

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

ZHIYONG FAN^{1,2}, WEI ZHENG², HUI LI², WUJUN WU², XIAOGANG LIU², ZHONGJIE SUN²,
HAITIAN HU², LIXUE DU², QINGAN JIA¹ and QINGGUANG LIU¹

¹Department of Hepatobiliary Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710061;

²Department of Hepatobiliary Surgery, Shaanxi Provincial People's Hospital, Xi'an, Shaanxi 710068, P.R. China

Received June 30, 2019; Accepted February 19, 2020

DOI: 10.3892/or.2020.7541

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 1 α (HIF-1 α) 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-1 α 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-1 α /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.

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-1 α subunit and a HIF-1 β subunit, activates the transcription of genes involved in proliferation and angiogenesis (9-11). HIF-1 β is stable under hypoxic or normoxic conditions. By contrast, HIF-1 α is an oxygen sensitive subunit and its expression is induced under hypoxic conditions (12). The degradation of HIF-1 α subunit is facilitated by ubiquitination following the hydroxylation of proline residues (13,14). The other post-translational modifications, such as acetylation and phosphorylation reactions, can also affect the stability of HIF-1 α (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-1 α target gene. VEGF has also been revealed to regulate cancer cell proliferation and migration (20).

HIF-1 α 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-1 α activity via direct interaction with the HIF-1 α 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

Correspondence to: Dr Qingguang Liu, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, 277 Yanta West Street, Xi'an, Shaanxi 710061, P.R. China
E-mail: liuqingguang@vip.sina.com

Key words: LOXL2, HIF-1 α , VEGF, Snail, FBP1, hepatocellular carcinoma

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 -aminopropionitrile (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-1 α /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 μ g/ml) in a humidified atmosphere of 5% CO₂ 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-1 α (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'-GAA ACCCTCCAGTCTATTATA-3'; sh2LOXL2, 5'-GGCAAT GAGAAGTCCATTATA-3'; sh1FBP1, 5'-CCACCATCAAAT GCTGTAGAA-3'; and sh2FBP1, 5'-CCACCATCAAATGCT GTAGAA-3'. The sequence of the scrambled hairpin lentiviral shRNA pLKO.1-puro control (PLK) was 5'-CCTAAG GTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAAC CTTAGG-3'.

Viral production and infection. 293T cells (2x10⁶/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- μ m). 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'-GCACCG TCAAGGCTGAGAAC-3' and 5'-TGGTGAAGACGCCAG TGGA-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 $\Delta\Delta$ Cq 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 2x10⁶ 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 FBPI in HCC cell lines. LOXL2 has been reported to promote EMT by

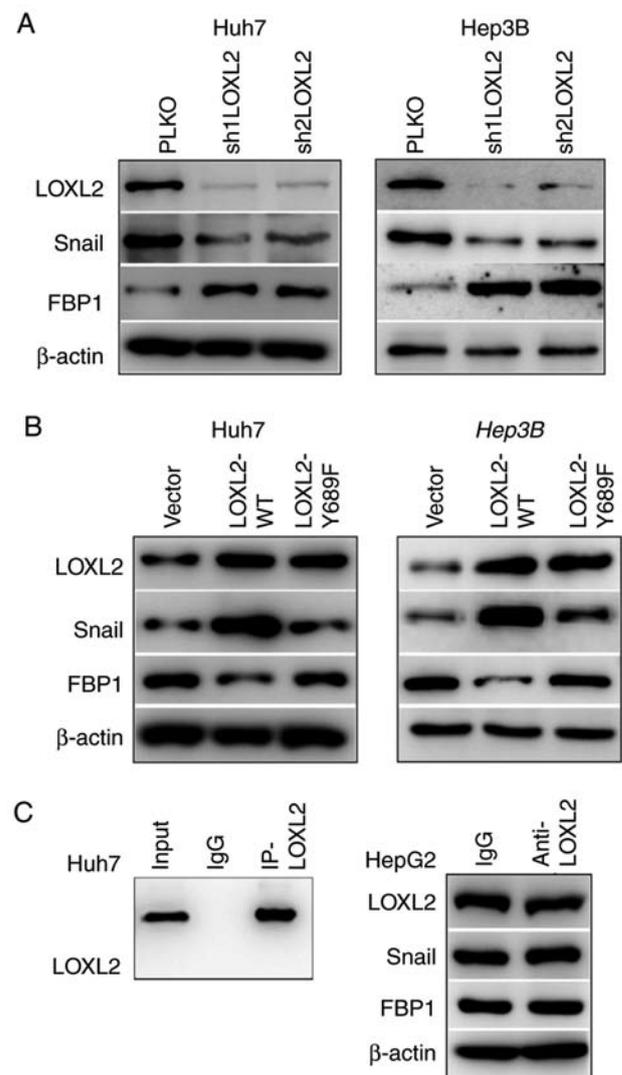


Figure 1. Intracellular LOXL2 inhibits the expression of FBPI. (A) Western blot detection of LOXL2, Snail and FBPI in LOXL2-knockdown Huh7 or Hep3B cells. β-actin was used as a loading control. (B) Overexpression of wild-type or point-mutated form of LOXL2 [LOXL2(Y689F)] lacking enzymatic activity in Huh7 or Hep3B cells. The expression levels of LOXL2, Snail and FBPI 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 FBPI were detected using western blotting. β-actin was used as a loading control. LOXL2, lysyl oxidase-like 2; FBPI, fructose-1, 6-biphosphatase.

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 FBPI expression, and thus enhances aerobic glycolysis (27). Therefore, the present study examined whether knockdown of LOXL2 can affect the expression level of FBPI in HCC cells. As presented in Fig. 1A, LOXL2-knockdown markedly inhibited the expression of Snail. In addition, the expression of FBPI was upregulated in LOXL2-knockdown Huh7 and Hep3B HCC cells (Fig. 1A). Furthermore, overexpression of wild-type LOXL2 increased

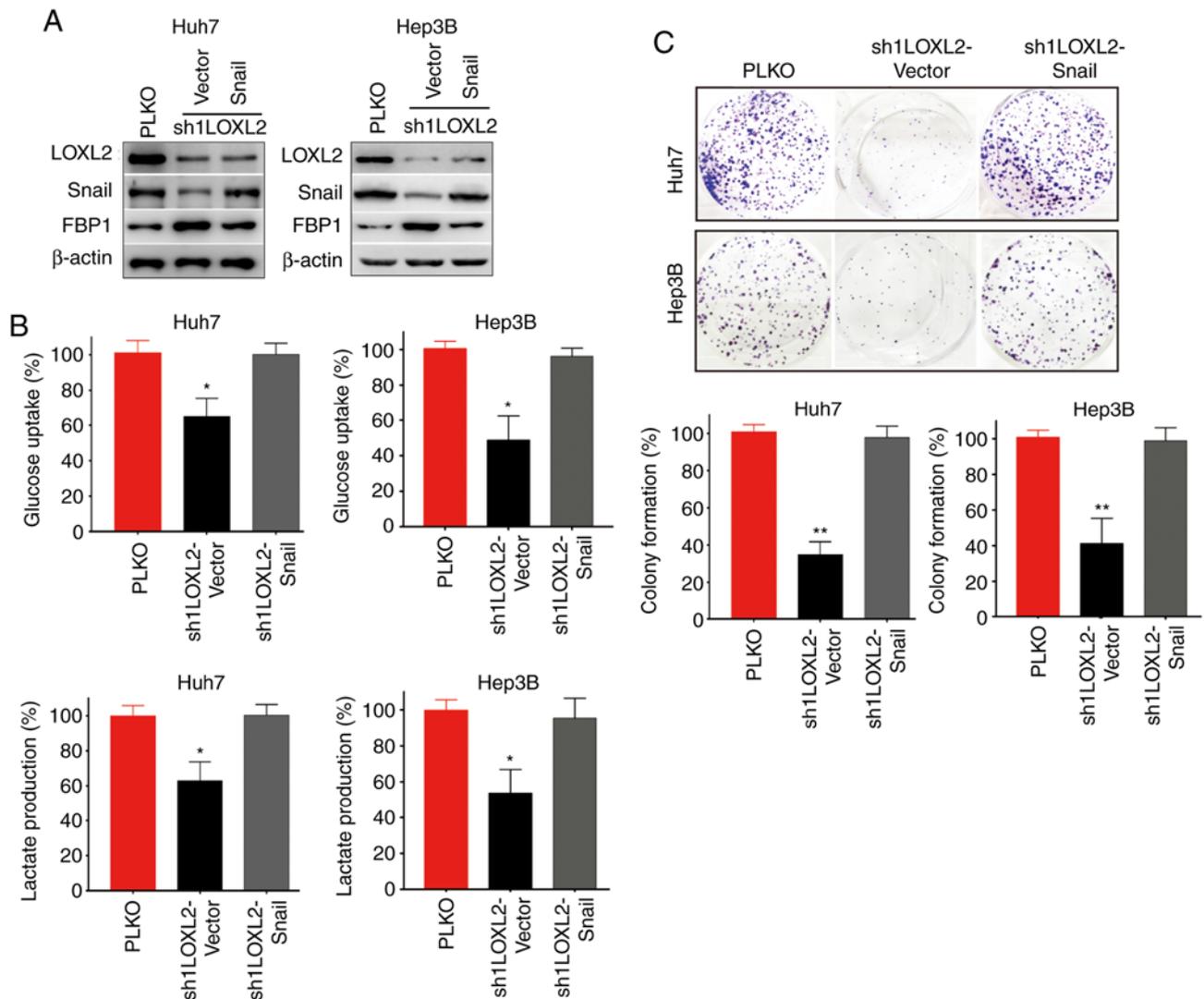


Figure 2. LOXL2 inhibits the expression of FBP1 and enhances glycolysis. (A) The expression of LOXL2, Snail and FBP1 were detected using western blot in vector control (PLKO), LOXL2 knockdown (sh1), and LOXL2 knockdown plus Snail-overexpressing Huh7 or Hep3B cells. β -actin was used as a loading control. (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-1 α . VEGF is known to be one of the target genes of HIF-1 α (45). The present study further investigated the role of LOXL2 in the regulation of HIF-1 α expression. As presented in Fig. 4A, overexpression of wild-type LOXL2 resulted in increased expression levels of HIF-1 α 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-1 α in Huh7 or Hep3B cells (Fig. 4A). FBP1 can inhibit HIF-1 α activity via direct interaction with the HIF-1 α C-terminus. Furthermore, FBP1 was reported to modulate cell metabolism of breast cancer cells by inhibiting the expression of HIF-1 α (46). It was also revealed that knockdown of FBP1 increased the expression of HIF-1 α (Fig. 4B). These data indicated that LOXL2 may increase the expression of HIF-1 α through inhibition of the expression of FBP1.

LOXL2 upregulates HIF-1 α /VEGF signaling pathways via the Snail-FBP1 axis. The present study further investigated the mechanism of LOXL2-induced regulation of HIF-1 α 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-1 α , 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-1 α 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-1 α and VEGF in FBP1-knockdown HCC cells compared to the DMSO group. HIF-1 α /VEGF signaling

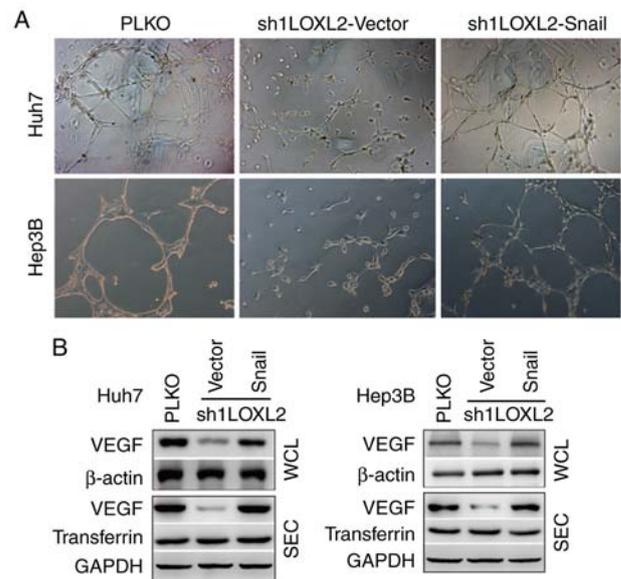


Figure 3. LOXL2 regulates angiogenesis and the expression of VEGF. (A) Confluent monolayers of HUVECs co-cultured with vector control, LOXL2 knockdown or LOXL2 knockdown plus Snail-overexpressing HCC cells. The formation of tubular networks was captured at 48 h. (B) The expression of VEGF from the whole-cell 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 endothelial growth factor.

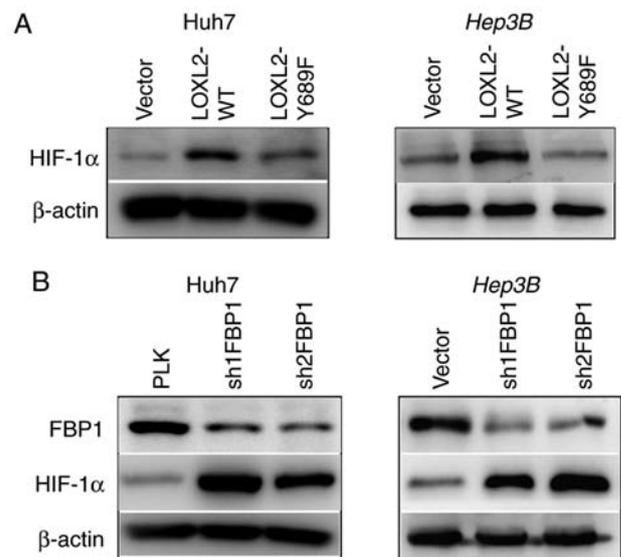


Figure 4. LOXL2 and FBP1 in the regulation of HIF-1 α expression. (A) Overexpression of wild-type or point-mutated form of LOXL2 (Y689F) in Huh7 or Hep3B cells. The expression levels of HIF-1 α were detected using western blotting. β -actin was used as a loading control. (B) The expression levels of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

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

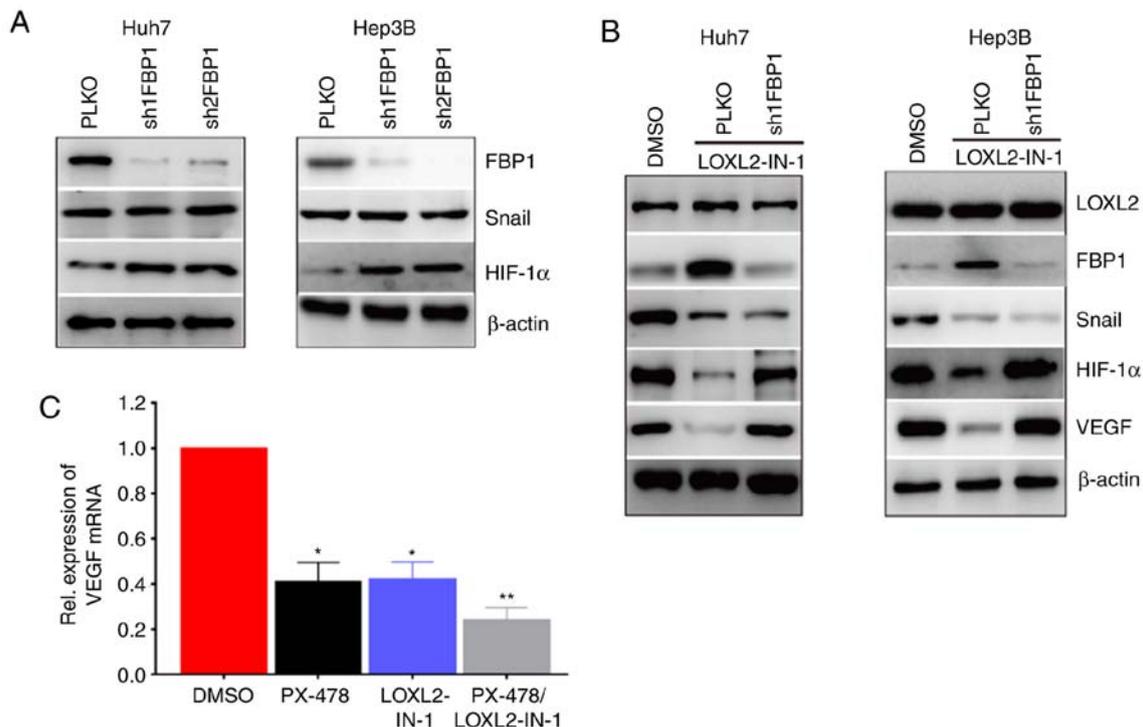


Figure 5. LOXL2 upregulates HIF-1 α /VEGF signaling pathways via the Snail-FBP1 axis. (A) The expression levels of HIF-1 α , Snail and FBP1 in vector control and FBP1-knockdown Huh7 or Hep3B cells were detected using western blotting. (B) Huh7 or Hep3B cells were treated with DMSO (the vehicle of LOXL2-IN-1); 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-1 α were detected using western blotting. β -actin was used as a loading control. (C) Huh7 cells were treated with DMSO, PX-478 (10 μ M), LOXL2-IN-1 (50 nM) or PX-478 (10 μ M) 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-1 α , hypoxia-inducible factor 1 α ; VEGF, vascular endothelial growth factor; FBP1, fructose-1, 6-biphosphatase.

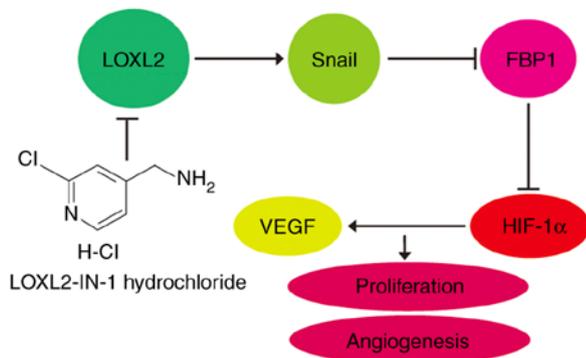


Figure 6. The diagram illustrates the mechanisms of LOXL2 upregulation of HIF-1 α /VEGF signaling pathways via the Snail-FBP1 axis. LOXL2, lysyl oxidase-like 2; FBP1, fructose-1, 6-biphosphatase; HIF-1 α , hypoxia-inducible factor 1 α ; VEGF, vascular endothelial growth factor.

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-1 α /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-1 α 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-1 α forms a heterodimer with HIF-1 β 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-1 α . 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-1 α and VEGF.

FBP1 has been reported to inhibit HIF-1 α activity or expression (27,46). The present study also revealed that knockdown of FBP1 increased the expression of HIF-1 α 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-1 α , 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-1 α and VEGF. By contrast, LOXL2-IN-1 did not alter the expression of Snail, HIF-1 α and VEGF in FBP1-knockdown HCC cells. Moreover, it was revealed that LOXL2-IN-1 inhibitor could exert a synergistic inhibitory effect with HIF-1 α 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. LOXL2 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-1 α /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.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Nature Science Foundation of China (grant no. 81502694).

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.

References

1. Hatzaras I, Bischof DA, Fahy B, Cosgrove D and Pawlik TM: Treatment options and surveillance strategies after therapy for hepatocellular carcinoma. *Ann Surg Oncol* 21: 758-766, 2014.
2. Morise Z, Kawabe N, Tomishige H, Nagata H, Kawase J, Arakawa S, Yoshida R and Isetani M: Recent advances in liver resection for hepatocellular carcinoma. *Front Surg* 1: 21, 2014.
3. Chan TH, Lin CH, Qi L, Fei J, Li Y, Yong KJ, Liu M, Song Y, Chow RK, Ng VH, *et al*: A disrupted RNA editing balance mediated by ADARs (Adenosine Deaminases that act on RNA) in human hepatocellular carcinoma. *Gut* 63: 832-843, 2014.
4. Raveh E, Matouk IJ, Gilon M and Hochberg A: The H19 long non-coding RNA in cancer initiation, progression and metastasis—a proposed unifying theory. *Mol Cancer* 14: 184, 2015.

5. Zhu AX, Kang YK, Rosmorduc O, Evans TR, Santoro A, Ross P, Gane E, Vogel A, Jeffers M, Meinhart G and Peña CE: Biomarker analyses of clinical outcomes in patients with advanced hepatocellular carcinoma treated with sorafenib with or without erlotinib in the SEARCH trial. *Clin Cancer Res* 22: 4870-4879, 2016.
6. Ikeda M, Shimizu S, Sato T, Morimoto M, Kojima Y, Inaba Y, Hagihara A, Kudo M, Nakamori S, Kaneko S, *et al*: Sorafenib plus hepatic arterial infusion chemotherapy with cisplatin versus sorafenib for advanced hepatocellular carcinoma: Randomized phase II trial. *Ann Oncol* 27: 2090-2096, 2016.
7. Lencioni R, Chen XP, Dagher L and Venook AP: Treatment of intermediate/advanced hepatocellular carcinoma in the clinic: How can outcomes be improved? *Oncologist* 15 (Suppl 4): S42-S52, 2010.
8. Finn RS, Zhu AX, Farah W, Almasri J, Zaiem F, Prokop LJ, Murad MH and Mohammed K: Therapies for advanced stage hepatocellular carcinoma with macrovascular invasion or metastatic disease: A systematic review and meta-analysis. *Hepatology* 67: 422-435, 2018.
9. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, *et al*: Role of HIF-1 α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 394: 485-490, 1998.
10. Eyries M, Siegfried G, Ciumas M, Montagne K, Agrapart M, Lebrin F and Soubrier F: Hypoxia-induced apelin expression regulates endothelial cell proliferation and regenerative angiogenesis. *Circ Res* 103: 432-440, 2008.
11. Semenza GL: Hypoxia-inducible factor 1 (HIF-1) pathway. *Sci STKE* 2007: cm8, 2007.
12. Zhang H, Lu H, Xiang L, Bullen JW, Zhang C, Samanta D, Gilkes DM, He J and Semenza GL: HIF-1 regulates CD47 expression in breast cancer cells to promote evasion of phagocytosis and maintenance of cancer stem cells. *Proc Natl Acad Sci USA* 112: E6215-E6223, 2015.
13. Botusan IR, Sunkari VG, Savu O, Catrina AI, Grünler J, Lindberg S, Pereira T, Ylä-Herttuala S, Poellinger L, Brismar K and Catrina SB: Stabilization of HIF-1 α is critical to improve wound healing in diabetic mice. *Proc Natl Acad Sci USA* 105: 19426-19431, 2008.
14. Kim DH, Sung B, Kim JA, Kang YJ, Hwang SY, Hwang NL, Suh H, Choi YH, Im E, Chung HY and Kim ND: HS-1793, a resveratrol analogue, downregulates the expression of hypoxia-induced HIF-1 and VEGF and inhibits tumor growth of human breast cancer cells in a nude mouse xenograft model. *Int J Oncol* 51: 715-723, 2017.
15. Masoud GN and Li W: HIF-1 α pathway: Role, regulation and intervention for cancer therapy. *Acta Pharm Sin B* 5: 378-389, 2015.
16. Choudhry H and Harris AL: Advances in hypoxia-inducible factor biology. *Cell Metab* 27: 281-298, 2018.
17. Semenza GL: HIF-1: Upstream and downstream of cancer metabolism. *Curr Opin Genet Dev* 20: 51-56, 2010.
18. Soga T: Cancer metabolism: Key players in metabolic reprogramming. *Cancer Sci* 104: 275-281, 2013.
19. Meijer TW, Kaanders JH, Span PN and Bussink J: Targeting hypoxia, HIF-1, and tumor glucose metabolism to improve radiotherapy efficacy. *Clin Cancer Res* 18: 5585-5594, 2012.
20. Fan C, Tang Y, Wang J, Xiong F, Guo C, Wang Y, Zhang S, Gong Z, Wei F, Yang L, *et al*: Role of long non-coding RNAs in glucose metabolism in cancer. *Mol Cancer* 16: 130, 2017.
21. Wang S, Wang X, Weng Z, Zhang S, Ning H and Li B: Expression and role of microRNA 18b and hypoxia inducible factor-1 α in placental tissues of preeclampsia patients. *Exp Ther Med* 14: 4554-4560, 2017.
22. Li B, Qiu B, Lee DS, Walton ZE, Ochocki JD, Mathew LK, Mancuso A, Gade TP, Keith B, Nissim I and Simon MC: Fructose-1,6-bisphosphatase opposes renal carcinoma progression. *Nature* 513: 251-255, 2014.
23. Sato R, Semba T, Saya H and Arima Y: Concise review: Stem cells and epithelial-mesenchymal transition in cancer: Biological implications and therapeutic targets. *Stem Cells* 34: 1997-2007, 2016.
24. May CD, Sphyris N, Evans KW, Werden SJ, Guo W and Mani SA: Epithelial-mesenchymal transition and cancer stem cells: A dangerously dynamic duo in breast cancer progression. *Breast Cancer Res* 13: 202, 2011.
25. Kalluri R and Weinberg RA: The basics of epithelial-mesenchymal transition. *J Clin Invest* 119: 1420-1428, 2009.
26. Shi S, Zhao J, Wang J, Mi D and Ma Z: HPIP silencing inhibits TGF- β 1-induced EMT in lung cancer cells. *Int J Mol Med* 39: 479-483, 2017.
27. Dong C, Yuan T, Wu Y, Wang Y, Fan TW, Miriyala S, Lin Y, Yao J, Shi J, Kang T, *et al*: Loss of FBP1 by Snail-mediated repression provides metabolic advantages in basal-like breast cancer. *Cancer Cell* 23: 316-331, 2013.
28. Jourdan-Le-Saux C, Tronecker H, Bogic L, Bryant-Greenwood GD, Boyd CD and Csiszar K: The LOXL2 gene encodes a new lysyl oxidase-like protein and is expressed at high levels in reproductive tissues. *J Biol Chem* 274: 12939-12944, 1999.
29. Choi SE, Jeon N, Choi HY, Shin JI, Jeong HJ and Lim BJ: Lysyl oxidase-like 2 is expressed in kidney tissue and is associated with the progression of tubulointerstitial fibrosis. *Mol Med Rep* 16: 2477-2482, 2017.
30. Wu L and Zhu Y: The function and mechanisms of action of LOXL2 in cancer (Review). *Int J Mol Med* 36: 1200-1204, 2015.
31. Cuevas EP, Eraso P, Mazón MJ, Santos V, Moreno-Bueno G, Cano A and Portillo F: LOXL2 drives epithelial-mesenchymal transition via activation of IRE1-XBP1 signalling pathway. *Sci Rep* 7: 44988, 2017.
32. Hutchinson JH, Rowbottom MW, Lonergan D, Darlington J, Prodanovich P, King CD, Evans JF and Bain G: Small molecule lysyl Oxidase-like 2 (LOXL2) inhibitors: The identification of an inhibitor selective for LOXL2 over LOX. *ACS Med Chem Lett* 8: 423-427, 2017.
33. Peinado H, Del Carmen Iglesias-de la Cruz M, Olmeda D, Csiszar K, Fong KS, Vega S, Nieto MA, Cano A and Portillo F: A molecular role for lysyl oxidase-like 2 enzyme in snail regulation and tumor progression. *EMBO J* 24: 3446-3458, 2005.
34. Chitty JL, Setargew YFI and Cox TR: Targeting the lysyl oxidases in tumour desmoplasia. *Biochem Soc Trans* 47: 1661-1678, 2019.
35. Leung L, Niculescu-Duvaz D, Smithen D, Lopes F, Callens C, McLeary R, Saturno G, Davies L, Aljarah M, Brown M, *et al*: Anti-metastatic Inhibitors of Lysyl Oxidase (LOX): Design and Structure-activity relationships. *J Med Chem* 62: 5863-5884, 2019.
36. Tang H, Leung L, Saturno G, Viros A, Smith D, Di Leva G, Morrison E, Niculescu-Duvaz D, Lopes F, Johnson L, *et al*: Lysyl oxidase drives tumour progression by trapping EGF receptors at the cell surface. *Nat Commun* 8: 14909, 2017.
37. Smithen DA, Leung LMH, Challinor M, Lawrence R, Tang H, Niculescu-Duvaz D, Pearce SP, Mcleary R, Lopes F, Aljarah M, *et al*: 2-Aminomethylene-5-sulfonylthiazole inhibitors of Lysyl Oxidase (LOX) and LOXL2 show significant efficacy in delaying tumor growth. *J Med Chem*: Sep 4, 2019 (Epub ahead of print). doi: 10.1021/acs.jmedchem.9b01112.
38. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
39. Harrison SA, Abdelmalek MF, Caldwell S, Shiffman ML, Diehl AM, Ghalib R, Lawitz EJ, Rockey DC, Schall RA, Jia C, *et al*: Simtuzumab is ineffective for patients with bridging fibrosis or compensated cirrhosis caused by nonalcoholic steatohepatitis. *Gastroenterology* 155: 1140-1153, 2018.
40. Altinbas A: A quick overview to the early phase clinical trials of Simtuzumab®: Are we losing the most promising anti-fibrotic product? *Med Hypotheses* 108: 159-160, 2017.
41. Lunt SY and Vander Heiden MG: Aerobic glycolysis: Meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol* 27: 441-464, 2011.
42. Brault C, Zerbib Y, Delette C, Marc J, Gruson B, Marolleau JP and Maizel J: The warburg effect as a type B lactic acidosis in a patient with acute myeloid leukemia: A diagnostic challenge for clinicians. *Front Oncol* 8: 232, 2018.
43. Qu Y, Xiao H, Xiao W, Xiong Z, Hu W, Gao Y, Ru Z, Wang C, Bao L, Wang K, *et al*: Upregulation of MIAT regulates LOXL2 expression by competitively binding MiR-29c in clear cell renal cell carcinoma. *Cell Physiol Biochem* 48: 1075-1087, 2018.
44. Zaffryar-Eilot S, Marshall D, Voloshin T, Bar-Zion A, Spangler R, Kessler O, Ghermazien H, Brekhan V, Suss-Toby E, Adam D, *et al*: Lysyl oxidase-like-2 promotes tumour angiogenesis and is a potential therapeutic target in angiogenic tumours. *Carcinogenesis* 34: 2370-2379, 2013.
45. Cheng L, Yu H, Yan N, Lai K and Xiang M: Hypoxia-inducible factor-1 α target genes contribute to retinal neuroprotection. *Front Cell Neurosci* 11: 20, 2017.
46. Shi L, He C, Li Z, Wang Z and Zhang Q: FBP1 modulates cell metabolism of breast cancer cells by inhibiting the expression of HIF-1 α . *Neoplasia* 64: 535-542, 2017.
47. Lee K and Kim HM: A novel approach to cancer therapy using PX-478 as a HIF-1 α inhibitor. *Arch Pharm Res* 34: 1583-1585, 2011.

48. Barone C, Koeberle D, Metselaar H, Parisi G, Sansonno D and Spinzi G: Multidisciplinary approach for HCC patients: Hepatology for the oncologists. *Ann Oncol* 24 (Suppl 2): ii15-ii23, 2013.
49. Ahn SG, Dong SM, Oshima A, Kim WH, Lee HM, Lee SA, Kwon SH, Lee JH, Lee JM, Jeong J, *et al*: LOXL2 expression is associated with invasiveness and negatively influences survival in breast cancer patients. *Breast Cancer Res Treat* 141: 89-99, 2013.
50. Bais MV and Goldring MB: LOXL2 as a protective in osteoarthritis cartilage. *Aging (Albany NY)* 9: 2024-2025, 2017.
51. Atala A: Re: Tumour-suppressive MicroRNA-29s directly regulate LOXL2 expression and inhibit cancer cell migration and invasion in renal cell carcinoma. *J Urol* 195: 1622, 2016.
52. Ye Y, Song YN, He SF, Zhuang JH, Wang GY and Xia W: GINS2 promotes cell proliferation and inhibits cell apoptosis in thyroid cancer by regulating CITED2 and LOXL2. *Cancer Gene Ther* 26: 103-113, 2019.
53. Park JS, Lee JH, Lee YS, Kim JK, Dong SM and Yoon DS: Emerging role of LOXL2 in the promotion of pancreas cancer metastasis. *Oncotarget* 7: 42539-42552, 2016.
54. Wu L, Zhang Y, Zhu Y, Cong Q, Xiang Y and Fu L: The effect of LOXL2 in hepatocellular carcinoma. *Mol Med Rep* 14: 1923-1932, 2016.
55. Barker HE, Chang J, Cox TR, Lang G, Bird D, Nicolau M, Evans HR, Gartland A and Erler JT: LOXL2-mediated matrix remodeling in metastasis and mammary gland involution. *Cancer Res* 71: 1561-1572, 2011.
56. Cuevas EP, Moreno-Bueno G, Canesin G, Santos V, Portillo F and Cano A: LOXL2 catalytically inactive mutants mediate epithelial-to-mesenchymal transition. *Biol Open* 3: 129-137, 2014.
57. Liu X, Wang X, Zhang J, Lam EK, Shin VY, Cheng AS, Yu J, Chan FK, Sung JJ and Jin HC: Warburg effect revisited: An epigenetic link between glycolysis and gastric carcinogenesis. *Oncogene* 29: 442-450, 2010.
58. Wong CC, Tse AP, Huang YP, Zhu YT, Chiu DK, Lai RK, Au SL, Kai AK, Lee JM, Wei LL, *et al*: Lysyl oxidase-like 2 is critical to tumor microenvironment and metastatic niche formation in hepatocellular carcinoma. *Hepatology* 60: 1645-1658, 2014.
59. Wang M, Zhao X, Zhu D, Liu T, Liang X, Liu F, Zhang Y, Dong X and Sun B: HIF-1 α promoted vasculogenic mimicry formation in hepatocellular carcinoma through LOXL2 up-regulation in hypoxic tumor microenvironment. *J Exp Clin Cancer Res* 36: 60, 2017.
60. Ninomiya G, Yamada S, Hayashi M, Takeda S, Suenaga M, Takami H, Kanda M, Iwata N, Niwa Y, Tanaka C, *et al*: Significance of Lysyl oxidaselike 2 gene expression on the epithelial-mesenchymal status of hepatocellular carcinoma. *Oncol Rep* 39: 2664-2672, 2018.
61. Yang MH, Chen CL, Chau GY, Chiou SH, Su CW, Chou TY, Peng WL and Wu JC: Comprehensive analysis of the independent effect of twist and snail in promoting metastasis of hepatocellular carcinoma. *Hepatology* 50: 1464-1474, 2009.
62. Wu TJ, Chang SS, Li CW, Hsu YH, Chen TC, Lee WC, Yeh CT and Hung MC: Severe hepatitis promotes hepatocellular carcinoma recurrence via NF- κ B pathway-mediated epithelial-mesenchymal transition after resection. *Clin Cancer Res* 22: 1800-1812, 2016.
63. Hirata H, Sugimachi K, Komatsu H, Ueda M, Masuda T, Uchi R, Sakimura S, Nambara S, Saito T, Shinden Y, *et al*: Decreased expression of Fructose-1,6-bisphosphatase associates with glucose metabolism and tumor progression in hepatocellular carcinoma. *Cancer Res* 76: 3265-3276, 2016.
64. Yang J, Jin X, Yan Y, Shao Y, Pan Y, Roberts LR, Zhang J, Huang H and Jiang J: Inhibiting histone deacetylases suppresses glucose metabolism and hepatocellular carcinoma growth by restoring FBP1 expression. *Sci Rep* 7: 43864, 2017.
65. Yang J, Wang C, Zhao F, Luo X, Qin M, Arunachalam E, Ge Z, Wang N, Deng X, Jin G, *et al*: Loss of FBP1 facilitates aggressive features of hepatocellular carcinoma cells through the Warburg effect. *Carcinogenesis* 38: 134-143, 2017.
66. Jiang H, Zhou Z, Jin S, Xu K, Zhang H, Xu J, Sun Q, Wang J and Xu J: PRMT9 promotes hepatocellular carcinoma invasion and metastasis via activating PI3K/Akt/GSK-3 β /Snail signaling. *Cancer Sci* 109: 1414-1427, 2018.
67. Liu GM, Li Q, Zhang PF, Shen SL, Xie WX, Chen B, Wu J, Hu WJ, Huang XY and Peng BG: Restoration of FBP1 suppressed Snail-induced epithelial to mesenchymal transition in hepatocellular carcinoma. *Cell Death Dis* 9: 1132, 2018.
68. Chen Z, Zuo X, Zhang Y, Han G, Zhang L, Wu J and Wang X: MiR-3662 suppresses hepatocellular carcinoma growth through inhibition of HIF-1 α -mediated Warburg effect. *Cell Death Dis* 9: 549, 2018.
69. Courtney R, Ngo DC, Malik N, Ververis K, Tortorella SM and Karagiannis TC: Cancer metabolism and the Warburg effect: The role of HIF-1 and PI3K. *Mol Biol Rep* 42: 841-851, 2015.
70. Takano S, Ishikawa E, Matsuda M, Sakamoto N, Akutsu H, Yamamoto T and Matsumura A: The anti-angiogenic role of soluble-form VEGF receptor in malignant gliomas. *Int J Oncol* 50: 515-524, 2017.
71. Zhu AX, Duda DG, Sahani DV and Jain RK: HCC and angiogenesis: Possible targets and future directions. *Nat Rev Clin Oncol* 8: 292-301, 2011.
72. Yang ZF and Poon RT: Vascular changes in hepatocellular carcinoma. *Anat Rec (Hoboken)* 291: 721-734, 2008.
73. Berretta M, Rinaldi L, Di Benedetto F, Lleshi A, De Re V, Facchini G, De Paoli P and Di Francia R: Angiogenesis inhibitors for the treatment of hepatocellular carcinoma. *Front Pharmacol* 7: 428, 2016.
74. Mossenta M, Busato D, Baboci L, Cintio FD, Toffoli G and Bo MD: New insight into therapies targeting angiogenesis in hepatocellular carcinoma. *Cancers (Basel)* 11: pii: E1086, 2019.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.