The pathological significance of LOXL2 in pre-metastatic niche formation of HCC and its related molecular mechanism

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Keywords: Hepatocellular carcinoma, Pre-metastatic niche, LOXL2

Posted Date: May 22nd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-30036/v1>

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Abstract

Background: The mechanisms underlying the contribution of primary tumor to pre-metastatic niche formation remains largely unknown in HCC. We previously reported that the released Lysyl oxidase like 2 (LOXL2) from HCC cells under higher stiffness stimulation facilitated the formation of lung pre-metastatic niche. Here we further clarified the pathological role of LOXL2 in promoting lung pre-metastatic niche formation in HCC and its relevant molecular mechanism.

Methods: Lung pre-metastatic niche nude mouse model and LOXL2-overexpressed xenograft HCC models were respectively developed to validate the significance of LOXL2 in pre-metastatic niche formation and pulmonary metastasis. Simultaneously, a gel-based cell culture system mirroring lung tissue stiffness in normal and pathological state was constructed to investigate the underlying mechanism of LOXL2-induced pre-metastatic niche formation.

Results: LOXL2 by tail vein injection had obvious inducing effects on CD11b⁺/CD45⁺ BMDCs recruitment and fibronectin expression in lung tissue during the development of lung pre-metastatic niche. Tumor-secreted LOXL2 also promoted the occurrence of pulmonary metastasis and the number of metastasis lesions remarkably in LOXL2-overexpressed xenograft HCC models. Applying a gel-based cell culture system, we discovered that LOXL2 and LOXL2-caused matrix stiffening all upregulated the expressions of MMP9 and fibronectin in lung fibroblasts significantly, but little effect on MMP2 expression. Moreover, the activation of PI3K-AKT pathway participated in the regulation of LOXL2-upregulated fibronectin. Additionally, LOXL2 and LOXL2-caused matrix stiffening also increased the number of adherent HCC cells evidently, as well as the expression of chemokine CXCL2. Clinical data analysis demonstrated that HCC patients in High-LOXL2 group had higher ratio of microvascular invasion (OR=10.563, P=0.005) and tumor recurrence (OR=8.556, P=0.013) than HCC patients in Low-LOXL2 group, also revealing a significance of LOXL2 in HCC progression and unfavorable outcome

Conclusion: Primary tumor-released LOXL2 contributes to the formation of lung pre-metastatic niche and the occurrence of lung metastasis. LOXL2 and LOXL2-caused matrix stiffening work together to induce pre-metastatic niche formation through influencing matrix remodeling and cell colonization. This study sheds light on the pathological significance of LOXL2 in lung pre-metastatic niche formation in HCC, also implicates a new intervention approach for HCC metastasis through reversal of lung pre-metastatic niche.

Background

Although three classical theories including seed and soil hypothesis [1-3], circulatory theory [3,4], and the cascade theory of metastatic spread [5] have well explained the metastasis mechanism of solid tumor from different aspects such as non-randomness of metastasis, metastasis pathway, multi-stages of metastasis, they are still unable to answer how primary tumor chooses the target organ and determine the formation of metastatic foci. The hypothesis of pre-metastatic niche, proposed by Kaplan in 2005 [6,7], builds an ideal bridge for the first time between primary tumor and metastatic lesion in the target

organ, and exhibits an obvious theoretical innovation in organotropism metastasis over three classical metastasis theories. Primary tumor-released soluble factors or exosomes enter the blood stream and reach distant target organ to induce a permissive environment before the arrival of metastatic tumor cells, termed the pre-metastatic niche. The induced pre-metastatic niche not only offers a favorable soil environment for the adherence and colonization of disseminated tumor cells, but also accelerates the completion of organ-specific metastasis. Currently, the existence of pre-metastatic niche has been validated in multiple tumor animal models including breast cancer, colon cancer, pancreatic cancer, melanoma and so on [8,9]. Matrix remodeling, Bone marrow derived cells (BMDCs) recruitment, inflammation, immunosuppressive and nutrient rich microenvironment, identified as typical pathological alterations during the development of pre-metastatic niche [10-13], have gradually become potential indicators to judge the occurrence of this pathological event. Some secreted factors from primary tumor such as CXCL17, G-CSF, OPN, CXCL12, TNF- α , TGF- β , VEGF-A, and PIGF have been characterized to contribute to the formation of pre-metastatic niche through influencing CD11b⁺/Gr-1⁺ myeloid-derived suppressor cells (MDSC)-mediated angiogenesis, inducing inflammation, remodeling extracellular matrix (ECM), altering niche cells, and recruiting immune cells [8,12,14]. Similarly, the released exosomes from primary tumor also reveal to trigger premetastatic niche formation via educating the resident cells in distant target organ [15]. MicroRNA-21-5p in small extracellular vesicles derived from colon cancer cells regulates macrophage polarization in the liver niche to drive an inflammatory pre-metastatic niche and then promote colon cancer liver metastasis [16]. In addition to tumor cells, other cells in tumor microenvironment like macrophages also participate in pre-metastatic niche formation in colorectal cancer liver metastases by producing CXCL1 to recruit MDSCs with CXCR2(+) [17]. Recently, some hypoxia-induced factors like Lysyl oxidase (LOX) present stronger abilities in inducing the formation of pre-metastatic niche [18], indicating an important pathological role of biophysical cue in pre-metastatic niche formation. Increased matrix stiffness is a remarkable physical property of Hepatocellular carcinoma (HCC) and its clinical significance has been highlighted in prediction of HCC progression and prognosis [19-21]. Other studies in vitro also suggest that higher matrix stiffness strengthens malignant characteristics of HCC cells and facilitates HCC invasion and metastasis by upregulating invasion/metastasis-associated gene expression [22,23], enhancing stemness characteristics [24], triggering the occurrence of epithelial-mesenchymal transition [25,26], promoting therapeutic resistance [27]. However, little effort has been dedicated to exploring the relationship between matrix stiffness and pre-metastatic niche in HCC. Our previous research demonstrated that the secreted Lysyl oxidase like 2 (LOXL2) from HCC cells under higher stiffness stimulation educated lung fibroblast and recruited BMDCs to induce lung pre-metastatic niche formation in vitro [28]. Here we further clarified the pathological roles of LOXL2 in premetastatic niche formation in two animal models and HCC tissues, simultaneously applied an in vitro system of mechanically tunable gel simulating lung tissue stiffness to investigate its underlying mechanism.

Materials And Methods

Cells and cell culture

MHCC97H was established at the Liver Cancer Institute of Zhongshan Hospital, Fudan University. Human embryo lung fibroblasts (HELFs) were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, CAS. MHCC97H cells and HELF cells were all cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Biowest) and 1% penicillin/streptomycin (Gibco).

Preparation of HCC cells with LOXL2 overexpression and their CM-LV-LOXL2-OE

MHCC97H cells with LV-LOXL2-OE and MHCC97H cells with LV-scramble were constructed by lentiviral-mediated RNA overexpression technology. After the transfected cells were grown in serum-free DMEM for 24h, their culture supernatants including CM-LV-LOXL2-OE and CM-LV-NC were collected, respectively. The detailed experiment procedures were same as the method reported previously [28].

Animal models

(1) Nude mouse model of pre-metastatic niche established by tail vein injection of CM-LV-LOXL2-OE.

BALB/C nude mice (male, 4 weeks old) were purchased from SLAC Laboratory Animal Co., Shanghai, China. All the nude mice were divided into 4 groups such as group 1 (CM-LV-NC), group 2 (CM-LV-LOXL2-OE), group 3 (Tumor/CM-LV-NC) and group 4 (Tumor/CM-LV-LOXL2-OE). Approximate 2×10^7 MHCC97H cells were inoculated subcutaneously into the right flank of each mouse in group 3 and group 4 to form subcutaneous tumor. On the 7th day after the inoculation, all the nude mice began to receive tail vein injection. The nude mice in group 1 and 3 were injected with CM-LV-NC (200 μ l/d, LOXL2 <0.0002 μ g/d), and group 2 and 4 with CM-LV-LOXL2-OE (200 μ l/d, LOXL2=0.045 μ g/d). Fifteen days later, the injection volume was changed to be 350 μ l/d. The injection was performed every other day. On the 14th, 22nd and 24th day after injection, the mice were executed by euthanasia. *Their lungs were douched slowly from the right ventricle with D-Hank's equilibrium solution. When the color of the lung turned to pale, the lung was collected and cut into small pieces of 1-2mm³. Subsequently, they were dissociated into single cell suspension in buffer containing type 1 collagenase (2mg/ml) using a GentleMACS Octo Dissociator (Miltenyi Biotec). The collected cells were filtered using a cell strainer with 70- μ m (Millipore) and treated in erythrocyte lysis buffer (Beyotime). After washed three times with PBS, the purified primary cells from lung tissue were used for flow cytometry analysis.* Simultaneously, the expression of the related molecules to pre-metastatic niche formation were detected in the collected lung tissues by immunohistochemistry, immunofluorescence and qRT-PCR. **(2) Xenograft HCC model** A subcutaneous HCC tumor derived from LV-LOXL2-OE-MHCC97H cells was established in nude mice by subcutaneous inoculation, and another subcutaneous HCC tumor from LV-NC-MHCC97H cells was also established as the control. Small tumor masses with the size of 2 $\times$ 2 $\times$ 2 mm³ obtained from two subcutaneous HCC tumors were orthotopically transplanted into the livers of new mice. Six weeks later, two groups of xenograft HCC models were formed successfully including LOXL2 overexpression group and the control group (6 nude mice in each group). HCC tissues, lung tissues and serum samples of xenograft HCC model were collected for evaluating the roles of LOXL2 in promoting HCC metastasis.

All animal care and experiment approved by Animal Care Ethical Committee of Fudan University (Permit Number: 2016-120) met the requirements of the guideline for the Care and Use of Laboratory Animals published by the National Academy of Science.

In vitro system of mechanically tunable gel simulating lung tissue stiffness

Taking use of dual biological function of LOXL2 in lung premetastatic niche formation and matrix crosslinking/tissue stiffness, we mixed COL1 (3.58 mg/ml, Corning), basic DMEM and CM-LV-LOXL2-OE (310 ng/ml) in 1:10:5 to construct high stiffness substrate mirroring the stiffness of lung premetastatic niche. Briefly, 2 ml of the mixture was first prepared, and then placed into a 6 well plate and incubated in 37°C incubator for 1.5 hours, subsequently the gel substrate was placed at 4°C for another 12 hours and form a gel-based culture platform with stiffness of 926.18 Pa. Simultaneously, we prepared a low stiffness substrate by mixing COL1 and basic DMEM in 1:20 as the control substrate (202.43 Pa) to mirror the stiffness of lung tissue. HELF cells were grown on different stiffness substrates mentioned above and treated with CM-LV-LOXL2-OE (310 ng/ml). Stiffness of the gel substrate was measured by TA.XTplusC Texture Analyzer (SMS, UK).

Detection of CD11b⁺/CD45⁺ BMDCs in lung tissue by Flow Cytometry (FCM)

Approximate 1×10^6 of the purified primary cells from lung tissue were prepared and resuscitated?? with 100ul of 1% BSA to block unspecific binding, then they were incubated with FITC-conjugated CD11b antibody (1:100, BD Pharmingen) and PE-conjugated CD45 antibody (1:100, BD Pharmingen) at 4°C for 30 minutes, ultimately the treated cells were subjected to flow cytometry analysis. Unstained cells, CD11b single-stained cells, and CD45 single-stained cells were set as gating in the experiment.

The levels of serum LOXL2 in xenograft HCC models detected by Enzyme-linked immunosorbent assay (ELISA)

The levels of serum LOXL2 in xenograft HCC model were measured by ELISA (human LOXL2 ELISA Kit, Boster China) in accordance with the manufacturer's instructions. Briefly, 100 µl of serum sample was added into each well and incubated at 37°C for 90 min, then reacted with 100 µl biotin-labeled anti-human LOXL2 antibody at 37°C for 60 min. Subsequently, the captured antigen-antibody complex was further incubated in 100 µl ABC working solution for 30 min at 37°C. Finally, substrate solution was used for color rendering. The absorbance was detected by a microplate reader (Thermo) at 450nm wavelength.

Cell adhesion assay

When they were grown on the gel substrate in each well to reach 60% confluence, lung fibroblasts were treated separately with CM-LV-LOXL2-OE and CM-LV-NC. Twenty-four hours later, 2×10^5 GFP-labeled HCC cells were evenly seeded into the well. After 24 hours culture, the attached HCC cells on the surface of

HELF cells were photographed using a fluorescent microscopy (Olympus). The average number of attached HCC cells was measured by counting six random microscope fields (×200).

Immunohistochemistry

The detailed procedure of immunohistochemistry sees our previous work [25]. Tissue slides were separately incubated with the diluted primary antibody against LOXL2 (1:200, Novus), Fibronectin (1:100, Proteintech), Collagen I (1:200, Affinity), LOX (1:100, Abcam) at 4°C overnight, and then reacted with the diluted secondary antibody(?). Photographs of representative sites were captured under high-power magnification (×100) using same setting parameters.

Double staining immunofluorescence

Fresh lung tissues derived from animal model were fixed in neutral formalin(?), and then embedded in paraffin and cut into 3µm thick sections for immunofluorescence analysis. After being treated with citric acid antigen repair solution (1:50, Beyotime), tissue slides were incubated with the both of rat monoclonal [M1/70] antibody to CD11b (1:200, Abcam) and rabbit polyclonal antibody to CD45 (1:200, Bioss) at 4°C overnight. Subsequently, they were reacted with two secondary antibodies including Alexa Fluor 488 goat anti-Rabbit IgG (H+L) antibody (1:300, YESEN) and Alexa Fluor 594 Donkey anti-Rat IgG (H+L) antibody (1:300, YESEN), respectively. Finally, the slides were stained with DAPI (1:3000, Beyotime). Six representative sites of photographs were captured by a confocal microscopy (Leica, SP8)

Western blot

Total protein form tissues or cells was extracted in RIPA buffer (Beyotime, China) containing protease inhibitors (Beyotime, China) and PhosStop (Roche). About 20ug total protein was loaded and separated by SDS-PAGE and then transferred onto a PVDF membrane (Millipore). Subsequently, the membrane was blocked in 5% BSA for 2 hours, and the incubated with the following diluted primary antibodies at 4°C overnight: GAPDH (1:1000, Proteintech), Fibronectin (1:1000, Proteintech), MMP2 (1:500, Cell Signal Technology), MMP9 (1:1000, Cell Signal Technology), p-AKT (1:1000, Cell Signal Technology), AKT (1:1000, Cell Signal Technology). Afterwards, the membrane was incubated at room temperature for 1h with HRP-conjugated secondary antibody (1:5000, Proteintech). Finally, the target band was detected using enhanced chemiluminescence (Tanon, China).

qRT-PCR

The detailed procedure of qRT-PCR was same as the methods described previously [28]. The primer sequences of the target genes are listed in **Table1**.

Table1. Primer sequence used for qRT-PCR

Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')
MMP2	GTTCAATTTGGCGGACTGT	AGGGTGCTGGCTGAGTAG
MMP9	GGGACGCAGACATCGTCATC	TCGTCATCGTCGAAATGGGC
CXCL12	GTTCAAAGCCAGCGTC	TAGTTCACCCCAAAGGA
Fibronectin	ATGCAACGATCAGGACACAAGGAC	GCCTCTCACACTTCCACTCTCC
GAPDH	CTCCTCCACCTTTGACGC	CCACCACCCTGTTGCTGT

HCC patients

Clinical data of 34 patients with HCC who received curative hepatectomy in Taizhou People's Hospital from March 2015 to March 2019 were retrospectively analyzed. All the patients did not receive any anticancer treatment before operation. Tumor differentiation was assessed according to the Edmondson grading system. Microvascular invasion was evaluated by the presence of tumor emboli within either the central hepatic vein, the portal, or the large capsular vessels [29]. Postoperative patients were followed up until September 2019, with a median follow-up of 18.8 months (range from 6 to 47 months). Tumor tissue samples of the patients were obtained from Department of Pathology, Taizhou People's Hospital. The expression levels of LOX, COL1 and LOXL2 in HCC tissues were analyzed by immunohistochemistry staining. The grey value of positive stained area in each photograph was measured by Image J v1.8.0. HCC patients were divided into two groups based on LOXL2 median expression value. The study was approved by the the Taizhou People's Hospital Research Ethics Committee.

Statistical analysis

Data analysis was performed with Grahpad Prism v8.2.1 statistical software and SPSS 20.0 statistical software (SPSS Inc., Chicago, IL). Classified variables were described using percentages frequency, continuous variables were reported using median (range) or mean (SD). Student's t-test was used to compare the mean of two continuous variables, and Fisher exact test was used to compare the ratio of classified variables. Spearman analysis was performed to analyze the correlation between LOXL2, LOX and COL1. $P < 0.05$ represented a statistical significance. All the experiments were performed in triplicate.

Results

The roles of LOXL2 in the formation of lung pre-metastatic niche in nude mouse

Previously, we reported that the released LOXL2 from HCC cells under higher stiffness stimulation educated lung fibroblast and recruited BMDCs to promote lung pre-metastatic niche formation in vitro [28]. Here we further validated the pathological significance of LOXL2 during the formation of pre-metastatic niche in animal model. We injected CM-LV-LOXL2-OE into tail vein, which simulated primary tumor-released TDSFs, to induce lung pre-metastatic niche formation in nude mouse. Based on the state of BMDCs recruitment in lung tissues at different time points, we found that CM-LV-LOXL2-OE injection resulted in an obvious increase in the proportion of CD11b⁺/CD45⁺ cells in lung tissues, regardless of the

presence of subcutaneous tumor (Fig.1B), highlighting a significant role of blood LOXL2 in lung BMDCs recruitment. Simultaneously, on the 24th day, lung BMDCs recruitment reached a peak value under CM-LV-LOXL2-OE induction, suggesting that tumor-derived LOXL2 present strong ability to induce lung pre-metastatic niche formation successfully in nude mouse. Analysis of double staining immunofluorescence also demonstrated that the number of cells with CD11b⁺/CD45⁺ in lung tissue section of the injected mice with CM-LV-LOXL2-OE was much more than that of the injected mice with CM-LV-NC (Fig.1C), in accordance with the results of FCM analysis. Considering that fibronectin production increase implicates occurrence of matrix remodeling in lung tissue, we continued to detect the expression of fibronectin in the lung tissues at both mRNA and protein level, and found that lung tissues of the injected mice with CM-LV-LOXL2-OE all exhibited higher expression in fibronectin as compared with the controls (Fig.1D-E), revealing that LOXL2 can increase the production of fibronectin in the lung tissues and in turn influence lung matrix remodeling. Additionally, the injected LOXL2 also showed to reach the lung tissue in immunohistochemistry analysis (Fig.1A). Taken together, the released LOXL2 from HCC cells participated in the formation of lung pre-metastatic niche by promoting BMDCs recruitment and matrix remodeling.

LOXL2 overexpression facilitated lung metastasis of xenograft HCC model

Subsequently, we established two groups of xenograft HCC nude mouse models using LV-LOXL2-OE-MHCC97H cells and LV-NC-MHCC97H cells to clarify the roles of LOXL2 in growth and metastasis of HCC. As shown in Fig.2A, there was no significant changes in the size of xenograft HCC tumors between the two groups, indicating that LOXL2 has little effects on the growth of xenograft HCC tumors. High expression of LOXL2 in tumor tissues (Fig.2B) and high level of LOXL2 in serum (Fig.2C) in LOXL2-overexpressed xenograft HCC model group all supported that LOXL2-overexpressed xenograft HCC models were successfully developed and tumor-secreted LOXL2 was released into the blood. Importantly, the pulmonary metastasis ration of LOXL2-overexpressed xenograft HCC models (6/6) was obviously higher than that of the controls (2/6) (Fig.2F), and the number of metastasis lesions in LOXL2-overexpressed xenograft HCC models (19 sites) was much more than that in the control group (7 sites). These results suggest that LOXL2 derived from primary tumor can promote lung metastasis in HCC remarkably, in agreement with the induction roles of LOXL2 in the formation of pre-metastatic niche. Meanwhile, we analyzed the state of BMDCs recruitment in lung tissues and found that the number of cells with CD11b⁺/CD45⁺ was increased evidently in LOXL2-overexpressed xenograft HCC models (Fig.2D), consistent with the results of nude mice model of pre-metastatic niche, further suggesting that the increase of CD11b⁺/CD45⁺ cell number is closely related to lung metastasis. Combining the results of pre-metastatic niche models and xenograft HCC models, we concluded that LOXL2 derived from primary HCC tumor participated in the formation of lung pre-metastatic niche and ultimately promoted the completion of lung metastasis in HCC.

LOXL2 and LOXL2-caused matrix stiffening participate in pre-metastatic niche formation through influencing matrix remodeling and cell colonization

The stiffness of lung tissue (approximate 200 Pa) is far lower than that of Petri dish (2-4 GPa)[30], thereby, the biological characteristics of cells grown on Petri dish are not able to exactly reflect the real pathological and physiological states of the resided cells in lung. Besides, matrix remodeling itself in pathological state also means the changes of matrix stiffness. Tumor-released LOXL2 can not only induce the formation of lung premetastatic niche formation by remodeling matrix and recruiting BMDCs [28], but also promote matrix crosslinking and increase matrix stiffness [31]. We here speculated that LOXL2 and LOXL2-induced matrix stiffening all participated in lung pre-metastatic niche formation. We respectively constructed high stiffness substrate (H, 926.18Pa, Fig. 3A (b), Supplementary Table 1) and low stiffness substrate (L, 202.434Pa, Fig.3A (a), Supplementary Table 1) to represent the stiffness of lung tissue in pathological state and physiological state. Using these two different stiffness substrates, we observed the morphological characteristics of lung fibroblasts and explored the effects of LOXL2 and LOXL2-caused matrix stiffening on the expressions of matrix remodeling-associated genes, and chemokine as well as the activation of related pathways in lung fibroblasts for better understand pre-metastatic niche formation.

Lung fibroblasts (HELFS) grew well on high or low stiffness substrates. Lung fibroblasts grown on substrate H exhibited an extension and expansion morphology, whereas on substrate L, they presented an appearance of round and small. Simultaneously, on same stiffness substrate, the size of lung fibroblasts treated with CM-LV-LOXL2-OE was more extended than that of the cells treated with CM-LV-NC (Fig.3B), implying that LOXL2 and LOXL2-caused matrix stiffening can obviously alter the morphology of lung fibroblasts and further influence their biological function.

The expression levels of matrix metalloproteinases (MMPs) and fibronectin generally indicate the state of matrix remodeling in lung tissue [6,32]. We further analyzed the expression levels of MMP9, MMP2, and fibronectin in lung fibroblasts grown on substrate H and L in the presence of CM-LV-LOXL2-OE or CM-LV-NC. MMP9 and fibronectin highly expressed in lung fibroblasts grown on substrate H compared with the cells on substrate L under same CM stimulation (Fig3C (a, c), Fig 3D (a, b)), demonstrating that LOXL2-caused matrix stiffening participates in regulation of matrix remodeling. Meanwhile, on the same stiffness substrate, CM-LV-LOXL2-OE stimulation significantly upregulated the expressions of MMP9 and fibronectin in lung fibroblasts (Fig. 3C (a, c), Fig 3D (a, b)), supporting the roles of LOXL2 in lung matrix remodeling. However, there was no significant changes in the expression of MMP2 among the four groups of cells (Fig. 3C (b), Fig.3D (a)), in consistence with the results of our previous work [28], indicating that MMP2 is not involved in the process of LOXL2-induced matrix remodeling. On the other hand, CM-LV-LOXL2-OE stimulation remarkably upregulated fibronectin expression and the phosphorylation level of AKT (Fig.3D (b)) in lung fibroblasts grown on substrate H and L. A recombinant protein LOXL2 (rhLOXL2) also showed same effects on the expression of fibronectin and p-AKT (Fig.3D (c)), indicating a linkage between PI3K-AKT pathway and LOXL2 expression. We applied PI3K inhibitor LY294002 to inactive phosphorylation of AKT, and found that it sharply attenuated the expression of fibronectin and p-AKT in lung fibroblasts grown on substrate H (Fig.3D (d)), validating that PI3K-AKT pathway participates in LOXL2-induced fibronectin upregulation.

Premetastatic niche created a suitable “soil” for colonization of circulating HCC cells. We used a modified cell adhesion assay to evaluate whether LOXL2-induced lung matrix remodeling was suitable for cell adhesion and colonization of HCC cells. No matter the lung fibroblasts were grown on high or low stiffness substrate, the numbers of adherent HCC cells on surface of lung fibroblasts treated with CM-LV-LOXL2-OE were all significantly higher than those of CM-LV-NC group (Fig.3E). Additionally, LOXL2-caused matrix stiffening also promoted the number of adherent HCC cells evidently (Fig.3E) These results indicates that LOXL2-induced matrix remodeling and LOXL2-caused matrix stiffening all assist HCC cell adhesion and colonization, ultimately promote the completion of lung metastasis. Except that, chemokine CXCL2 highly expressed in lung fibroblasts grown on substrate H treated with CM-LV-LOXL2-OE, which will be beneficial to the chemotaxis of HCC cells to site of lung pre-metastatic niche. Accordingly, the above data suggest that LOXL2 and LOXL2-caused matrix stiffening all contribute to pre-metastatic niche formation through influencing matrix remodeling and cell colonization.

Clinical significance of LOXL2 expression in HCC cohort

We first defined median expression level of LOXL2 in tumor tissues as a threshold to classify HCC patients into LOXL2 high expression group (High-LOXL2 group) and LOXL2 low expression group (Low-LOXL2 group). By a retrospective analysis of pathological parameters of HCC patients between High-LOXL2 group and Low-LOXL2 group, we found that HCC patients in High-LOXL2 group had higher ratio of microvascular invasion (OR=10.563, *P*=0.005) and tumor recurrence (OR=8.556, *P*=0.013) than HCC patients in Low-LOXL2 group (Table 2). The above results validate a clinical significance of LOXL2 upregulation in HCC progression and unfavorable outcome. Given that the expression levels of LOX and COL1 can indicate the grade of liver matrix stiffness, we further stratified HCC patients into LOX^{high}/COL1^{high} group (group H) and LOX^{low}/COL1^{low} group (group L) for analyzing the relationship between LOXL2 and matrix stiffness. HCC patients in High-LOXL2 had higher matrix stiffness (OR=5.76, *P*=0.038). As shown in Fig.4 (A-C), the expression of LOXL2 was obviously increased in HCC tissue in LOX^{high}/COL1^{high} group, as compared with that in LOX^{low}/COL1^{low} group, and positively correlated with the expression of LOX and COL1, respectively (Figure.4D), revealing that LOXL2 expression is positively correlated with matrix stiffness, in agreement with the results described previously[28].

Table 2 Clinic significance of LOXL2 in HCC cohort

Variables		Low-LOXL2 (%)	High-LOXL2 (%)	OR (95%CI)	P value
Age	≤59	8(47.06%)	8(47.06%)	1.000 (0.260-3.845)	1.000
	>59	9(52.94%)	9(52.94%)		
Sex	Male	14((82.35%)	14((82.35%)	1.000 (0.171-5.833)	1.000
	Female	3(21.45%)	3(21.45%)		
HBsAg	Negative	14(82.35%)	15(88.24%)	0.433 (0.068-2.764)	0.656
	Positive	3(21.45%)	2(11.76%)		
ALT (U/L)	≤40	12(70.59%)	11(64.71%)	1.309 (0.310-5.533)	1.000
	>40	5(29.41%)	6(35.29%)		
AST (U/L)	<35	10(58.82%)	8(47.06%)	1.607 (0.414-6.240)	0.732
	≥35	7(41.18%)	9(52.94%)		
AFP (ng/ml)	≤20	11(64.71%)	6(35.29%)	3.361 (0.823-13.722)	0.084
	>20	6(35.29%)	11(64.71%)		
Tumor size (cm)	<3*3	6(35.29%)	2(11.76%)	4.091 (0.690-24.244)	0.225
	≥3*3	11(64.71%)	15(88.24%)		
Microvascular invasion	no	13(76.47%)	4(23.53%)	10.563 (2.165-51.529)	0.005
	yes	4(23.53%)	13(76.47%)		
Tumor differentiation	high	8(47.06%)	5(29.41%)	2.133 (0.519-8.761)	0.481
	low	9(52.94%)	12(70.59%)		
Tumor recurrence	no	11(64.71%)	3(21.45%)	8.556 (1.736-42.169)	0.013
	yes	6(35.29%)	14(82.35%)		
Stiffness	Low (L)	12(70.59%)	5(29.41%)	5.76 (1.317-25.387)	0.038
	High (H)	5(29.41%)	12(70.59%)		

Discussion

Metastasis is still the major obstacle to improving therapeutic effect and prognosis of HCC patients. It is undoubtedly of great significance to identify the metastasis driver molecules for development of novel treatment strategy against HCC metastasis. Despite some progress has been made in the understanding of tumor metastasis mechanism during the last two decades, how primary tumor influences and determines the occurrence of metastatic lesion in the target organ remains largely dismal. The theory of pre-metastatic niche, which builds up a bridge between primary tumor and metastatic foci, offers a new

research direction for better understand organotropism metastasis [9,33]. The formation of pre-metastatic niche can facilitate the completion of distant metastasis obviously through constructing a favorable soil environment for colonization and growth of circulating tumor cells (CTC) [15], and gradually become an important speed-limiting step at the late stage of metastasis. So far, the identified tumor-derived soluble factors are still very limited for triggering pre-metastatic niche formation in HCC. LOXL2 upregulation has been identified to promote metastasis of gastric, breast and colorectal cancer [34-36], and implicates in less differentiation [37] and poor prognosis [35,38], implying a positive correlation between LOXL2 level and tumor metastasis. Except that, hypoxia-associated LOXL2 and LOX also exhibit a potential induction role in the formation of pre-metastatic niche in HCC and breast cancer [39,40]. High expression of LOXL2 alters tissue stiffness to promote the occurrence of intrahepatic metastasis in HCC, simultaneously enhances recruitment of BMDCs to metastatic site and facilitates completion of extrahepatic metastasis [40]. Our previous research demonstrated that the released LOXL2 from HCC cells under high stiffness stimulation facilitated the formation of lung pre-metastatic niche in vitro [28], highlighting the pathological significance of LOXL2 during matrix stiffness-induced pre-metastatic niche formation. Besides, LOXL2 presents an important biological function in matrix remodeling and tissue stiffening due to its catalyzing ability in the cross-linking of collagen and elastin [41]. Fibronectin deposition and MMPs all appear to be critical factors for remodeling matrix and regulating the formation of lung pre-metastatic niche [6, 15,33] On the other hand, fibronectin matrix modulates LOX catalytic activity by providing specific microenvironments [42]. Therefore, in addition to LOXL2 itself, we speculate that LOXL2-caused matrix stiffening may be also involved in the formation of lung pre-metastatic niche.

BMDCs recruitment and matrix remodeling are the most common pathological alterations during the formation of pre-metastasis niche. Firstly, we injected CM-LV-LOXL2-OE into the blood by tail vein simulating primary tumor-released TDSFs and validate the induction roles of LOXL2 in the formation of lung pre-metastatic niche formation. Our results showed that the injected LOXL2 significantly enhanced CD11b⁺/CD45⁺ BMDCs recruitment and fibronectin expression in lung tissue, suggesting that the injected LOX2 can induce the formation of lung pre-metastatic niche successfully. Subsequently, we applied LOXL2-overexpressed xenograft HCC models to further evaluate the the pathological role of LOXL2 in promoting lung metastasis in HCC. Our data demonstrated that high level of serum LOXL2 released from primary tumor obviously promoted the occurrence of pulmonary metastasis and the number of metastasis lesions, meanwhile increased the number of CD11b⁺/CD45⁺ BMDCs, in agreement with the induction roles of LOXL2 in the formation of pre-metastatic niche and the results of other study [40], implying that the increase of BMDCs recruitment is closely associated with the occurrence of lung metastasis. Accordingly, the above data in vivo confirmed that LOXL2 derived from primary tumor participated in the formation of lung pre-metastatic niche and accelerate the occurrence of lung metastasis.

To investigate whether LOXL2-caused matrix stiffening also contribute to the formation of lung pre-metastatic niche, we constructed high and low stiffness substrate gel mirroring lung tissue stiffness in pathological state and normal state to explore the relevant mechanism by which LOXL2-caused matrix

stiffening participate in lung pre-metastatic niche formation. We mainly analyzed the effects of LOXL2 and LOXL2-caused matrix stiffening on the expressions of matrix remodeling-associated molecules, and chemokine as well as the activation of related pathways in lung fibroblasts, and found that both LOXL2 and LOXL2-caused matrix stiffening prominently upregulated MMP9 and fibronectin expressions in lung fibroblasts, supporting that they participate in regulation of matrix remodeling together. Like CM-LV-LOXL2-OE stimulation, recombinant protein LOXL2 intervention also showed same effects on the expression of fibronectin and p-AKT, indicating a linkage between LOXL2 expression and PI3K-AKT pathway. PI3K inhibitor evidently suppressed the expressions of fibronectin and p-AKT in lung fibroblasts grown on high stiffness substrate, further illustrating that LOXL2 stimulation promotes fibronectin upregulation in lung fibroblasts via activating PI3K-AKT pathway. Additionally, no significant roles were found for MMP2 in LOXL2-induced matrix remodeling, in consistence with the results of our previous work [28]

Our results also indicated that LOXL2-induced matrix remodeling and LOXL2-caused matrix stiffening provide a favorable soil environment for adhesion and colonization of HCC cells. Except that, LOXL2 stimulation obviously increased the expression of chemokine CXCL2 in lung fibroblasts grown on high stiffness substrate, which will be beneficial to the chemotaxis of liver cancer cells to site of lung pre-metastatic niche. Clinical significance of LOXL2 upregulation in HCC progression and unfavorable outcome was validated in a retrospective analysis of pathological parameters in HCC patients between High-LOXL2 group and Low-LOXL2 group. Additionally, the expression of LOXL2 was positively correlated with matrix stiffness, in agreement with in vitro findings described previously [28].

Conclusion

Primary tumor-released LOXL2 contributes to the formation of lung pre-metastatic niche and the occurrence of lung metastasis. LOXL2 and LOXL2-caused matrix stiffening work together to induce lung pre-metastatic niche formation through influencing matrix remodeling and cell colonization. This study sheds light on the pathological significance of LOXL2 in lung pre-metastatic niche formation in HCC, also implicates a new intervention approach for HCC metastasis through reversal of lung pre-metastatic niche.

List Of Abbreviations

LOXL2: Lysyl oxidase like 2; TDSF: Tumor derived soluble factor; BMDC: Bone marrow derived cells; HELF: Human embryo lung fibroblast; CM: Conditioned medium; COL1: Collagen 1; CTC: Circulating tumor cell; CXCL12: C-X-C motif chemokine ligand 12; ECM: Extracellular matrix; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HCC: Hepatocellular carcinoma; MDSC: Myeloid-derived suppressor cell; LOX: Lysyl oxidase; MMP: Matrix metalloproteinase; NC: Negative control; OPN: Osteopontin; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; rhLOXL2: Recombinant human Lysyl oxidase like 2; TGF- β : Transforming growth factor beta; VEGF: Vascular endothelial growth factor; G-CSF: Granulocyte colony stimulating factor; TNF- α : Tumor necrosis factor alpha.

Declarations

Ethics approval and consent to participate

The study was approved by the the Taizhou People's Hospital Research Ethics Committee. All animal protocols were approved by the Ethical Committee on Animal Experiments of Zhongshan Hospital, Fudan University (Permit Number:2016- 120).

Consent for publication

All authors consented to the publication of this manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that there are no competing interests.

Funding

This study was sponsored by grants from the National Nature Science Foundation of China (81972910, 81573019) and Jiangsu Province Medical Youth Talent (QNRC2016513).

Authors' contributions

Conceived and designed the experiments: JFC, KZZ, SFW. Performed the experiments: SFW, XXX. Analyzed data: SFW, XXX, YHW, XZ. Contributed reagents/materials/analysis tools: ML, MMW, RXC, ZMW, JC, DMG, YZ, ZGR. Wrote the paper: SFW, JFC. All authors read and approved the final manuscript.

References

1. Paget S. The distribution of secondary growths in cancer of the breast. Lancet 1889; 1:99-101.
2. Paget S. The distribution of secondary growths in cancer of the breast. 1889. Cancer Metastasis Rev 1989; 8: 98-101.
3. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2002;2: 563-572.
4. Ewing J. Neoplastic Diseases. A Treatise on Tumors. Philadelphia, PA: W.B. Saunders Co.; 1928. pp. 77-89.
5. Bross IDJ, Blumenson LE. Metastatic sites that produce generalized cancer: identification and kinetics of generalizing sites. In: Weiss L(ed). Fundamental aspects of metastasis. Amsterdam:

North-Holland Publishing Company, 1976: 359-375

6. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA, Zhu Z, Hicklin D, Wu Y, Port JL, Altorki N, Port ER, Ruggero D, Shmelkov SV, Jensen KK, Rafii S, Lyden D. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 2005; 438: 820-827
7. Kaplan RN, Rafii S, Lyden D. Preparing the "soil": the premetastatic niche. *Cancer Res.* 2006;66:11089–11093.
8. Sceneay J, Smyth M J, Möller A. The pre-metastatic niche: finding common ground. *Cancer & Metastasis Reviews*, 2013, 32(3-4):449-464.
9. Yang L, Cao X. Characteristics and Significance of the Pre-metastatic Niche. *Cancer Cell*, 2016, 30(5):668-681.
10. McAllister SS, Weinberg RA. The tumour-induced systemic environment as a critical regulator of cancer progression and metastasis. *Nat Cell Biol.* 2014;16(8):717-27.
11. Jiang T, Chen L, Huang Y, Wang J, Xu M, Zhou S, Gu X, Chen Y, Liang K, Pei Y, Song Q, Liu S, Ma F, Lu H, Gao X, Chen J. Metformin and Docosahexaenoic Acid Hybrid Micelles for Premetastatic Niche Modulation and Tumor Metastasis Suppression. *Nano Lett.* 2019;19(6):3548-3562.
12. Chin AR, Wang SE. Cancer Tills the Premetastatic Field: Mechanistic Basis and Clinical Implications. *Clin Cancer Res.* 2016 ;22(15):3725-33.
13. Doglioni G, Parik S, Fendt SM. Interactions in the (Pre)metastatic Niche Support Metastasis Formation. *Front Oncol.* 2019;9:219.
14. Hsu YL, Yen MC, Chang WA, Tsai PH, Pan YC, Liao SH, Kuo PL. CXCL17-derived CD11b+Gr-1+ myeloid-derived suppressor cells contribute to lung metastasis of breast cancer through platelet-derived growth factor-BB. *Breast Cancer Res.* 2019;21(1):23
15. Peinado H, Lavotshkin S, Lyden D. The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts. *Semin Cancer Biol.* 2011;21(2):139–146.
16. Shao Y, Chen T, Zheng X, Yang S, Xu K, Chen X, Xu F, Wang L, Shen Y, Wang T, Zhang M, Hu W, Ye C, Yu X, Shao J, Zheng S. Colorectal cancer-derived small extracellular vesicles establish an inflammatory premetastatic niche in liver metastasis. *Carcinogenesis.* 2018;39(11):1368-1379
17. Wang D, Sun H, Wei J, Cen B, DuBois RN. CXCL1 Is Critical for Premetastatic Niche Formation and Metastasis in Colorectal Cancer. *Cancer Res.* 2017;77(13):3655-3665.
18. Cox TR, Erler JT, Rumney RMH. Established Models and New Paradigms for Hypoxia-Driven Cancer-Associated Bone Disease. *Calcif Tissue Int.* 2018;102(2):163–173
19. Kim, M.N.; Kim, S.U.; Kim, B.K.; Park, J.Y.; Kim, D.Y.; Ahn, S.H.; Song, K.J.; Park, Y.N.; Han, K.H. Increased risk of hepatocellular carcinoma in chronic hepatitis B patients with transient elastography-defined subclinical cirrhosis. *Hepatology* 2015, 61, 1851–1859. 27.
20. Jung KS, Kim SU, Choi GH, et al. Prediction of recurrence after curative resection of hepatocellular carcinoma using liver stiffness measurement (FibroScan®). *Ann Surg Oncol.* 2012;19(13):4278–

21. Wang, Q.; Fiel, M.I.; Blank, S.; Luan, W.; Kadri, H.; Kim, K.W.; Manizate, F.; Rosenblatt, A.G.; Labow, D.M.; Schwartz, M.E.; et al. Impact of liver fibrosis on prognosis following liver resection for hepatitis B-associated hepatocellular carcinoma. *Br. J. Cancer* 2013, 109, 573–581.
22. Y. You, Q. Zheng, Y. Dong, Y. Wang, L. Zhang, T. Xue, X. Xie, C. Hu, Z. Wang, R. Chen, Y. Wang, J. Cui, Z. Ren, Higher Matrix Stiffness Upregulates Osteopontin Expression in Hepatocellular Carcinoma Cells Mediated by Integrin beta1/GSK3beta/beta-Catenin Signaling Pathway, *PLoS One*, 2015, 10(8): e0134243.
23. Y. Dong, X. Xie, Z. Wang, C. Hu, Q. Zheng, Y. Wang, R. Chen, T. Xue, J. Chen, D. Gao, W. Wu, Z. Ren, J. Cui, Increasing matrix stiffness upregulates vascular endothelial growth factor expression in hepatocellular carcinoma cells mediated by integrin beta1, *Biochem Biophys Res Commun*, 2014, 444(3): 427-432
24. Y. You, Q. Zheng, Y. Dong, X. Xie, Y. Wang, S. Wu, L. Zhang, Y. Wang, T. Xue, Z. Wang, R. Chen, Y. Wang, J. Cui, Z. Ren, Matrix stiffness-mediated effects on stemness characteristics occurring in HCC cells, *Oncotarget*, 2016, 7(22): 32221-32231.
25. Y. Dong, Q. Zheng, Z. Wang, X. Lin, Y. You, S. Wu, Y. Wang, C. Hu, X. Xie, J. Chen, D. Gao, Y. Zhao, W. Wu, Y. Liu, Z. Ren, R. Chen, J. Cui, Higher matrix stiffness as an independent initiator triggers epithelial-mesenchymal transition and facilitates HCC metastasis, *J Hematol Oncol*, 2019 12(1):112.
26. Tang RZ, Gu SS, Chen XT, He LJ, Wang KP, Liu XQ. Immobilized Transforming Growth Factor-Beta 1 in a Stiffness-Tunable Artificial Extracellular Matrix Enhances Mechanotransduction in the Epithelial Mesenchymal Transition of Hepatocellular Carcinoma. *ACS Appl Mater Interfaces*. 2019;11(16):14660–14671
27. Liu C, Liu Y, Xie HG, et al. Role of three-dimensional matrix stiffness in regulating the chemoresistance of hepatocellular carcinoma cells. *Biotechnol Appl Biochem*. 2015; 62(4):556–562
28. S. Wu, Q. Zheng, X. Xing, Y. Dong, Y. Wang, Y. You, R. Chen, C. Hu, J. Chen, D. Gao, Y. Zhao, Z. Wang, T. Xue, Z. Ren, J. Cui, Matrix stiffness-upregulated LOXL2 promotes fibronectin production, MMP9 and CXCL12 expression and BMDCs recruitment to assist pre-metastatic niche formation, *J Exp Clin Cancer Res*, 2018, 37(1):99.
29. Vauthey JN, Lauwers GY, Esnaola NF, et al. Simplified staging for hepatocellular carcinoma. *J Clin Oncol*. 2002;20(6):1527–1536.
30. Butcher DT, Alliston T, Weaver VM. A tense situation: forcing tumour progression. *Nat Rev Cancer*. 2009;9(2):108–122
31. Kim YM, Kim EC, Kim Y. The human lysyl oxidase-like 2 protein functions as an amine oxidase toward collagen and elastin. *Mol Biol Rep*. 2011;38(1):145–149.
32. Psaila B, Lyden D. The metastatic niche: adapting the foreign soil. *Nat Rev Cancer*. 2009;9(4):285–293
33. Peinado H, Zhang H, Matei IR, et al. Pre-metastatic niches: organ-specific homes for metastases. *Nat Rev Cancer*. 2017;17(5):302–317

34. Peng L, Ran YL, Hu H, et al. Secreted LOXL2 is a novel therapeutic target that promotes gastric cancer metastasis via the Src/FAK pathway. *Carcinogenesis*. 2009;30(10):1660–1669.
35. Ahn SG, Dong SM, Oshima A, et al. LOXL2 expression is associated with invasiveness and negatively influences survival in breast cancer patients. *Breast Cancer Res Treat*. 2013;141(1):89–99.
36. Park PG, Jo SJ, Kim MJ, et al. Role of LOXL2 in the epithelial-mesenchymal transition and colorectal cancer metastasis. *Oncotarget*. 2017;8(46):80325–80335.
37. Fong SF, Dietzsch E, Fong KS, et al. Lysyl oxidase-like 2 expression is increased in colon and esophageal tumors and associated with less differentiated colon tumors. *Genes Chromosomes Cancer*. 2007;46(7):644–655.
38. Cao C, Lin S, Zhi W, et al. LOXL2 Expression Status Is Correlated With Molecular Characterizations of Cervical Carcinoma and Associated With Poor Cancer Survival via Epithelial-Mesenchymal Transition (EMT) Phenotype. *Front Oncol*. 2020;10:284.
39. Erler JT, Bennewith KL, Cox TR, et al. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell*. 2009;15(1):35–44.
40. Wong CC, Tse AP, Huang YP, et al. Lysyl oxidase-like 2 is critical to tumor microenvironment and metastatic niche formation in hepatocellular carcinoma. *Hepatology*. 2014;60(5):1645–1658.
41. Kim YM, Kim EC, Kim Y. The human lysyl oxidase-like 2 protein functions as an amine oxidase toward collagen and elastin. *Mol Biol Rep*. 2011;38(1):145–149.
42. Fogelgren B, Polgár N, Szauter KM, et al. Cellular fibronectin binds to lysyl oxidase with high affinity and is critical for its proteolytic activation. *J Biol Chem*. 2005;280(26):24690–24697.

Figures

Figure 1

The roles of LOXL2 in the formation of lung pre-metastatic niche in nude mouse A. The expression of LOXL2 in lung tissues of lung pre-metastatic niche nude mice models with tumor-free or tumor-bearing. B. FCM analysis of CD11b⁺/CD45⁺ BMDCs recruitment in lung tissues during the formation of lung pre-metastatic niche. C. The state of CD11b⁺/CD45⁺ BMDCs recruitment in lung tissues of lung pre-metastatic niche nude mice models with tumor-free or tumor-bearing. Red arrow indicates BMDCs with

CD11b+/CD45+. mRNA expression level (D) and protein expression (E) of fibronectin in lung tissues of lung pre-metastatic niche nude mice models with tumor-free or tumor-bearing.

Figure 2

LOXL2 overexpression promote lung metastasis occurrence of xenograft HCC model A. Size of xenograft HCC tumors. B. The expression of LOXL2 in xenograft HCC tumors. C. Levels of serum LOXL2 in xenograft HCC models. D. The state of CD11b+/CD45+ BMDCs recruitment in lung tissues of xenograft

HCC models. Red arrow indicates BMDCs with CD11b+/CD45+. E. Analysis of pulmonary metastasis in xenograft HCC models.

Figure 3

LOXL2 and LOXL2-caused matrix stiffening participate in pre-metastatic niche formation through influencing matrix remodeling and cell colonization A. Stiffness analysis of the gel substrate by TA. XTPLUSC Texture Analyzer. (a) 202.434Pa of low stiffness substrate (b) 926.18Pa of high stiffness substrate. B. The morphological characteristics of lung fibroblasts grown on high stiffness substrate (H)

and low stiffness substrate (L). C. The effects of LOXL2 and LOXL2-caused matrix stiffening on the expressions of matrix remodeling-associated genes (MMP9, MMP2, Fibronectin), and chemokine CXCL12. D. The effects of LOXL2 and LOXL2-caused matrix stiffening on the expressions of matrix remodeling-associated proteins and the activation of related pathways in lung fibroblasts E. A modified cell adhesion assay shows the numbers of adherent HCC cells on surface of lung fibroblasts treated with CM-LV-LOXL2-OE.

Figure 4

Different expression analysis of LOXL2, LOX and Collagen 1 in HCC tissues between LOXhigh/ COL1high group and LOXlow/ COL1low group A.Different expression of Collagen 1 between LOXhigh/ COL1high group and LOXlow/ COL1low group B..Different expression of LOX between between LOXhigh/ COL1high group and LOXlow/ COL1low group. C. Different expression of LOX between between LOXhigh/ COL1high group and LOXlow/ COL1low group. D. A correlation analysis between LOXL2 and Collage 1 or LOX.

Figure 5

Schematic diagram of the mechanism underlying LOXL2-induced lung pre-metastatic formation in HCC.