL2 protein were examined by immunoprecipitation assays in Huh7 cells following immunoblotting (western blotting) of LOXL2. Right, after incubation of Huh7 cells with simtuzumab antibody for 12 h in Huh7 cells, the expression levels of LOXL2, Snail and FBP1 were detected using western blotting. β-actin was used as a loading control. LOXL2, lysyl oxidase-like 2; FBP1, fructose-1, 6-biphosphatase.

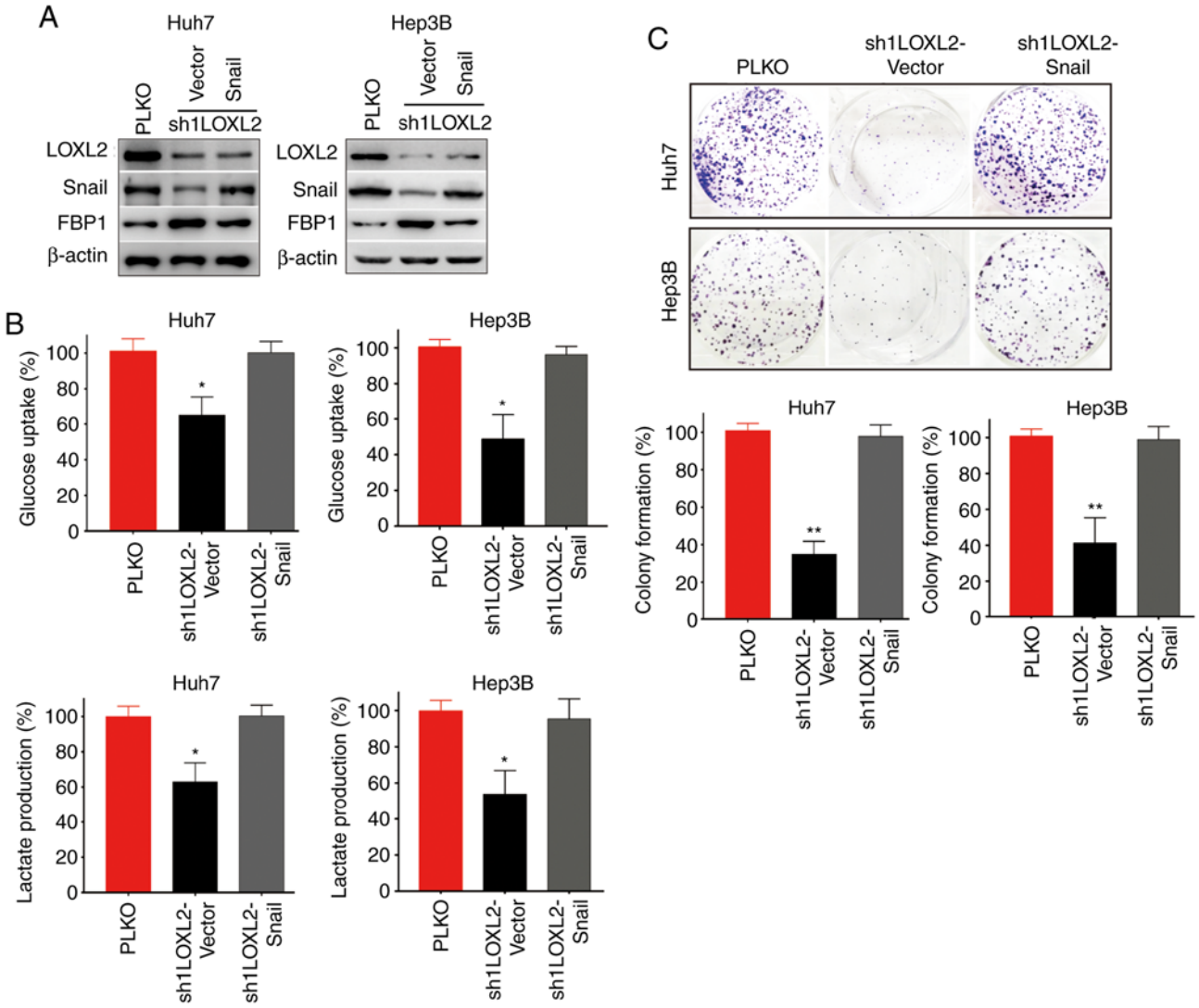

Figure 2. LOXL2 inhibits the expression of FBP1 and enhances glycolysis. (A) The expression of LOXL2, Snail and FBP1 were detected using western blot in vector control (PLKO), LOXL2 knockdown (sh1), and LOXL2 knockdown plus Snail-overexpressing Huh7 or Hep3B cells. β-actin was used as a loading control. (B) Glucose uptake and lactate production were detected in culture medium or the lysates and normalized to total cellular protein amount in vector control, LOXL2 knockdown, and LOXL2 knockdown plus Snail-overexpressing Huh7 or Hep3B cells. *P<0.05 vs. PLKO or the sh1LOXL2-Snail groups. (C) Colony formation assay was used to estimate the cell proliferation in vector control, LOXL2 knockdown, and LOXL2 knockdown plus Snail-overexpressing Huh7 or Hep3B cells. Representative images of the colony formation assay and quantification of colony formation efficiency in various types of HCC cells. **P<0.01 vs. PLKO or the sh1LOXL2-Snail groups. LOXL2, lysyl oxidase-like 2; FBP1, fructose-1, 6-biphosphatase.

the expression of Snail and inhibited the expression of FBP1 (Fig. 1B). Notably, overexpression of the point-mutated form of LOXL2 [LOXL2(Y689F)], which lacks enzymatic activity, did not affect the expression of Snail1 or FBP1 (Fig. 1B). LOXL2 is a member of the lysyl oxidase family, with both intracellular and extracellular functions. To examine whether the intracellular LOXL2 or extracellular LOXL2 plays a requisite role in regulation of Snail or FBP1 expression, the anti-human LOXL2 therapeutic antibody simtuzumab was used. Simtuzumab is a humanized monoclonal antibody designed for the treatment of fibrosis (39,40). It binds to LOXL2 and acts as an immunomodulator. The binding between simtuzumab and extracellular LOXL2 protein was first evaluated with immunoprecipitation assays. The data demonstrated good binding between extracellular LOXL2 protein of Huh7 cells and simtuzumab (Fig. 1C). Following incubation of Huh7 cells with simtuzumab antibody, the expression of Snail and FBP1 was examined by western blotting. Simtuzumab did not affect the expression levels of Snail and FBP1 (Fig. 1C). Therefore, intracellular LOXL2 was responsible for the regulation of Snail and FBP1 expression.

LOXL2 inhibits the expression of FBP1 and enhances the glycolysis in a Snail-dependent manner. To further determine the mechanism of LOXL2-regulated expression of FBP1, Snail was overexpressed in LOXL2 stable knockdown HCC cells. As presented in Fig. 2A, LOXL2 knockdown did not significantly affect the expression of FBP1 in Snail-overexpressing HCC cells. Loss of FBP1 is reported to increase glycolysis, including cellular glucose uptake and lactate generation (27). To examine whether LOXL2 affects glycolysis in HCC cells, the present study assessed glucose uptake and lactate generation, and it was revealed that LOXL2-knockdown significantly decreased the glucose uptake and lactate generation, whereas Snail-overexpressing Huh7 and Hep3B cells had normal

glucose uptake and lactate generation compared with their vector control cells (Fig. 2B). Tumor cells exhibit high aerobic glycolysis during rapid proliferation (27,41,42). LOXL2 has also been reported to upregulate cell proliferation in clear cell renal cell carcinoma (43). The present study revealed that LOXL2-knockdown suppressed the proliferation of HCC cells but not Snail-overexpressing HCC cells (Fig. 2C). These results indicated that LOXL2 inhibited the expression of FBP1 and enhanced the glycolysis in a Snail-dependent manner.

LOXL2 regulates angiogenesis and expression of VEGF. A previous study has reported that LOXL2 can promote bFGF-induced tumor angiogenesis (44). In the present study, HUVECs were co-cultured along with LOXL2-knockdown or the vector control HCC cells. As presented in Fig. 3A, HUVECs co-cultured with LOXL2-knockdown HCC cells reduced the generation of tubular networks. By contrast, HUVECs co-cultured with LOXL2-knockdown and Snail-overexpressing HCC cells exhibited a normal generation of tubular networks (Fig. 3A). It was then revealed that knockdown of LOXL2 in Huh7 or Hep3B cells inhibited the expression of VEGF but not in the Snail-overexpressing HCC cells (Fig. 3B). These findings indicated that LOXL2 may be involved in the regulation of angiogenesis in HCC.

Overexpression of LOXL2 or knockdown of FBP1 increases the expression of HIF-1α. VEGF is known to be one of the target genes of HIF-1α (45). The present study further investigated the role of LOXL2 in the regulation of HIF-1α expression. As presented in Fig. 4A, overexpression of wild-type LOXL2 resulted in increased expression levels of HIF-1α in Huh7 and Hep3B cells. However, overexpression of the point-mutated form LOXL2 (Y689F), which lacks enzymatic activity, failed to regulate the expression of HIF-1α in Huh7 or Hep3B cells (Fig. 4A). FBP1 can inhibit HIF-1α activity via direct interaction with the HIF-1α C-terminus. Furthermore, FBP1 was reported to modulate cell metabolism of breast cancer cells by inhibiting the expression of HIF-1α (46). It was also revealed that knockdown of FBP1 increased the expression of HIF-1α (Fig. 4B). These data indicated that LOXL2 may increase the expression of HIF-1α through inhibition of the expression of FBP1.

LOXL2 upregulates HIF-1α/VEGF signaling pathways via the Snail-FBP1 axis. The present study further investigated the mechanism of LOXL2-induced regulation of HIF-1α expression. It was revealed that knockdown of FBP1 in Huh7 or Hep3B cells did not alter the expression levels of Snail (Fig. 5A). This suggests that FBP1 only regulates the expression of HIF-1α, and Snail is the upstream suppressor of FBP1 (Fig. 2A). LOXL2-IN-1 hydrochloride (LOXL2-IN-1) is a selective LOXL2 inhibitor. Similar to LOXL2 knockdown, LOXL2-IN-1 treatment resulted in a similar effect on the expression of Snail, FBP1, HIF-1α and VEGF in Huh7 or Hep3B cells (Fig. 5B). Inhibition of LOXL2 by LOXL2-IN-1 increased the expression of FBP1. Therefore, the effect of LOXL2-IN-1 in FBP1-knockdown HCC cells was examined. As presented in Fig. 5B, LOXL2-IN-1 did not affect the expression of HIF-1α and VEGF in FBP1-knockdown HCC cells compared to the DMSO group. HIF-1α/VEGF signaling

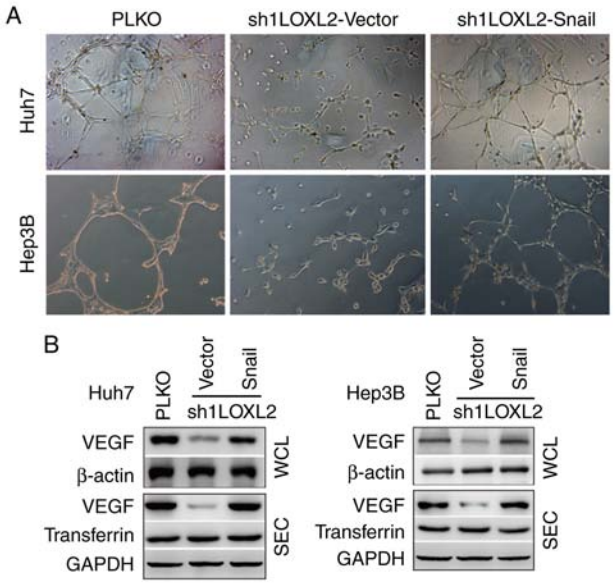

Figure 3. LOXL2 regulates angiogenesis and the expression of VEGF. (A) Confluent monolayers of HUVECs co-cultured with vector control, LOXL2 knockdown or LOXL2 knockdown plus Snail-overexpressing HCC cells. The formation of tubular networks was captured at 48 h. (B) The expression of VEGF from the whole-cell lysates or the secreted culture medium of Huh7 or Hep3B cells was detected using western blotting in vector control, LOXL2 knockdown or LOXL2 knockdown plus Snail-overexpressing Huh7/Hep3B cells. β-actin was used as a loading control of the whole-cell lysates. Transferrin and GAPDH were used as loading control of serum. LOXL2, lysyl oxidase-like 2; VEGF, vascular endolthelial growth factor.

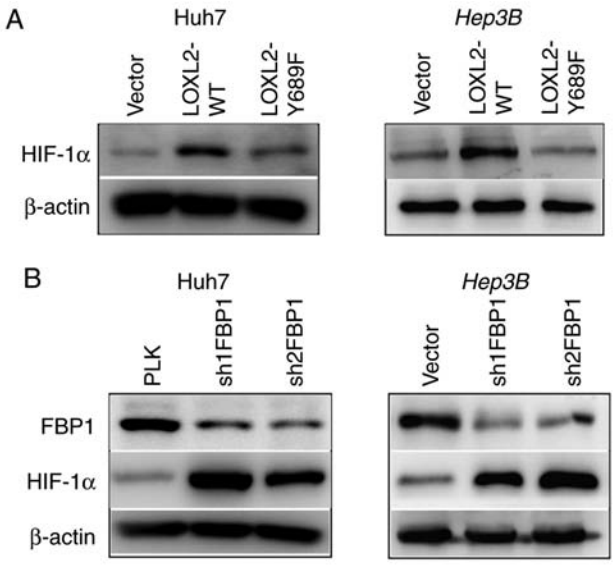

Figure 4. LOXL2 and FBP1 in the regulation of HIF-1α expression. (A) Overexpression of wild-type or point-mutated form of LOXL2 (Y689F) in Huh7 or Hep3B cells. The expression levels of HIF-1α were detected using western blotting. β-actin was used as a loading control. (B) The expression levels of HIF-1α and FBP1 in vector control and FBP1-knockdown Huh7 or Hep3B cells were detected using western blotting. LOXL2, lysyl oxidase-like 2; FBP1, fructose-1, 6-biphosphatase; HIF-1α, hypoxia-inducible factor 1α.

was upregulated by LOXL2. The effect of LOXL2 inhibitor in combination with HIF-1α inhibitor PX-478, the first novel HIF-1α inhibitor in clinical stage for the treatment of solid

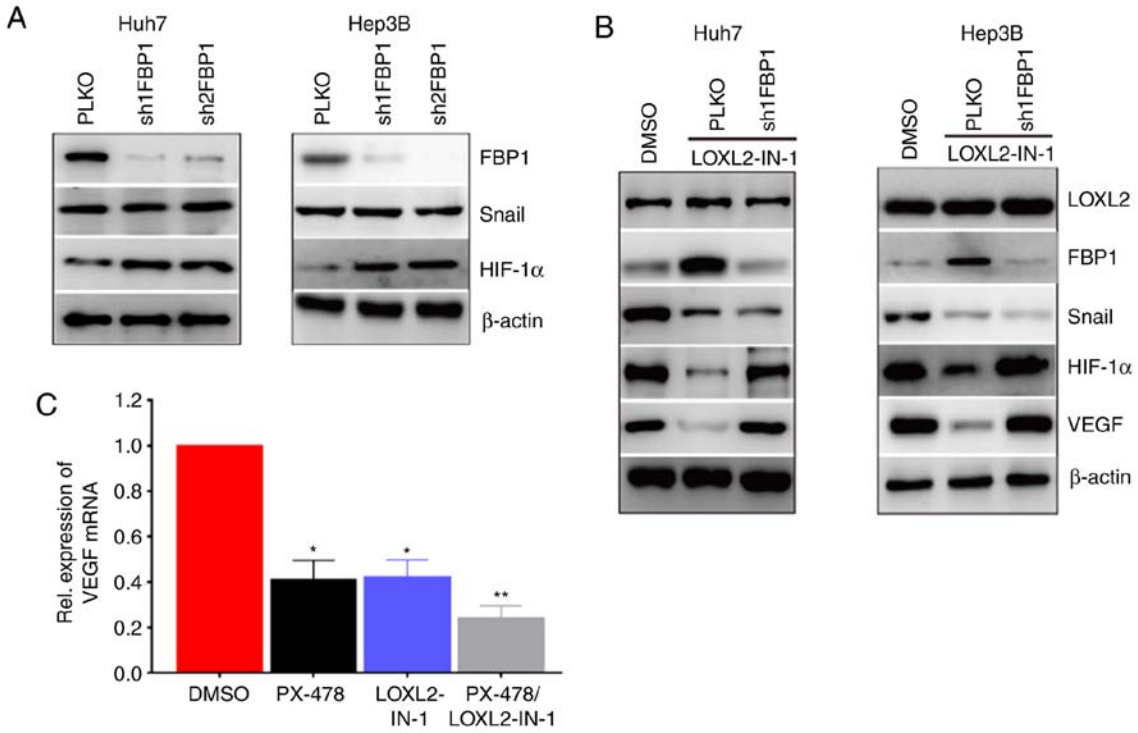

Figure 5. LOXL2 upregulates HIF-1α/VEGF signaling pathways via the Snail-FBP1 axis. (A) The expression levels of HIF-1α, Snail and FBP1 in vector control and FBP1-knockdown Huh7 or Hep3B cells were detected using western blotting. (B) Huh7 or Hep3B cells were treated with DMSO (the vehicle of LOXL2-IN-1); the vector control (PLKO) or FBP1-knockdown Huh7 or Hep3B cells were treated with LOXL2-IN-1 hydrochloride (LOXL2-IN-1, 50 nM). All these HCC cells were treated with DMSO or LOXL2-IN-1 for 12 h. The expression of LOXL2, Snail, FBP1 and HIF-1α were detected using western blotting. β-actin was used as a loading control. (C) Huh7 cells were treated with DMSO, PX-478 (10 μM), LOXL2-IN-1 (50 nM) or PX-478 (10 μM) in combination with LOXL2-IN-1 (50 nM) for 12 h. VEGF mRNA expression was confirmed by quantitative RT-PCR assay. *P<0.01 vs. the DMSO treatment group, **P<0.05 vs. PX-478 or LOXL2-IN-1 treatment group. LOXL2, lysyl oxidase-like 2; HIF-1α, hypoxia-inducible factor 1α; VEGF, vascular endolthelial growth factor; FBP1, fructose-1, 6-biphosphatase.

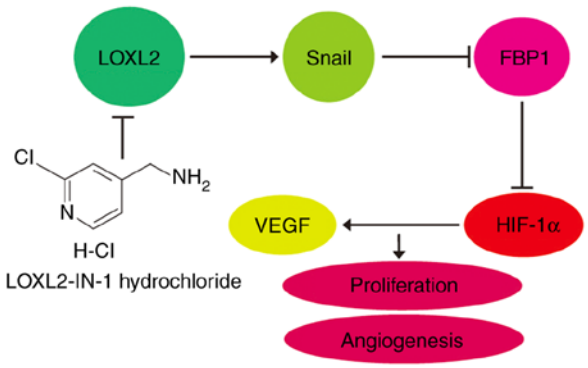

Figure 6. The diagram illustrates the mechanisms of LOXL2 upregulation of HIF-1α/VEGF signaling pathways via the Snail-FBP1 axis. LOXL2, lysyl oxidase-like 2; FBP1, fructose-1, 6-biphosphatase; HIF-1α, hypoxia-inducible factor 1α; VEGF, vascular endolthelial growth factor.

tumors, was therefore assessed (47). A synergistic inhibitory effect of LOXL2-IN-1 inhibitor with PX-478 inhibitor on the expression of VEGF was demonstrated in Fig. 5C. In summary, these results indicated that LOXL2 upregulates HIF-1α/VEGF signaling pathways via the Snail-FBP1 axis (Fig. 6).

Discussion

HCC is the most common type of liver malignancy and its progression is a multistep process involving transformation, survival, proliferation, invasion, angiogenesis and metastasis (3,8). Presently, there is no marked and effective medical therapy for patients with advanced HCC (48). Therefore, it is necessary and crucial to develop more efficient therapeutic strategies and targets for patients with HCC.

LOXL2 is a member of the LOX gene family, which encodes a copper-dependent amine oxidase that catalyzes the first step in the formation of crosslinks in collagens and elastin (49,50). It is involved in the progression of tumor progression and metastasis (31,49,51-53). Upregulated expression of LOXL2 has been revealed in several types of cancer, including HCC (54). Extracellularly, LOXL2 can catalyze the covalent cross-linkages of collagen and elastin in the extracellular matrix. Intracellularly, LOXL2 modifies histone tails and reduces cell polarity, which increases metastatic potential of tumors (55,56). Interacting with intracellular Snail protein, intracellular LOXL2 has been reported to stabilize Snail protein and promote EMT in certain breast cancer cells (33). The present study revealed that knockdown of LOXL2 inhibited the expression of Snail. Furthermore, overexpression of wild-type LOXL2 increased the expression of Snail. Notably, overexpression of the point-mutated form of LOXL2 (Y689F), which lacks enzymatic activity, did not affect the expression of Snail. Furthermore, targeting extracellular LOXL2 of HCC cells with a therapeutic antibody was unable to abolish its regulation to the expression of Snail. These results indicated that intracellular activity of LOXL2 is responsible for Snail upregulation.

FBP1, a rate-limiting enzyme in gluconeogenesis, catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate (27,57). Snail interacts with G9a and Dnmt1 to suppress FBP1 expression, and thus enhances aerobic glycolysis. The present study revealed that LOXL2-knockdown did not significantly affect the expression of FBP1 in Snail-overexpressing HCC cells. Similar results were obtained in the analysis of cellular glucose uptake and lactate generation in Snail-overexpressing HCC cells. Knockdown of FBP1 in HCC cells did not alter the expression levels of Snail. Thus, Snail is an upstream suppressor of FBP1. These results indicated that LOXL2 inhibited the expression of FBP1 and enhanced the glycolysis in a Snail-dependent manner. There have been numerous studies that have revealed the expression of LOXL2, Snail and FBP1 in human HCC tissues. For example, LOXL2 was revealed to be frequently overexpressed in human HCC and displayed poor prognosis (54,58-60). High expression of Snail or decreased expression of FBP1 was correlated with poor outcomes in HCC patients (61-66). Notably, HCC patients with high Snail but low FBP1 expression were identified with the worst prognosis. Conversely, patients with low Snail but high FBP1 expression were identified with the best prognosis (67).

HIF-1α is a subunit of the heterodimeric transcription factor HIF-1, which is considered to be a master transcriptional regulator of cellular and developmental responses to hypoxia (68). In accordance with its dynamic biological role, HIF-1α forms a heterodimer with HIF-1β and thus associates with HREs of promoters of hypoxia-responsive genes to induce transcription (69). VEGF is known to be a target gene of HIF-1α. VEGF is one of the most important angiogenic factors in cancer progression and is important to regulate cancer cell proliferation and migration (70). The present study indicated that LOXL2 could induce cell proliferation and angiogenesis of HCC cells. Accordingly, it also indicated that LOXL2 was responsible for the upregulation of HIF-1α and VEGF.

FBP1 has been reported to inhibit HIF-1α activity or expression (27,46). The present study also revealed that knockdown of FBP1 increased the expression of HIF-1α in HCC cells. Additionally, knockdown of FBP1 in HCC cells did not alter the expression levels of Snail. This indicated that FBP1 only regulated the expression of HIF-1α, and Snail was an upstream suppressor of FBP1. Inhibition of the activity of LOXL2 by LOXL2-IN-1 also demonstrated a similar effect in LOXL2-knockdown HCC cells to the expression of Snail, FBP1, HIF-1α and VEGF. By contrast, LOXL2-IN-1 did not alter the expression of Snail, HIF-1α and VEGF in FBP1-knockdown HCC cells. Moreover, it was revealed that LOXL2-IN-1 inhibitor could exert a synergistic inhibitory effect with HIF-1α inhibitor PX-478 on the expression of VEGF. HCC is a highly angiogenic cancer (71,72), and therefore antiangiogenic therapy is an effective strategy in the treatment of HCC. LOX-L2 inhibitors could be potent combination partners for the antiangiogenic drugs approved for the treatment of HCC such as Cabometyx, Cyramza, Lenvatinib Mesylate, Ramucirumab and Sorafenib Tosylate (73,74). The present study revealed a potential role of LOXL2 in HCC, which may provide new insights into the treatment of HCC. However, a limitation of the present study was that the effect of LOXL2 therapeutic antibody or LOXL2 inhibitor was not verified in an *in vivo* study. We will further investigate the effect of LOXL2 therapeutic antibody or LOXL2 inhibitor in animal models of HCC.

In conclusion, the present study demonstrated that LOXL2 upregulated HIF-1α/VEGF signaling pathways via the Snail-FBP1 axis, and this phenomenon could be inhibited by LOXL2 inhibition. Therefore, these findings further support that LOXL2 serves an important role in the progression of HCC. Considering the high expression level of LOXL2 in HCC observed in several studies, LOXL2 may be considered a promising candidate for novel treatment strategies against HCC.

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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

All the authors participated in data collection and analysis. ZF, QJ and QL participated in the design of the study. ZF and QL participated in the writing of the manuscript and data interpretation. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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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