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Forkhead box C1 promotes metastasis and invasion of non–small cell lung cancer by binding directly to the lysyl oxidase promoter

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Abstract

Increasing evidence indicates that human forkhead box C1 (FOXC1) plays important roles in tumor development and metastasis. However, the underlying molecular mechanism of FOXC1 in non–small cell lung cancer (NSCLC) metastasis remains unclear. Here, we identified FOXC1 as an independent prognostic factor in NSCLC and showed clear biological implications in invasion and metastasis. FOXC1 overexpression enhanced the proliferation, migration and invasion of NSCLC cells, whereas FOXC1 silencing impaired the effects both in vitro and in vivo. Importantly, we found a positive correlation between FOXC1 expression and lysyl oxidase (LOX) expression in NSCLC cells and patient samples. Downregulation of LOX or LOX activity inhibition in NSCLC cells inhibited the FOXC1-driven effects on cellular migration and invasion. Xenograft models showed that inhibition of LOX activity by β-aminopropionitrile monofumarate decreased the number of lung metastases. Mechanistically, we demonstrated a novel FOXC1-LOX mechanism that was involved in the invasion and metastasis of NSCLC. Dual-luciferase assay and ChIP identified that FOXC1 bound directly in the LOX promoter region and activated its transcription. Collectively, the present study offered new insight into FOXC1 in the mediation of NSCLC metastasis through interaction with the LOX promoter and further revealed that targeted inhibition of LOX protein activity could prevent lung metastasis in murine xenograft models. These data implicated FOXC1 as a potential therapeutic strategy for the treatment of NSCLC metastasis.

KEYWORDS
FOXC1, invasion, LOX, metastasis, NSCLC

1 | INTRODUCTION

Primary lung cancer is the predominant cause of cancer-related deaths worldwide,[1,2] among which non–small cell lung cancer (NSCLC) is expected to account for 80%-85%.[3] Recent innovations in diagnosis and treatment, including anti–angiogenesis agents and anti–epidermal growth factor receptor (EGFR) agents, did not extend the age-standardized overall 5-year survival rate beyond the range of 10%-20% in most countries between 2000 to 2014.[4] This abysmal survival rate indicates the necessity to develop an understanding of

the mechanisms of NSCLC progression and metastasis for targeting the drivers of lung cancer.

The human forkhead box (FOX) family comprises a group of evolutionarily conserved transcription factors which are characterized by a distinct DNA-binding forkhead domain.[5] FOXC1 (Mf1, FKHL7, FREAC3), a member of the FOX family, plays an important role in brain,[6,7] eye[8,9] and heart formation[10,11] during embryonic development. Recent studies have demonstrated that FOXC1 is substantially elevated in several aggressive human carcinomas, including basal-like breast cancer,[12-14] pancreatic ductal adenocarcinoma,[15,16] gastric cancer,[17] hepatocellular carcinoma[18,19] and acute myeloid leukemia,[20] and it is postulated to be a marker of poor prognosis. Several previous studies have reported that FOXC1 is involved in multiple steps of tumor progression in breast cancer, including cell proliferation, migration, invasion, and epithelial to mesenchymal transition (EMT).[21-25] Few studies have focused on FOXC1 function in NSCLC. The precise mechanism of FOXC1 in regulating NSCLC progression remains unknown.

Lysyl oxidase is a copper-dependent enzyme involved in post-translational cross-linking of both collagen and elastin, which, in turn, stabilizes the extracellular matrix (ECM), allowing for tissue maintenance and structural homeostasis.[26,27] Accumulating data have indicated that LOX can create a stiffer microenvironment for tumor metastasis[28,29] and induce pre–metastatic niche formation.[30,31] It is associated with poor progression in various types of tumors, including non–small cell lung cancer[32-34] and is regarded as a targetable cancer metastasis molecule.[35] In our preliminary work, when FOXC1 was overexpressed, we identified 15 dramatic enrichment ECM-related genes by gene DNA microarray, among which LOX was especially upregulated (52-fold).

In this study, we first identified FOXC1 as an important prognostic factor in NSCLC and showed clear biological implications in invasion and metastasis. We showed a correlation between FOXC1 expression and LOX expression and implicated LOX activity in the metastatic phenotype. Furthermore, we explored for the first time the mechanism of FOXC1 by directly binding in the promoter of LOX. Finally, we showed that genetic silencing or pharmacologic inhibition of LOX can decrease metastasis in vitro and in vivo. This offers a new mechanistic insight into FOXC1 regulation in the invasion and metastasis of NSCLC and implicates FOXC1 as a potential therapeutic strategy for the treatment of NSCLC metastasis.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

A total of 105 NSCLC patients who underwent surgery in the Department of Thoracic Surgery, Jinan Central Hospital from January 2010 to December 2015 were included in this study. The patients who had received neoadjuvant chemotherapy or radiotherapy before surgery were excluded. In addition, 40 biopsies from benign disease patients were used as control. Patients were contacted by phone to check on their health status and the last censor date was on 30 December 2017. Informed written consent was obtained from the patients who participated in the study and the study was approved by the hospital's institutional review board.

2.2 | Immunohistochemical staining

Immunohistochemistry (IHC) was performed as described previously.[36] Anti–FOXC1 pAb (1:100; Abcam) or anti–LOX mAb (1:300; Abcam) was used in the IHC. Normal mouse or rabbit IgG instead of primary antibodies were used as negative control.

Immunohistochemical analysis was performed by two independent pathologists. The proportion score represented the estimated fraction of positive staining tumor cells (0 = none; 1 = less than 25%; 2 = 25%-75%; 3 = greater than 75%). The intensity score represented the average staining intensity of positive tumor cells (0 = none; 1 = week; 2 = intermediate; 3 = strong). The two scores were multiplied to generate the immunoreactivity score (IS) for each case (range = 0-9). FOXC1 expression was defined as either high expression (score ≥ 3) or low expression (score < 3).

2.3 | Cell culture

Human NSCLC cell lines A549, H226, H1975, H1650 and H1299 and normal pulmonary epithelial cell line BEAS-2B were purchased from the Cell Resource Center of the Chinese Academy of Sciences. H226, H1299, H1650 and H1975 cells were cultured in RPMI 1640 medium (Gibco, Life Technologies) with 10% FBS (Gibco, Life Technologies); A549 and BEAS-2B cells were cultured in DMEM (Gibco, Life Technologies) with 10% FBS. All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2 incubator.

2.4 | Cell transfection

Lentiviral particles expressing FOXC1 were built by Genechem. Briefly, H1299/H1650 cells were cultured in 6-well plates for 24 h; 500 μL fresh medium containing 10 μL lentivirus (1×10^9 TU/mL) was then added to each well. After 12 h, the medium was refreshed and the infection rate was observed using a fluorescence microscope 72 h later. The stable cell lines were selected by puromycin (Invitrogen) and collected for later assays (after 4 weeks). The MOCK was a negative control. FOXC1 expression was detected by quantitative RT-PCR (qRT-PCR) and western blot.

Plasmid shFOXC1 (Genepharma) was transfected into the cells using X-treme GENE HP Reagents (Roche) according to the manufacturer's instructions; non–targeting plasmid (shNC) was used as a negative control. A549/H226 cells (2×10^5) were transfected with 2.5 μg plasmid in 6-well plates. The medium was refreshed after 12 h and the cells were collected 48 h later. After observing the infection rate using a fluorescence microscope 72 h later, the stable cell lines were selected by puromycin and collected for later assays after 4-6 weeks. FOXC1 expression was detected by qRT-PCR and western blot. The shRNA sequences are listed as follows:

shFOXC1-1:5′-GGGAATAGTAGCTGTCAAATG-3′;
shFOXC1-2:5′-GGAGCTTTCGTCTACGACTGT-3′;
shNC: 5′-GTTCTCCGAACGTGTCACGT-3′.

To silence LOX expression, siRNA against human LOX (Genepharma) was transfected into NSCLC cells. The siRNA sequences are listed in Table S1.

2.5 | Western blot assay

Total proteins were loaded on a 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Millipore). Subsequently, the membrane was blocked by TBST containing 5% non–fat milk for 1 hour and incubated overnight at 4°C with the primary antibodies against FOXC1 (1:1000; Abcam), LOX (1:1000; Abcam) and anti–GAPDH mAb (1:5000; Proteintech) as an internal control. Afterwards, the blots were labeled for 1 h with HRP-conjugated secondary antibody (1:10 000; Proteintech). Finally, the blots were exposed to the ChemiDoc XRS + system (Bio-Rad).

2.6 | Quantitative RT-PCR assay

Total RNA was extracted from NSCLC cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNAs were synthesized from total RNA (2 μg) using the PrimeScript RT Reagent Kit with gDNA eraser (Perfect Real Time) (TaKaRa). QRT-PCR was carried out with an ABI7500 sequence detector (Applied Biosystems) using SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa). The forward and reverse primer sequences are listed in Table S2. Gene expression was determined by the $2^{-\Delta\Delta Ct}$ method using GAPDH as an internal control. All experiments were repeated at least three times.

2.7 | Cell proliferation assay in vitro

Cell proliferation was assessed with the Cell Counting Kit-8 (CCK8) assay. Cells were seeded in 96-well plates at 1×10^3 cells per well, then treated with 100 μL medium without FBS and 10 μL CCK8 solution and incubated for 2 h at 37°C. The Microplate Absorbance Reader was used to read the absorbance at 450 nm at 24, 48, 72, 96 and 120 h, respectively. Assays were repeated at least three times.

2.8 | Colony formation assay in vitro

Cells were plated in 6-well plates at a starting number of 2×10^2 cells. Cell colonies were stained with Giemsa (Solarbio) and counted after 2-3 weeks of culture. Each experiment was performed at least three times.

2.9 | Wound-healing assay

Cells were seeded at a density of 1×10^5 cells/mL in 6-well plates. A scratch wound was created across the center of the monolayer of cells in each well with a sterile 200-μL pipette tip. Images of the cells that had migrated into the cell-free scratch wound area were acquired and the migration area was measured under an inverted microscope. The scratch wound area was determined by the relative percentage compared to untreated control cells. Assays were repeated at least three times.

2.10 | Cell migration and invasion assays in vitro

Migration and invasion assays were performed with transwell chambers containing 8-μm pore membranes (Corning). Approximately 1×10^5 cells were seeded into the upper chamber uncoated or Matrigel-coated membrane (BD Transduction Laboratories) with serum-free medium. Then the medium with 10% FBS was added to the lower chamber. Finally, the migrated or invasive cells on the bottom of the insert were fixed, stained and calculated.

2.11 | Whole genome DNA microarray

Cells transfected with FOXC1 and negative control were collected using TRIzol Reagent (Invitrogen, Life Technologies) according to protocol. Then, the samples were amplified and labeled using the RNeasy Mini Kit (Qiagen p/n 74 104). Next, the samples were hybridized using the Agilent Gene Expression Hybridization Kit (Agilent Technologies) in Agilent SureHyb hybridization chambers. After hybridization and washing, the slides were scanned with the Agilent DNA microarray scanner using the settings recommended of Agilent Scan Control software. The data were collected using the Agilent Feature Extraction Software (version 11.0.1.1) and analyzed using GeneSpring GX software (version 11.5.1).

2.12 | Bioinformatics prediction tools

The transcription factor targeted gene was analyzed by using the online database and tools in the Eukaryotic Promoter Database (EPDnew, http://epd.vitalit.ch/index.php) and the Gene Tranion Regulation Database (GTRD, http://gtrd.biouml.org/). The JASPAR CORE vertebrata database (http://jaspar.genereg.net/) was used to predict the well-characterized activators binding sites of FOXC1 gene with a relative profile score threshold of 90%.

2.13 | Luciferase reporter assay

The pGL4.10-Lox promoter (wild-type or mutant) was cloned. Cells were cultured in 24-well plates and co–transfected with pGL4.10-Lox promoter or pGL4.10-Lox promoter mutant vectors (200 ng) and mimics (100 ng) of the FOXC1 plasmid using Lipofectamine 2000. Luciferase activity was measured 24 h later with a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity, and the effect of FOXC1 on luciferase reporter with LOX promoter region was then normalized with that on luciferase reporter without LOX promoter region.

2.14 | ChIP assay

ChIP assays were performed as described in the protocol with modifications.[37] FOXC1 antibody was used for the CHIP assay to recognize endogenous FOXC1 (1:1000; Abcam). IgG was the negative control. A 127-bp fragment in the LOX was amplified using standard PCR conditions. Primer sequences are described in Table S3. The amplified fragments were analyzed in a 2% agarose gel.

2.15 | Subcutaneous xenograft and tail vein-lung metastasis tumor models

Female nude BALB/c mice, aged 6-8 weeks, were purchased from Beijing HFK Bioscience (Beijing). Animal experiment protocols were approved by the Institutional Animal Care and Use Committee of FMMU. Mice were randomized into groups named H1299-MOCK, H1299-FOXC1, H1299-FOXC1 + BAPN, H1299-FOXC1 + Control or A549-shNC, A549-shFOXC1, A549 + Control and A549 + BAPN. Each group has 6 mice; 1×10^6 cells (H1299-MOCK, H1299-FOXC1 or A549-shNC, A549-shFOXC1) were subcutaneously inoculated into the left axillary of each mouse. Seven days after tumor cell inoculation, each mouse was treated daily with or without I; 100 mg/kg; Sigma-Aldrich) for the last four weeks. BAPN were used to inhibit the LOX role. PBS was used as control. Tumor sizes were monitored every 3 days using calipers and tumor volumes were calculated according to the formula: length × width^2 × 0.5.

To observe the role of FOXC1 in distant metastasis, FOXC1 overexpressing or silencing cells and their corresponding controls were injected intravenously with 5×10^5 cells in 0.1 mL DMEM medium via tail vein. Seven days later, each mouse was treated daily with or without BAPN for the last four weeks. Mice were killed and visible lung surface micrometastatic white spots were counted using a dissecting microscope (Nikon) at 36 days. Histological analyses were used to detect metastasis in lungs which were embedded in paraffin and dyed with H&E. Animal studies were conducted in accordance with the NIH animal use guidelines and current Chinese regulations and standards for laboratory animal use.

2.16 | Lysyl oxidase activity assay

The original fluorescence-based method was used to assess LOX enzymatic activity as described previously.[38,39] For the in vivo assay, terminal blood was taken from mice at the end of the experiment described above. Plasma (10 μL) was detected and fluorescence was plotted, where 0 meant medium and 500 μM BAPN (complete LOX inhibition). For the in vitro data, 50 μL of phenol-red-free

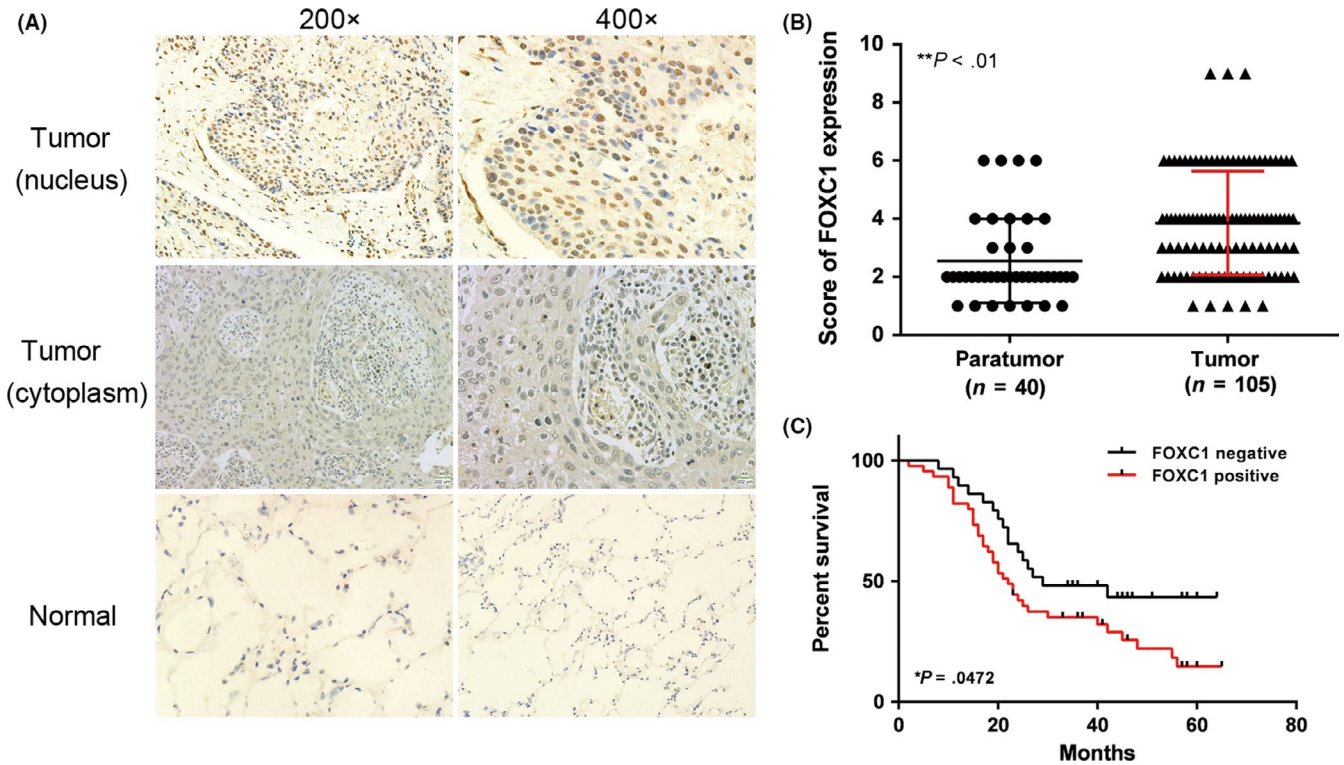

FIGURE 1 Forkhead box C1 (FOXC1) was highly expressed in non–small cell lung cancer (NSCLC) tissues. (A) FOXC1 expression in tumor specimens and normal lung bronchus tissues. Positive FOXC1 expression was identified as brown staining in the nucleus and cytoplasm of NSCLC cells (magnification of 200× and 400×). (B) FOXC1 highly expressed in 59 of 105 (56.19%) NSCLC specimens, whereas FOXC1 expression was detected in 9 of 40 (22.5%) adjacent nontumorous specimens ($P < .01$). (C) Survival analysis of NSCLC patients with high or low FOXC1 expression by Kaplan-Meier survival analysis (long-rank test). Patients with high expression of FOXC1 showed poorer overall survival than those with low expression ($P = .0472$)

medium taken from cells was incubated overnight at 37°C with different concentrations of BAPN. The fluorescent emission was read at 590 nm using a BMG Lab Technologies Polarstar Optima.

2.17 | Statistical analysis

Statistical analysis was carried out with SPSS 15.0. Values are presented as the mean ± SEM. Statistical differences between groups were identified using Student's *t* test, ANOVA, χ^2 or Fisher's exact test, and Pearson's correlation test, as appropriate. Overall survival curves were calculated using the Kaplan-Meier method and significance was determined using the log-rank test. $P < .05$ was considered statistically significant. All the experiments were conducted in triplicate.

3 | RESULTS

3.1 | Highly expressed forkhead box C1 in human non–small cell lung cancer correlated with poor prognosis

To determine FOXC1 expression in NSCLC, IHC was performed in 105 primary human NSCLC tissues and 40 adjacent nontumorous tissues. As presented in Figure 1A, FOXC1 displayed either the cytoplasm or the nucleus in tumor cells, while little staining of FOXC1 was identified in the stromal cells. FOXC1 was highly expressed in 59 of 105 (56.19%) NSCLC specimens and showed stronger brownish yellow to brown particles, whereas FOXC1 expression was detected in 9 of 40 (22.5%) adjacent nontumorous specimens and FOXC1 staining was too weak or not observed ($P < .01$) (Figure 1B).

We further analyzed the association between FOXC1 expression and the clinicopathological parameters. As presented in Table 1, the high expression of FOXC1 was observed more often in patients with positive lymph node status (N0, 37.8%; N1 + N2, 66.2%, $P = .005$) and in non–squamous NSCLC patients (squamous NSCLC, 42.6%; non–squamous NSCLC, 67.2%; $P = .011$). Its expression was higher in stage II + III (61.8%) but did not approach significance ($P = .059$). No statistically significant correlations were identified between FOXC1 expression and gender, age, smoking history, differentiation or tumor size. More importantly, patients with high expression of FOXC1 showed poorer overall survival than those with low expression ($P = .0472$) (Figure 1C). Univariate Cox proportional hazards analysis of OS revealed that high expression of FOXC1 ($P = .009$), T2 + T3 ($P = .042$), positive lymph node metastasis ($P = .009$) and stage II + III ($P = .02$) were associated with poor outcome in NSCLC patients. In multivariate analysis, only FOXC1 expression ($P = .043$) remained as an independent prognostic factor of overall survival (hazard ratio, 1.988, 95% confidence interval, 1.022-3.860) (Table 2). The result indicated that FOXC1 high expression was negatively correlated with patient survival.

TABLE 1 Correlations between forkhead box C1 (FOXC1) expression and the clinicopathological parameters of 105 non–small cell lung cancer (NSCLC) patients

	FOXC1 expression			
Clinicopathological factor	High	Low	χ^2	*P* value
Gender				
Male	38	36	2.384	.123
Female	21	10		
Age (year)				
<60	30	25	0.127	.722
≥60	29	21		
Smoking history (year)				
<30	24	16	0.381	.537
≥30	35	30		
Histology				
Squamous NSCLC	20	27	6.428	.011*
Non–squamous NSCLC	39	19		
Differentiation				
Well and moderate	43	28	1.703	.192
Poorly	16	18		
Tumor size				
T1	20	17	1.494	.474
T2	30	24		
T3	9	5		
Regional lymph node involvement				
N0	14	23	7.817	.005*
N1-N2	45	23		
TNM staging				
I	12	17	3.57	.059
II+III	47	29		

*$P < 0.05$

3.2 | Forkhead box C1 promoted proliferation, migration and invasion of non–small cell lung cancer cells in vitro

To investigate the role of FOXC1 in NSCLC progression, we first determined FOXC1 expression in five NSCLC cell lines (A549, H226, H1975, H1650 and H1299) and the normal lung/bronchial epithelial cell line (BEAS-2B). We found that FOXC1 expression was significantly higher in five NSCLC cell lines compared with that in BEAS-2B (Figure S1). Then, we selected H1299 and H1650 with endogenous low FOXC1 expression to be constructed two FOXC1 overexpression cell lines. The FOXC1 expression increased in FOXC1 transfected cells both at mRNA and at protein levels compared with control cells (Figure 2A,B). The role of FOXC1 on proliferation was evaluated by CCK-8 assay and colony formation assay, and the growth of the FOXC1-overexpression H1299 and H1650 cells was significantly accelerated (Figure 2C–F). We further found that FOXC1 overexpression cells closed scratch wounds

TABLE 2 Univariate analysis and multivariate analysis

Clinicopathological factor	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Gender						
Male	1.362	0.696-2.665	.367	1.217	0.504-2.934	.662
Female						
Age (year)						
>60	0.593	0.336-1.049	.073	0.684	0.376-1.246	.214
≤60						
Smoking history (year)						
<30	0.880	0.507-1.544	.655	0.672	0.237-1.911	.456
≥30						
Histology						
Squamous NSCLC	1.031	0.59-1.8	.915	0.745	0.303-1.831	.520
Non–squamous NSCLC						
Differentiation						
Well and moderate	1.004	0.548-1.841	.989	1.110	0.575-2.143	.756
Poorly						
Tumor size						
T1	1.946	1.025-3.610	.042*	1.422	0.649-3.115	.380
T2+T3						
Regional lymph node involvement						
N0	2.469	1.259-4.831	.009*	1.672	0.475-5.882	.423
N1-N2						
TNM staging						
I	2.457	1.151-5.263	.02*	1.114	.254-4.902	.887
II+III						
FOXC1 expression						
High expression	2.237	1.220-4.098	.009*	1.988	1.022-3.860	.043*
Low expression						

*$P < 0.05$

more quickly than control cells (Figure 2G,H) and significantly promoted the migration and invasion of lung cancer cells (Figure 2I to L).

Meanwhile, we also selected A549 and H226 with endogenous high FOXC1 expression to be constructed two FOXC1-silenced cell lines. The FOXC1 expression significantly decreased in the cells transfected with FOXC1 shRNA vector compared to those with negative control transfection (Figure 3A,B). Silence of FOXC1 inhibited the cell proliferation and reduced the colony formation ability (Figure 3C to F). The cell migration and invasion were also significantly suppressed when A549 and H226 were silenced by FOXC1 shRNA vector (Figure 3G to L).

Taken together, these findings suggested that FOXC1 can promote proliferation, migration and invasion of NSCLC cells in vitro.

3.3 | Forkhead box C1 overexpression promoted non–small cell lung cancer progression in vivo

To assess the contribution of FOXC1 in tumor progression in vivo, we established subcutaneous xenograft and tail vein-lung metastasis tumor models. First, to examine whether FOXC1 promotes tumor growth, stable transfected H1299-FOXC1 or A549-shFOXC1 cells as well as their corresponding control cells were implanted into the mice. The tumor volumes and the tumor weight were dramatically increased in mice with injection of H1299-FOXC1 cells (Figure 4A to C) and significantly decreased in mice with injection of A549-shFOXC1 cells at the assigned day, compared with their corresponding control groups (Figure 4F to H).

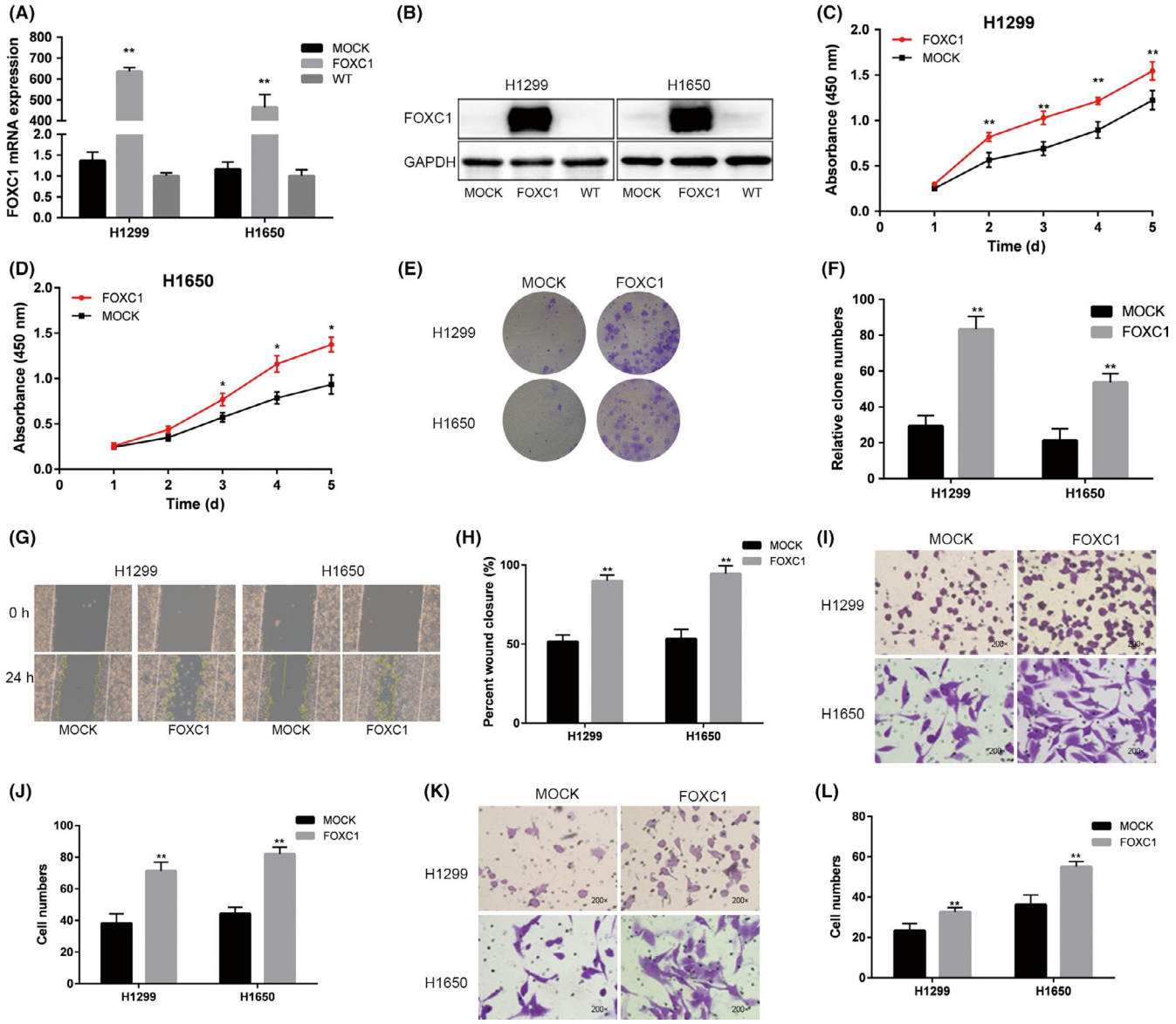

FIGURE 2 Overexpression of forkhead box C1 (FOXC1) promoted cell proliferation, migration and invasion of non–small cell lung cancer (NSCLC) cells in vitro. (A) and (B) The mRNA and protein expression of FOXC1 significantly increased in lentivirus-infected H1299 and H1650 cells compared with vector-infected cells (MOCK) by RT-PCR and western blot. (C and D) Cell proliferation was assessed with the Cell Counting Kit-8 (CCK8) assay at 24, 48, 72, 96 and 120 h, respectively. High FOXC1 overexpression enhanced cell proliferation of lentivirus-infected H1299 and H1650 cells. (E and F) Cell proliferation rates of lentivirus-infected H1299 and H1650 cells and their control groups were determined via colony formation assay as described. (G and H) Representative outcomes and statistical analysis of cell migration by wound-healing assay. FOXC1 overexpression cells closed scratch wounds more quickly than control cells. (I and J) Representative outcomes and statistical analysis of cell migration by transwell migration assay. FOXC1 overexpression significantly promoted the migration of lung cancer cells. (K and L) Representative outcomes and statistical analysis of cell invasion by transwell invasion assay. FOXC1 overexpression significantly promoted the invasion of lung cancer cells (magnification of 200×). MOCK vector was used as negative control. The error bars indicate ±SEM. $^{*}P < .05$, $^{**}P < .01$ by Student's *t* test. All the results were repeated thrice

Meanwhile, to evaluate whether FOXC1 promotes metastasis in vivo, H1299-FOXC1 and A549-shFOXC1 cells as well as their corresponding control cells were also injected into the mice via the tail vein, respectively. At day 36, the number of lung metastases was much greater in H1299-FOXC1-treated mice (Figure 4D,E), while the opposite effect occurred in A549-shFOXC1-treated mice compared with their corresponding control groups (Figure 4I,J).

3.4 | Lysyl oxidase was a downstream target of forkhead box C1

To define the mechanism of FOXC1 promoting tumor progression, we examined transcriptome changes mediated by FOXC1 overexpression in H1299 cells on gene expression profiles. Gene Ontology analysis revealed that several biological processes were altered (Figure 5A). Metastasis is a complicated multistep that involves

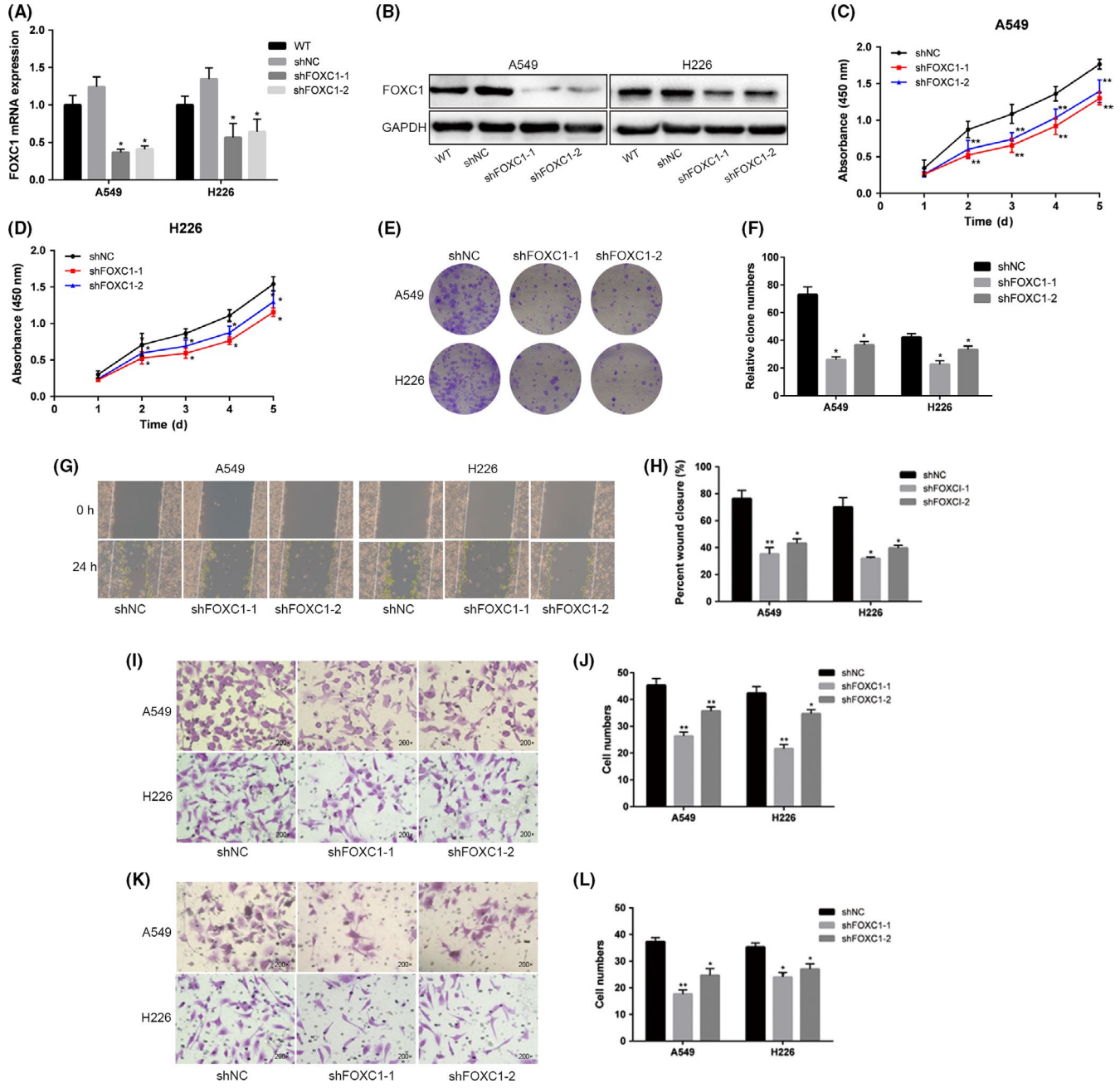

FIGURE 3 Knockdown of forkhead box C1 (FOXC1) inhibited cell proliferation, migration and invasion of non–small cell lung cancer (NSCLC) cells in vitro. (A and B) The mRNA and protein expression of FOXC1 significantly decreased in A549 and H226 cells transfected with FOXC1-shRNA vector compared with negative control (shNC) by RT-PCR and western blot. (C and D) Cell proliferation was assessed with the Cell Counting Kit-8 (CCK8) assay and FOXC1 knockdown attenuated cell proliferation of A549 and H226 cells transfected with FOXC1-shRNA vector. (E and F) Silence of FOXC1 reduced the colony formation ability. (G and H) Representative outcomes and statistical analysis of cell migration by wound-healing assay. The cell migration was significantly suppressed when A549 and H226 was silenced by FOXC1 shRNA vector. (I and J) Representative outcomes and statistical analysis of cell migration by transwell migration assay. Silence of FOXC1 significantly inhibited the migration of lung cancer cells. (K and L) Representative outcomes and statistical analysis of cell invasion by transwell invasion assay. The cell invasion was also significantly suppressed when A549 and H226 was silenced by FOXC1 shRNA vector (magnification of 200×). The error bars indicate ±SEM. **P* < .05, ***P* < .01 by Student's *t* test. All the results were repeated thrice

attachment to, degradation of and detachment from an extracellular matrix, and, finally, active migration away from the primary tumor. Hence, we focused on several ECM-related genes, including FN1, MMP7, MMP1, LOX, COL1A1, ITGA2, ANK3, IGFBP3 and CD24 when FOXC1 overexpression changed (Table S4). Among these genes, we focused on LOX, which were strongly upregulated by FOXC1 overexpression (Figure 5B), and demonstrated that LOX protein was upregulated in invasive lung adenocarcinoma.[32]

To further demonstrate the association between LOX and FOXC1, LOX expression was measured in FOXC1-overexpressed

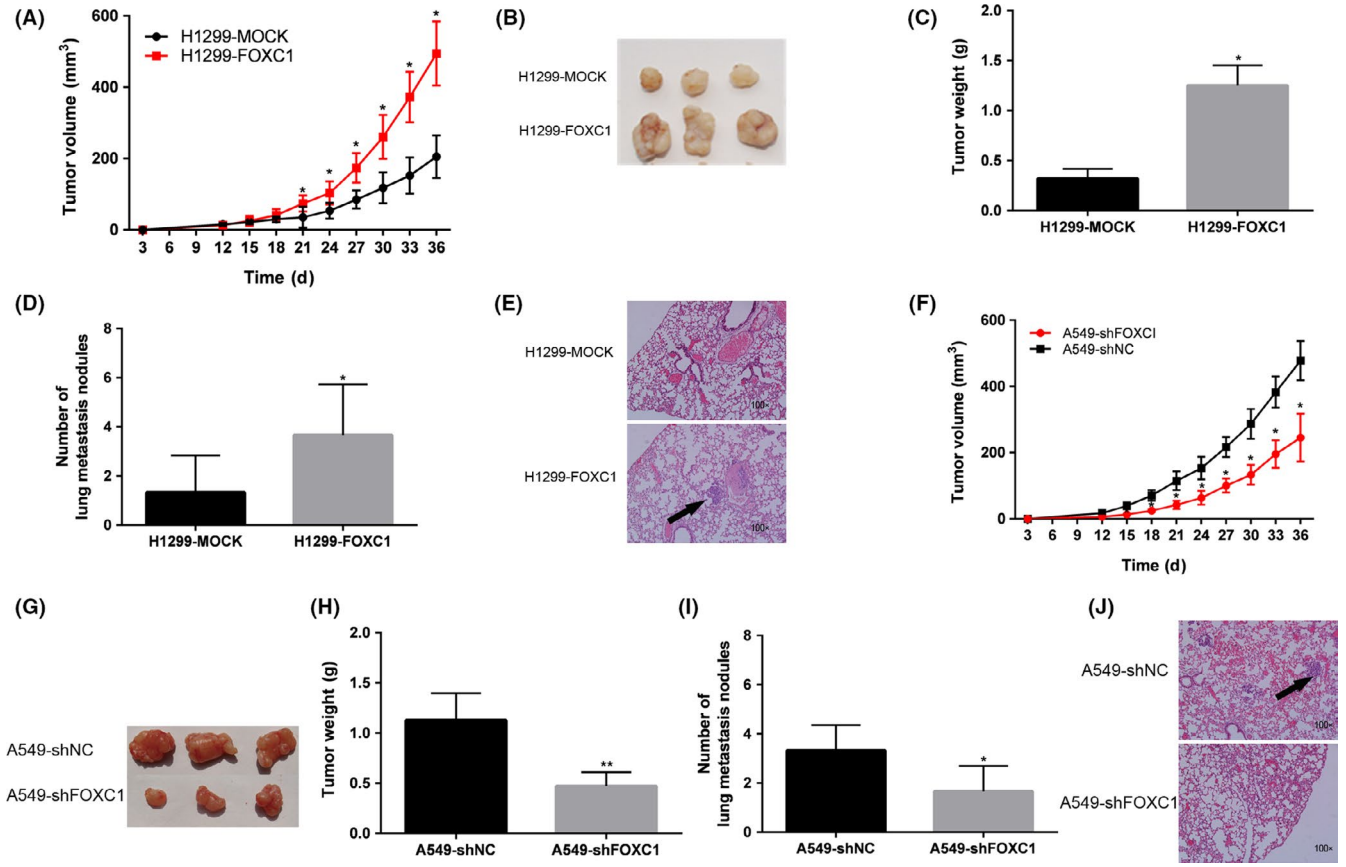

FIGURE 4 Forkhead box C1 (FOXC1) facilitated non–small cell lung cancer (NSCLC) tumor growth and metastasis in vivo. (A and B) Tumor size and (C) tumor weight of mice after subcutaneous injection with H1299-MOCK and H1299-FOXC1 cells. The tumor volumes and the tumor weight were dramatically increased in mice with injection H1299-FOXC1 cells at the assigned day, compared with their corresponding control groups. (D and E) Comparison of lung metastasis in mice after intravenous injection with H1299-MOCK and H1299-FOXC1 cells. At day 36, the number of lung metastasis was much greater in H1299-FOXC1-treated mice compared with their corresponding control groups. Lung metastases were defined as gross lesions of at least 25 cells. (F and G) Tumor size and (H) tumor weight of mice after subcutaneous injection with cells named A549-shNC and A549-shFOXC1. The tumor volumes and the tumor weight were significantly decreased in mice with injection of A549-shFOXC1 cells at the assigned day, compared with their corresponding control groups. (I and J) Microscopic quantification of metastasis in lungs after intravenous injection with A549-shNC and A549-shFOXC1 cells. At day 36, the number of lung metastases was much lower in A549-shFOXC1-treated mice compared with their corresponding control groups (magnification of 100×). The error bars indicate ±SEM. $^{*}P < .05$, $^{**}P < .01$ by Student's *t* test

or FOXC1-silenced lung cancer cells by qRT-PCR and western blot. Overexpression of FOXC1 in H1299 and H1650 cells dramatically increased LOX mRNA and protein expression (Figure 5C,D), while the FOXC1-silenced cells provided the opposite results (Figure 5E,F).

To explore the molecular mechanism by which FOXC1 regulates LOX, online analysis of bioinformation revealed that the FOXC1 may combine in the LOX promoter. We examined the LOX promoter sequence and detected five putative FOXC1 binding sites in the LOX promoter (Figure S2). Thereafter, luciferase reporter plasmids carrying the wild type (WT) or mutant LOX promoter regions were co–transfected with the Renilla luciferase reporter plasmid into H1299 cells. As the reporter assays showed, overexpression of FOXC1 and LOX-luc significantly enhanced the activity of luciferase reporters driven by LOX promoter compared with the three controls (Figure 5G). Consistent with this result, CHIP analysis further confirmed that FOXC1 could bind directly in the LOX promoter region in cells (Figure 5H,5I).

3.5 | Lysyl oxidase was essential for forkhead box C1-mediated non–small cell lung cancer invasion and metastasis

To determine whether the effects of FOXC1 in NSCLC metastasis are mediated by LOX, we first assessed the function of LOX in A549 and H226 cells transfected with LOX-siRNA (Figure 6A). The results showed that the cell proliferation was not significantly attenuated in siLOX-transfected A549 and H226 cells (Figure S3A), but LOX knockdown significantly inhibited the migration and invasion of A549 and H226 cells (Figures 6,7B to D Figure S3B to D). Then we performed the LOX functional assays in FOXC1-overexpressed H1299 and H1650 cells (Figure 6E). Silence of LOX inhibited the

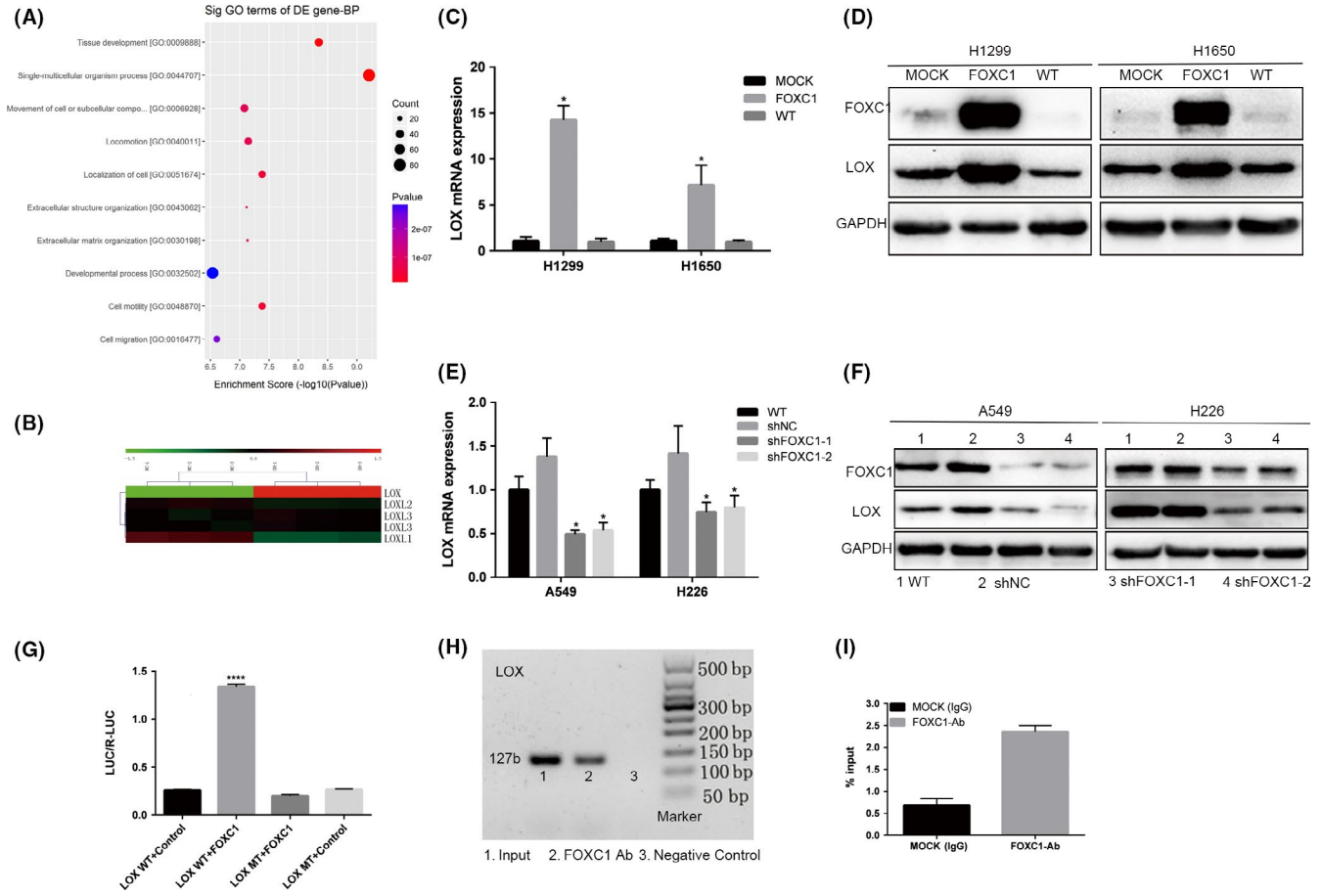

FIGURE 5 Lysyl oxidase (LOX) is a novel downstream target of forkhead box C1 (FOXC1). (A) A functional annotation of clustering of genes regulated by FOXC1. Enriched groups were named by the gene ontology term of the group member with the significant difference and were ranked by the group enrichment score dot plot. (B) Gene expression of LOX family in H1299 cells transfected with FOXC1 or MOCK vector. Red: high expression; green: low expression. (C and D) The mRNA and protein expression of LOX significantly increased when FOXC1 was overexpressed in lentivirus-infected H1299 and H1650 cells. (E and F) The mRNA and protein expression of LOX significantly decreased when FOXC1 was silenced in A549 and H226 cells transfected with FOXC1-shRNA vector. (G) The luciferase activity of the promoter variants was determined in the presence of FOXC1 overexpression or negative control by Dual-Luciferase Reporter Assay System. (H and I) CHIP assay showed OXC1 directly binding to the LOX promoter in H1299 cells. Results were expressed as percentage of input. The error bars indicate ±SEM. *P < .05, **P < .01, ***P < .005, ****P < .001 by Student's t test

migratory and invasive behaviors of the cells compared with the control cells (Figure 6F to H, Figure S3F to H). However, LOX down-regulation in those cells had no significant effect on cell proliferation (Figure S3E). These results demonstrated that LOX was essential for FOXC1-mediated NSCLC invasion and metastasis but not proliferation in vitro.

In addition, to assess the role of LOX in FOXC1-mediated NSCLC progression in vivo, we also analyzed the tumor growth and metastasis in subcutaneous xenograft and tail vein-lung metastasis tumor models. Consist with the result in vitro, the number of lung micrometastasis was much less in the BAPN group compared with the control group (Figure 6I to L), but there was no significance for either tumor volumes or weight. (Figure S3I to N). The data indicated that LOX was the main reason contributing to FOXC1-derived tumor metastasis in vivo.

Furthermore, to evaluate the LOX function, H1299-FOXC1 cells were treated with BAPN, a specific and irreversible inhibitor of LOX enzymatic activity. Consistent with previous reports, LOX inhibition significantly inhibited the migration and invasion of BAPN-treated H1299-FOXC1 cells but had no significant effect on cell proliferation (Figure S4A to G). As for the in vivo assay, some mice were treated daily with 100 mg/kg BAPN as described for the last weeks and terminal bleeds were taken from mice at the end of the experiment. We observed that the LOX activity level in the blood of BAPN-treated mice was obviously reduced, which was confirmed in an in vitro assay (Figure S4H). All these results provided powerful evidence that LOX was essential for FOXC1-mediated NSCLC invasion and metastasis in vitro as well as in vivo.

3.6 | Correlation between forkhead box C1 and lysyl oxidase expression in vivo

The positive correlation between FOXC1 and LOX in NSCLC cells reminded us to investigate whether such a relationship also exists in NSCLC patients and xenograft models. We first determined

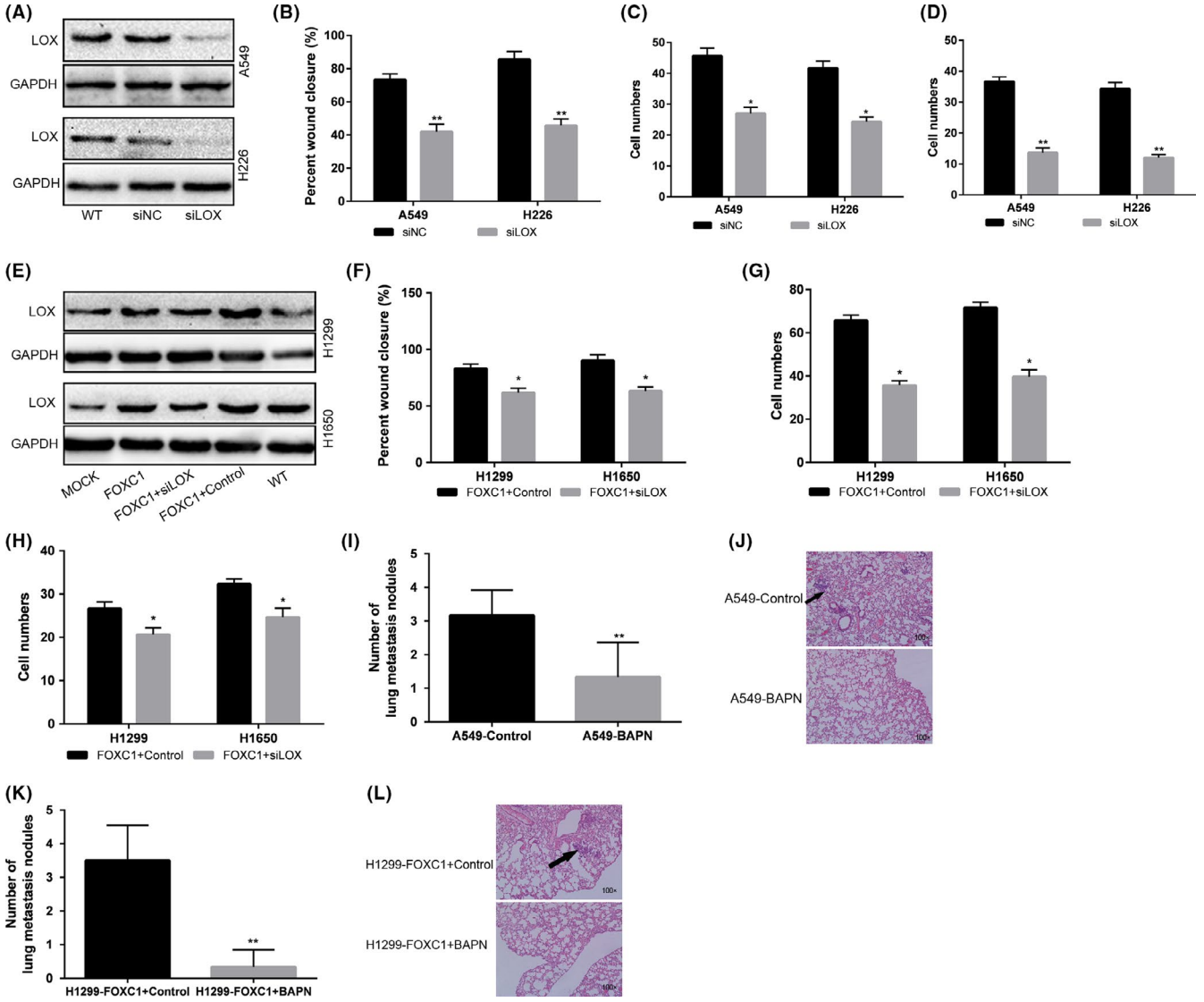

FIGURE 6 Lysyl oxidase (LOX) was essential for forkhead box C1 (FOXC1)-mediated non–small cell lung cancer (NSCLC) invasion and metastasis. (A) The LOX expression significantly decreased in A549 and H226 cells transfected with siLOX and negative control (siNC). (B and C) Comparison of cell migration by wound-healing assay and transwell migration assay. LOX knockdown significantly inhibited the migration of A549 and H226 cells. (D) Comparison of cell invasion by transwell invasion assay and LOX knockdown significantly inhibited the invasive ability of A549 and H226 cells. (E) The LOX expression significantly decreased in FOXC1 overexpression H1299 and H1650 cells transfected with siLOX and negative control (control). (F and G) Comparison of cell migration by wound-healing assay and transwell migration assay in FOXC1 overexpression H1299 and H1650 cells transfected with siLOX and negative control (control), respectively. Silence of LOX inhibited the migratory behaviors of the cells compared with the control cells. (H) Comparison of cell invasion by transwell invasion assay and LOX downexpression inhibited the invasive behaviors of the cells compared with the control cells. (I and J) Comparison of lung metastasis in mice after intravenous injection with A549 cells and β-aminopropionitrile monofumarate (BAPN) or control. (K and L) Microscopic quantification of metastasis in lungs after intravenous injection with H1299-FOXC1 cells and BAPN or control (magnification of 100×). The error bars indicate ±SEM. **P* < .05, ***P* < .01 by Student's *t* test. All the results were repeated thrice

FOXC1 and LOX expression in the former 105 primary human NSCLC tissues by IHC staining, and representative images are shown in Figure 7A. The data showed that LOX expression in the tumor was higher than in paratumor tissues ($P < .01$) (Figure S5A). Furthermore, NSCLC patients with LOX high expression have shorter overall survival than patients without LOX expression (Figure S5B, $P = .0456$). The upregulation of LOX was also positively correlated with the level of its master regulator FOXC1 (Figure 7B, $P < .01$, $r = .6257$). Finally, we found that positive co-expression of FOXC1/LOX predicted the lowest overall survival in NSCLC patients (Figure 7C). Similarly, the positive correlation between FOXC1 and LOX expression was also observed in subcutaneous xenograft models (Figure S5C). The specificity of FOXC1 and LOX antibodies have been verified (Figure S6).

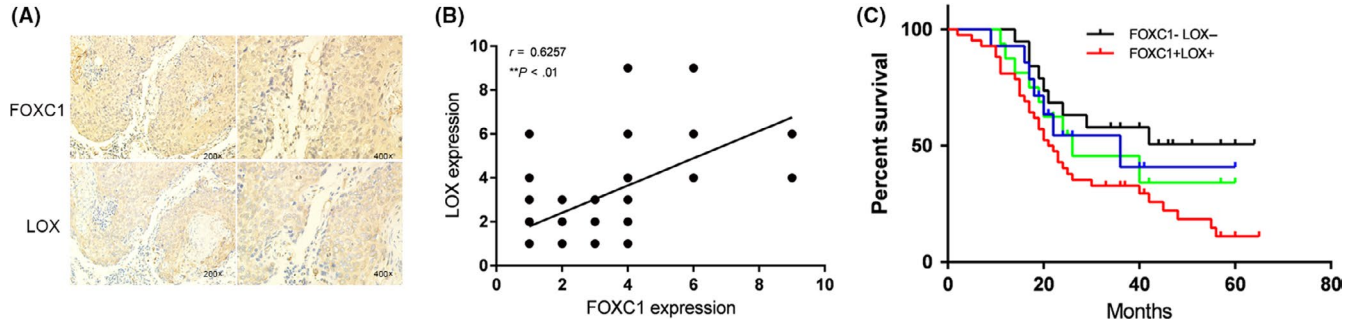

FIGURE 7 Lysyl oxidase (LOX) expression was closely correlated with forkhead box C1 (FOXC1) expression in non–small cell lung cancer (NSCLC) tissues. (A) Immunohistochemistry (IHC) images of FOXC1 and LOX expression in primary human NSCLC tissues were positively correlated. (B) The positive correlation between FOXC1 expression and the expression of its target gene LOX in NSCLC tissues ($P < .01$, $r = .6257$). (C) The correlation analysis between overall survival and the expression of FOXC1 and LOX (log-rank test)

4 | DISCUSSION

In our study, we found that FOXC1 was highly expressed in NSCLC patients and identified FOXC1 as an important prognostic factor in NSCLC. FOXC1 overexpression promoted the proliferation, metastasis and invasion of NSCLC, whereas FOXC1 silencing inhibited these effects. In particular, we first found a positive correlation between FOXC1 expression and LOX expression in NSCLC cancer samples and implicated LOX activity in the metastatic phenotype. We further confirmed that FOXC1 directly interacted within the promoter of LOX and showed that pharmacologic inhibition or genetic silencing of LOX can decrease metastasis in vivo. Our study provided new interventional strategies to inhibit and treat metastasis of NSCLC.

Recent studies have demonstrated that FOXC1 is highly expressed in various cancers,[40-42] including NSCLC,[43] and is postulated to be a marker of poor prognosis[17,18] In the present study, we identified that high FOXC1 expression in NSCLC patients was more frequently associated with adverse clinical parameters and poor OS independent of other clinicopathological prognostic factors, including lymph node status in NSCLC patients. This result was consistent with the conclusion of the study of Cao et al, in which FOXC1 expression was found to be elevated in NSCLC tissues and negatively correlated with patient survival.[44] In addition, FOXC1 was expressed in the membrane and cytoplasm, as well as in the cell nucleus. It appeared that a different location of FOXC1 in NSCLC cells might promote tumor progression through different patterns, and further extensive study is needed. We also detected high expression of FOXC1 (67.2%) in non–squamous NSCLC patients, which was significantly higher than that in squamous NSCLC patients, and not consistent with the result of Wei et al.[43] This was possibly owing to the smaller number of specimens, and further study is needed to identify whether FOXC1 expression depends on the cell types in NSCLC patients. Moreover, we found that FOXC1 was implicated with cell phenotype, such as proliferation, metastasis and invasion in vitro, together with tumor growth and metastasis in vivo. It is worth mentioning that as one of the few original articles on FOXCI in NSCLC, the finding of Chen et al identified that silencing FOXC1 inhibited the proliferation and migration of A549 cells.[45] Taken together, these results indicated that FOXC1 was involved in tumorigenesis and progression of the NSCLC through promoting tumor cell growth and metastasis.

FOXC1 promoted metastasis and invasion through inducing EMT,[25] increasing breast cancer stem cell properties,[46] and was involved in the repression of ER expression[47] in breast cancer cells. Lin et al reported that hypoxia activated FOXC1 transcription via direct binding of hypoxia-inducible factor-1α (HIF-1α) to the hypoxia-responsive element (HRE) in the FOXC1 promoter and FOXC1 gain-of-function in lung cancer cells promoted cell proliferation, migration and invasion.[48] However, the precise function and mechanism by which FOXC1 exerts its functions in NSCLC remains to be elucidated. Our study showed, for the first time, that FOXC1 promoted migration and invasion through directly targeted LOX expression in NSCLC.

LOX, a member of a five-member family of amine oxidases, has been shown to be an important regulator of the ECM.[49] Emerging evidence implicates LOX as being strongly associated with poor progression in various tumors, including non–small cell lung cancer, and it is regarded as a targetable secreted molecule involved in cancer metastasis.[26,30,32,34,50] Downregulation of LOX in H1650 and H1299 cells transfected with FOXC1 vector or inhibition of LOX activity using BAPN inhibited the FOXC1-driven effects on cell migration and invasion in vitro, and inhibition of LOX activity using BAPN decreased the number of lung metastases in vivo, which suggested that FOXC1-LOX axis was involved in the metastasis and invasion of NSCLC and FOXC1 promoted metastasis by regulating LOX expression. In this study, we also found that LOX knockdown failed to influence the proliferation in those cells. This conclusion is different from the findings of Kanapathipillai et al.[51] The reason might be that the modulation of other cellular pathways or proteins induced by FOXC1 compensated for the inhibition of cell growth mediated by LOX downregulation. However, the above conclusion is consistent with the results of Erler et al, who found that inhibition of LOX (with siRNA or BAPN) did not have a major effect on tumor growth, and there was no association between tumor size and the number of metastases.[28] After all, homeostasis is a complex regulated network.

Weaver and colleagues demonstrated LOX-mediated collagen crosslinking as a contributor to tumor matrix stiffening, which led to enhanced integrin signaling and invasive behavior in tumors.[49,52] Cox et al identified LOX as a novel regulator of the formation of focal pre–metastatic lesions, which provided a platform for circulating tumor cells to colonize and form bone metastases.[53] Tang et al found that LOX regulated the EGFR to drive tumor progression.[29] However, the exact downstream target of LOX and the signal pathway of the LOX-induced metastasis and invasion in NSCLC cells needs to be further characterized.

In summary, our study found a positive correlation between FOXC1 and LOX expression in NSCLC patients and explored, for the first time, the mechanism of FOXC1 by binding in the promoter of LOX. This offers a new mechanistic insight into FOXC1 regulation in the invasion and metastasis of NSCLC and implicates FOXC1 as a potential therapeutic strategy for the treatment of NSCLC metastasis.

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DISCLOSURE STATEMENT

The authors have no conflict of interest to declare. All authors have read the journal's authorship agreement. The manuscript has been reviewed by and approved by all named authors.

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REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin*. 2018;68:7-30.
2. Chen W, Sun K, Zheng R, et al. Cancer incidence and mortality in China, 2014. *Chin J Cancer Res*. 2018;30:1-12.
3. Tsim S, O'Dowd CA, Milroy R, Davidson S. Staging of non–small cell lung cancer (NSCLC): a review. *Respir Med*. 2010;104:1767-1774.
4. Claudia Allemani TM, Di Carlo V, Harewood R et al. Global surveillance of trends in cancer survival 2000–14 (CONCORD-3): analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. *Lancet*. 2018;391:1023–1075.
5. Katoh M, Igarashi M, Fukuda H, Nakagama H, Katoh M. Cancer genetics and genomics of human FOX family genes. *Cancer Lett*. 2013;328:198-206.
6. Aldinger KA, Lehmann OJ, Hudgins L, et al. FOXC1 is required for normal cerebellar development and is a major contributor to chromosome 6p25.3 Dandy-Walker malformation. *Nat Genet*. 2009;41:1037-1042.
7. French CR, Seshadri S, Destefano AL, et al. Mutation of FOXC1 and PITX2 induces cerebral small-vessel disease. *J Clin Invest*. 2014;124:4877-4881.
8. Ito YA, Goping IS, Berry F, Walter MA. Dysfunction of the stress-responsive FOXC1 transcription factor contributes to the earlier-onset glaucoma observed in Axenfeld-Rieger syndrome patients. *Cell Death Dis*. 2014;5:e1069.
9. Berry FB, Skarie JM, Mirzayans F, et al. FOXC1 is required for cell viability and resistance to oxidative stress in the eye through the transcriptional regulation of FOXO1A. *Hum Mol Genet*. 2008;17:490-505.
10. Fatima A, Wang Y, Uchida Y, et al. Foxc1 and Foxc2 deletion causes abnormal lymphangiogenesis and correlates with ERK hyperactivation. *J Clin Invest*. 2016;126:2437-2451.
11. Lambers E, Arnone B, Fatima A, Qin G, Wasserstrom JA, Kume T. Foxc1 regulates early cardiomyogenesis and functional properties of embryonic stem cell derived cardiomyocytes. *Stem Cells*. 2016;34:1487-1500.
12. Han B, Bhowmick N, Qu Y, Chung S, Giuliano AE, Cui X. FOXC1: an emerging marker and therapeutic target for cancer. *Oncogene*. 2017;36:3957-3963.
13. Ray PS, Wang J, Qu Y, et al. FOXC1 Is a potential prognostic biomarker with functional significance in basal-like breast cancer. *Can Res*. 2010;70:3870-3876.
14. Muggerud AA, Ronneberg JA, Warnberg F, et al. Frequent aberrant DNA methylation of ABCB1, FOXC1, PPP2R2B and PTEN in ductal carcinoma in situ and early invasive breast cancer. *Breast Cancer Res*. 2010;12:R3.
15. Subramani R, Camacho FA, Levin CI, et al. FOXC1 plays a crucial role in the growth of pancreatic cancer. *Oncogenesis*. 2018;7:52.
16. Wang L, Gu F, Liu CY, Wang RJ, Li J, Xu JY. High level of FOXC1 expression is associated with poor prognosis in pancreatic ductal adenocarcinoma. *Tumour Biol*. 2013;34:853-858.
17. Xu Y, Shao QS, Yao HB, Jin Y, Ma YY, Jia LH. Overexpression of FOXC1 correlates with poor prognosis in gastric cancer patients. *Histopathology*. 2014;64:963-970.
18. Xia L, Huang W, Tian D, et al. Overexpression of forkhead box C1 promotes tumor metastasis and indicates poor prognosis in hepatocellular carcinoma. *Hepatology*. 2013;57:610-624.
19. Huang W, Chen Z, Zhang L, et al. Interleukin-8 Induces expression of FOXC1 to promote transactivation of CXCR1 and CCL2 in hepatocellular carcinoma cell lines and formation of metastases in mice. *Gastroenterology*. 2015;149(1053–67):e14.
20. Somerville TD, Wiseman DH, Spencer GJ, et al. Frequent derepression of the mesenchymal transcription factor gene FOXC1 in acute myeloid leukemia. *Cancer Cell*. 2015;28:329-342.
21. Wang J, Ray PS, Sim MS, et al. FOXC1 regulates the functions of human basal-like breast cancer cells by activating NF-kappaB signaling. *Oncogene*. 2012;31:4798-4802.
22. Yanli Jin P, Bingchen Han P, Jiongyu Chen BS, et al. FOXC1 is a critical mediator of EGFR function in human basal-like breast cancer. *Ann Surg Oncol*. 2014;21:S758-S766.
23. Sizemore ST, Keri RA. The forkhead box transcription factor FOXC1 promotes breast cancer invasion by inducing matrix metalloprotease 7 (MMP7) expression. *J Biol Chem*. 2012;287:24631-24640.
24. Han B, Zhou B, Qu Y, et al. FOXC1-induced non–canonical WNT5A-MMP7 signaling regulates invasiveness in triple-negative breast cancer. *Oncogene*. 2018;37:1399-1408.
25. Hopkins A, Coatham ML, Berry FB. FOXC1 regulates FGFR1 isoform switching to promote invasion following TGFbeta-induced EMT. *Mol Cancer Res*. 2017;15:1341-1353.
26. Trackman PC. Lysyl oxidase isoforms and potential therapeutic opportunities for fibrosis and cancer. *Expert Opin Ther Targets*. 2016;20:935-945.

27. Johnston KA, Lopez KM. Lysyl oxidase in cancer inhibition and metastasis. *Cancer Lett*. 2018;417:174-181.
28. Erler JT, Bennewith KL, Nicolau M, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature*. 2006;440:1222-1226.
29. Tang H, Leung L, Saturno G, et al. Lysyl oxidase drives tumour progression by trapping EGF receptors at the cell surface. *Nat Commun*. 2017;8:14909.
30. Osawa T, Ohga N, Akiyama K, et al. Lysyl oxidase secreted by tumour endothelial cells promotes angiogenesis and metastasis. *Br J Cancer*. 2013;109:2237-2247.
31. Cox TR, Gartland A, Erler JT. Lysyl oxidase, a targetable secreted molecule involved in cancer metastasis. *Cancer Res*. 2016;76:188-192.
32. Wilgus ML, Borczuk AC, Stoopler M, et al. Lysyl oxidase: a lung adenocarcinoma biomarker of invasion and survival. *Cancer*. 2011;117:2186-2191.
33. Min C, Kirsch KH, Zhao Y, et al. The tumor suppressor activity of the lysyl oxidase propeptide reverses the invasive phenotype of Her-2/neu-driven breast cancer. *Cancer Res*. 2007;67:1105-1112.
34. Wu M, Min C, Wang X, et al. Repression of BCL2 by the tumor suppressor activity of the lysyl oxidase propeptide inhibits transformed phenotype of lung and pancreatic cancer cells. *Cancer Res*. 2007;67:6278-6285.
35. Villanueva MT. Metastasis: LOX does some prepping. *Nat Rev Cancer*. 2015;15:384–385.
36. Liu Huang ZH, Fan YI, He L, et al. FOXC1 promotes proliferation and epithelial-mesenchymal transition in cervical carcinoma through the PI3K-AKT signal pathway. *Am J Transl Res*. 2017;9(3):1297-1306.
37. Tamimi Y, Skarie JM, Footz T, Berry FB, Link BA, Walter MA. FGF19 is a target for FOXC1 regulation in ciliary body-derived cells. *Hum Mol Genet*. 2006;15:3229-3240.
38. Palamakumbura AH, Trackman PC. A fluorometric assay for detection of lysyl oxidase enzyme activity in biological samples. *Anal Biochem*. 2002;300:245-251.
39. 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;280:24690-24697.
40. Liu J, Zhang Z, Li X, et al. Forkhead box C1 promotes colorectal cancer metastasis through transactivating ITGA7 and FGFR4 expression. *Oncogene*. 2018;37:5477-5491.
41. Xiaoming Zhu LW, Yangqiu B, Sen W, Shuangyin H. FoxC1 promotes epithelial-mesenchymal transition through PBX1 dependent transactivation of ZEB2 in esophageal cancer. *Am J Cancer Res*. 2017;7(8):1642-1653.
42. Xu YZ, Chen FF, Zhang Y, et al. The long noncoding RNA FOXCUT promotes proliferation and migration by targeting FOXC1 in nasopharyngeal carcinoma. *Tumour Biol*. 2017;39:1010428317706054.
43. Wei LX, Zhou RS, Xu HF, Wang JY, Yuan MH. High expression of FOXC1 is associated with poor clinical outcome in non–small cell lung cancer patients. *Tumour Biol*. 2013;34:941-946.
44. Cao S, Wang Z, Gao X, et al. FOXC1 induces cancer stem cell-like properties through upregulation of beta-catenin in NSCLC. *J Exp Clin Cancer Res*. 2018;37:220.
45. Chen S, Jiao S, Jia Y, Li Y. Effects of targeted silencing of FOXC1 gene on proliferation and in vitro migration of human non–small-cell lung carcinoma cells. *Am J Transl Res*. 2016;8(8):3309-3318.
46. Han B, Qu Y, Jin Y, et al. FOXC1 activates smoothened-independent hedgehog signaling in basal-like breast cancer. *Cell Rep*. 2015;13:1046-1058.
47. Yu-Rice Y, Jin Y, Han B, et al. FOXC1 is involved in ERalpha silencing by counteracting GATA3 binding and is implicated in endocrine resistance. *Oncogene*. 2016;35:5400-5411.
48. Lin YJ, Shyu WC, Chang CW, et al. Tumor hypoxia regulates forkhead box C1 to promote lung cancer progression. *Theranostics*. 2017;7:1177-1191.
49. Levental KR, Yu H, Kass L, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell*. 2009;139:891-906.
50. Baker AM, Cox TR, Bird D, et al. The role of lysyl oxidase in SRC-dependent proliferation and metastasis of colorectal cancer. *J Natl Cancer Inst*. 2011;103:407-424.
51. Kanapathipillai M, Mammoto A, Mammoto T, et al. Inhibition of mammary tumor growth using lysyl oxidase-targeting nanoparticles to modify extracellular matrix. *Nano Lett*. 2012;12:3213-3217.
52. Ng MR, Brugge JS. A stiff blow from the stroma: collagen crosslinking drives tumor progression. *Cancer Cell*. 2009;16:455-457.
53. Cox TR, Rumney R, Schoof EM, et al. The hypoxic cancer secretome induces pre–metastatic bone lesions through lysyl oxidase. *Nature*. 2015;522:106-110.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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