ESM1/HIF-1α pathway modulates chronic intermittent hypoxia-induced non-small-cell lung cancer proliferation, stemness and epithelial-mesenchymal transition

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Abstract. Obstructive sleep apnea (OSA) is a sleep-related disorder characterized by chronic intermittent hypoxia (CIH). Previous studies have found that intermittent hypoxia promotes drug resistance, cell proliferation, migration and invasion in non-small cell lung cancer (NSCLC). Endothelial cell-specific molecule-1 (ESM1) is a molecule shown to be overexpressed in several types of tumors. The purpose of this study was to investigate the correlation between CIH and ESM1 and their potential roles in the progression of NSCLC. Tumorspheres, cell viability and colony formation assays were used to evaluate cell proliferation. The expression levels of cancer stem cell (CSC) markers CD44, CD133, OCT4 and SOX2 were measured with western blotting and/or RT-qPCR. Transwell assays were applied to assess cell migration and invasion. Changes in the expression levels of epithelial-mesenchymal transition (EMT)-associated proteins were also detected by western blotting. The results indicated that CIH enhanced lung cancer stem cell (LCSC) NSCLC progression by promoting stemness, drug resistance, cell proliferation, migration and invasion via the ESM1/HIF-1α pathway. Unexpectedly, inhibition of ESM1 reversed the CIH-involved negative effects on LCSCs and in a mouse model. ESM1 therefore appears to be crucial mediator of CIH-mediated lung cancer progression.

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Abbreviations: OSA, obstructive sleep apnea; CIH, chronic intermittent hypoxia; NSCLC, non-small cell lung cancer; siRNA, small interfering RNA; ESM1, endothelial cell-specific molecule-1; HIF-1α, hypoxia inducible factor-1α; CSC, cancer stem cell; EMT, epithelial-mesenchymal transition; RT-qPCR, quantitative reverse transcription polymerase chain reaction

Key words: non-small cell lung cancer, chronic intermittent hypoxia, cancer stem cell, ESM1, HIF-1α, epithelial-mesenchymal transition

Introduction

Obstructive sleep apnea (OSA) is a sleep-related breathing disorder that has increased in prevalence in recent years. OSA is a serious public health issue associated with multiple health problems including cerebrovascular disease, cardiovascular disease and pulmonary disease (1-6). Characterized by chronic intermittent hypoxia (CIH), which results from the upper airway being recurrently obstructed during sleep, OSA is estimated to affect 3-7% of the adult population (7,8). In addition, OSA has been associated with increased cancer mortality and cancer incidence in some studies (9-11).

Lung cancer is a major cause of cancer-related deaths worldwide, accounting for 27 and 25% of cancer deaths among men and women, respectively (12). Three major forms of hypoxia are present in solid tumors: Chronic, acute and intermittent (13). The correlation between OSA-related CIH and the development of lung cancer has been studied, and accumulating evidence indicates that intermittent hypoxia is a key regulator of the interplay between cancer cells and endothelial cells in tumors, and it enhances cancer progression and metastasis (14-17). Furthermore, previous studies have shown that hypoxia in tumors is correlated with a greater risk of metastasis, increased invasiveness, and resistance to systemic and radiation therapy (18). The mechanisms underlying these systemic and cellular responses remain unknown and warrant further investigation.

Endothelial cell-specific molecule-1 (ESM1) is a newly reported molecule that is expressed in the lung, regulated by inflammatory cytokines and associated with endothelial dysfunction (19). Recent studies have found that ESM-1 is markedly overexpressed in several types of cancer, including clear cell renal cell carcinoma, gastric cancer and lung cancer, thus making ESM1 a potential endothelial cell marker and a possible target for cancer therapy (19-22). Furthermore, silencing of ESM1 was found to inhibit cell survival, migration and invasion, and to modulate cell cycle progression in hepatocellular carcinoma (23). According to recent research, ESM1 enhances adhesion between

monocytes and endothelial cells under intermittent hypoxic conditions (24). An investigation of the modulation of ESM1 under hypoxia has indicated that ESM-1 expression is induced by hypoxia-inducible factor-1α (HIF-1α) (20). HIF-1α is correlated with apoptosis in lung cancer and is commonly overexpressed in non-small cell lung cancer (NSCLC) (25-27). However, the interactions among CIH, ESM1 and HIF-1α require further study.

In the present study, we constructed a lung cancer stem cell (LCSC) model and mouse model with tumors *in situ*. The expression levels of ESM1 and HIF-1α under CIH were investigated. We detected whether CIH exposure enhanced lung cancer cell survival, drug resistance and stemness. Cell proliferation, migration and cell invasion were analyzed after CIH preconditioning. We further identified the expression of related indicators after small interfering (si)-ESM1 plasmid transfection or lentivirus infection. The results showed that CIH promoted ESM1 and HIF-1α expression and led to drug resistance, cell proliferation, migration and invasion. After ESM-1 suppression, these CIH-induced effects were reversed. Overall, our results suggest that ESM-1 may be a potential target in OSA-associated lung cancer progression.

Materials and methods

Cell culture. Human lung cancer cells (PC-9 and A549 cells) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in 90% Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin in a 37˚C humidified incubator with 5% CO_2.

LCSC construction. PC9 and A549 cisplatin-resistant cells were obtained through cisplatin repeated treatment with increasing doses (1 to 10 μmol/l), and cells were cultured in serum-free stem cell culture medium (DMEM-F12 medium; insulin, 4 U/l; B27, 1X; EGF, 20 ng/ml; and bFGF, 20 ng/ml) to construct the spheres.

Cell CIH exposure The cells were exposed to 5 sec of 14 to 15% O_2 in every 60 sec cycle for 24 or 48 h. All cells were cultured for 48 h for further experiments.

Total RNA isolation and quantitative reverse transcription-polymerase chain reaction (RT-qPCR). RNA was isolated with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and was reverse-transcribed with a miScript Reverse Transcription kit (Qiagen, Germany). RT-qPCR was performed with a SYBR Premium Ex Taq II kit (Takara) on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The amplification reactions were run with 30 thermocycles of 30 sec at 95˚C, 30 sec at 55˚C and 30 sec at 72˚C. The expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method. All reactions were performed in triplicate, and the mean value was used to calculate expression levels after normalization to β-actin. The primer sequences are shown in Table I.

Protein extraction and western blot analysis. The lung tissue and LCSCs were lysed with RIPA buffer, and the protein concentration was determined with a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Approximately 30 μg of protein from each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. Membranes were blocked in 5% skim milk in TBST and incubated with primary antibodies to one of the following (all from Abcam) overnight at 4˚C: CD44 (ab157107, 1:1,000), CD133 (ab226355, 1:1,000), OCT4 (ab18976, 1:1,000), SOX2 (ab97959, 1:1,000), E-cadherin (ab40772, 1:1,000), N-cadherin (ab18203, 1:1,000), Snail (ab53519, 1:1,000), Vimentin (ab92547, 1:1,000), ESM1 (ab103590, 1:1,000), HIF-1α (ab51608, 1:1,000) or β-actin (ab8226, 1:1,500). Membranes were incubated with the corresponding secondary antibody for 1 h at room temperature and washed in TBST. Protein signals were detected with Super ECL Plus Detection Reagent (Merck).

Cell viability assay. The Cell Counting Kit-8 (CCK-8) assay was used to detect cell survival rates. Transfected cells were seeded into 96-well plates at a density of 5,000 cells/well in triplicate. Cell viability was measured with the CCK-8 system (Gibco; Thermo Fisher Scientific, Inc.) at different cisplatin concentrations after seeding, according to the manufacturer's instructions.

For colony formation assays, transfected cells were seeded into 6-well plates at a density of 2,000 cells/well and maintained in DMEM containing 10% FBS for 10 days. The colonies were fixed with 70% ethanol for 15 min at room temperature and stained with 2% crystal violet. The positive colonies (more than 50 cells or 3 mm2 per colony) were counted. Colonies were imaged under a light microscope (Olympus, Tokyo, Japan) and Results were expressed as the average number of cells in every visual field after they were fixed and stained.

Sphere formation assay. Cells were grown in MammoCult medium (Stem Cell Technologies) supplemented with MammoCult Proliferation Supplements (Stem Cell Technologies) and plated in 24-well plates with ultra-low attachment at a density of 10,000 viable cells/ml and grown for 10 days. Spheres were counted and photographed under a light microscope (Olympus).

Transwell migration assay. Cell migration was analyzed with Transwell chambers (Corning Inc.) in accordance with the manufacturer's protocol. After incubation at 37˚C for 48 h, cells on the upper surfaces of the Transwell chambers were removed with cotton swabs, and cells located on the lower surfaces were fixed with methanol for 10 min, then stained with 0.1% crystal violet. Stained cells were imaged under a light microscope (Olympus) and counted in five randomly selected fields. In invasion experiments, chamber inserts were coated with 200 mg/ml Matrigel and dried overnight under sterile conditions.

Mouse orthotopic assay. A total of 24 male BALB/c-nu nude mice (6 weeks old, weight 20±2 g) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). After anesthesia, a 5-mm incision was cut at 1.5 cm above the arcus

Table I. Primers for the RT-qPCR analysis.

Name	Sequences (5'-3')
CD44	F: GACACATATTGTTTCAATGCTTCAGC
	R: GATGCCAAGATGATCAGCCATTCTGGAAT
CD133	F: TACAACGCCAAACCACGACTGT
	R: TCTGAACCAATGGAATTCAAGACCCTTT
ESM1	R: GCCATGTCATGCTCTTTGCAG
	F: GGCGGCACCACCATGTACCCT
β-actin	R: AGGGGCCGGACTCGTCATACT
	F: CTTGCTACCGCACAGTCTCA

F, forward; R, reverse; ESM1, endothelial cell-specific molecule-1.

costarum of the left anterior axillary line in each male nude mouse, and tissues were pulled apart until the chest wall was exposed; a PC-9 suspension with a density of $1x10^7$ ml was prepared, and then 50 μl cell suspension and 50 ml Matrigel were mixed and subsequently injected into the left lung of the mice through the chest wall at a depth of 3 mm. After the injection, the needle was held in place for 5 sec and was then removed, and the incision was sutured. At 1 week after the operation, magnetic resonance imaging examination was performed to determine whether the tumor had grown. On day 7 after cell injection, CIH exposure was initiated. The oxygen content in the CIH exposure chamber was measured over the course of several cycles with an oxygen sensor placed on the bottom of the chamber. Animals were exposed to 5 sec of 14 to 15% O_2 in every 60 sec cycle. Each challenge lasted 8 h during the daytime and was repeated on days 7, 14, 21 and 28 and 2 days after the last challenge. si-ESM1 lentivirus was purchased from HanBio. One hundred microliters of filter-purified lentivirus cocktail ($1x10^5$ IU/μl) was administered by intravenous injection weekly on days 7, 14, 21 and 28 and 2 days after the last challenge. All mice were treated for 5 weeks and sacrificed by cervical dislocation following anesthesia with an intraperitoneal injection of sodium pentobarbital (60 mg/kg).

This study was performed in strict accordance with Guide for the Care and Use of Laboratory Animals (8th edition, 2011, published by The National Academies Press, https://www.nap.edu/catalog/12910/guide-for-the-care-and-use-of-laboratory-animals-eighth). The protocol was reviewed and approved by the Shanghai Ninth People's Hospital Institutional Review Board (permit no. HKDL2013001b). They were raised in a constant temperature (22±1˚C) and humidity (65±5%), with a 12-h light/dark cycle, with standard diet and water. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering.

Enzyme-linked immunosorbent assay (ELISA). The concentrations of ESM1 in mouse serum were determined with an ELISA kit (Nanjing Jiancheng Bioengineering Institute), according to standard protocols.

Immunohistochemistry. Tumor tissue samples were fixed in 10% formalin and embedded in paraffin. Sections (5-μm) were stained with Ki67 to evaluate proliferation at 4˚C overnight. The sections were then washed in PBS and incubated at room temperature for 20 min with biotinylated secondary antibody (Qiagen, Germany). Sections were examined with an Axiophot light microscope at x200 magnification (Zeiss) and imaged with a digital camera.

Statistical analysis. All results in this study are presented as the mean ± SD and were statistically analyzed using GraphPad Prism 9.0 (GraphPad Software, Inc.). One-way ANOVA with post hoc test was applied among the various groups. $P<0.05$ was considered to indicate statistical significance.

Results

ESM1 is overexpressed at both the protein and gene levels in PC-9 and A549 cells after culture for 7 days. Repeated cisplatin treatments with increasing doses (1 to 10 μmol/l) were applied to obtain PC-9 and A549 cisplatin-resistant cells. After 7 days in culture, the cell proliferation led to a progressive increase in diameters and sphere numbers, and the tumorspheres formed from NSCLC cell lines were identified under a microscope (Fig. 1A). To further identify the cultured LCSCs, the CSC markers CD44 and CD133 were used. Western blotting and RT-qPCR results together showed that the cultured tumorigenic lung tumorspheres exhibited significantly increased expression of CD133 and CD44 at both the protein and mRNA levels (Fig. 1B and C).

Previous studies have identified that ESM1 is markedly overexpressed in cancer cells (21,22,28). In the present study, western blotting confirmed that the ESM1 expression increased significantly at the protein level in PC-9 and A549 cells (Fig. 1D). RT-PCR analysis further showed that ESM1 expression was significantly enhanced at the mRNA level (Fig. 1E). These results indicated that the cisplatin-resistant LCSCs obtained from PC-9 and A549 cell lines displayed stem-like features and had enhanced expression of ESM1 at both the protein and mRNA levels.

CIH exposure leads to drug resistance, and promotes cell proliferation, migration and invasion in vitro. Accumulating studies show that hypoxia in several tumor types is associated with a greater risk of metastasis, increased invasiveness and resistance to systemic and radiation therapy (14-16). To investigate the cellular changes caused by CIH, we exposed the cells to 5 sec of 14 to 15% O_2 in every 60 sec cycle for 24 or 48 h. All groups were cultured for a total of 48 h. The viability of cisplatin-resistant PC-9 and A549 cells was assessed with CCK-8 assays, and the results showed greater cell viability in the 24-h CIH exposure group and much higher cell viability in the 48-h group compared with the control group (Fig. 2A). To further detect the correlation between drug resistance and CIH, we added different doses of cisplatin during the cell culture. Although the cell viability was significantly decreased as the dose increased, among the groups cultured with the same cisplatin dose, the groups exposed to CIH for 24 h presented significantly higher cell viability, and those exposed for 48 h showed the highest cell viability (Fig. 2B).

Colony formation assay results showed that in the PC-9 and A549 LCSC groups, cell proliferation increased markedly

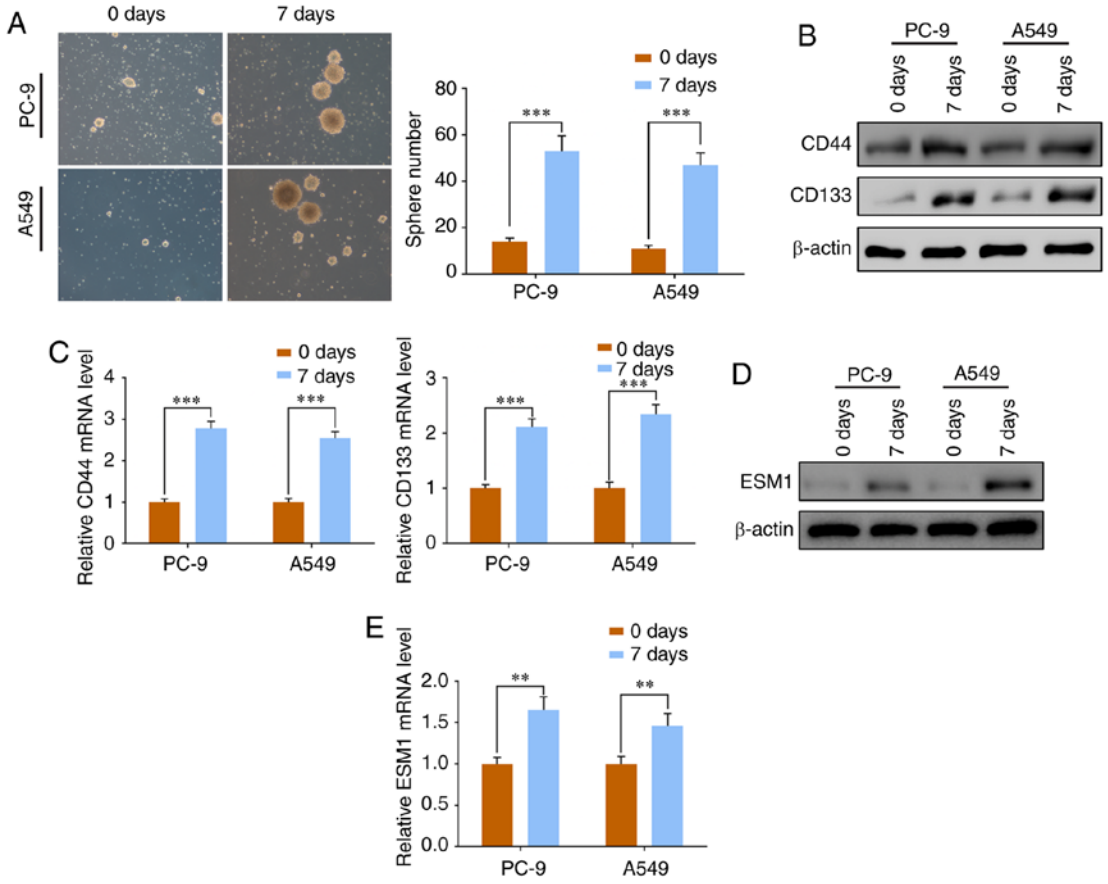

Figure 1. ESM1 is overexpressed at both the protein and gene levels in PC-9 and A549 cells after stem cell culture for 7 days. Repeated cisplatin treatments with increasing doses (1 to 10 μmol/l) were applied to obtain PC-9 and A549 cisplatin-resistant cells, which were cultured for 7 days. (A) Images of PC-9 and A549 tumor spheres under a microscope and quantitative results (magnification, x100). (B) Western blotting results of the CSC markers CD44 and CD133 in the PC-9 and A549 groups, before and after 7 days of culture. (C) RT-qPCR results of the CD44 and CD133 mRNA levels in the PC-9 and A549 groups. (D) Western blotting results of ESM1 in the PC-9 and A549 groups. (E) RT-qPCR confirmation of changes in ESM1 at the mRNA level *in vitro*. Data are expressed as the means ± SEM (n=3); **P<0.01, ***P<0.001 vs. 0 days. ESM1, endothelial cell-specific molecule-1; CSC, cancer stem cell.

after a 24-h CIH exposure and was further enhanced after CIH exposure for 48 h (Fig. 2C). Photographs of the tumorspheres provided additional confirmation of the increasing sphere numbers (Fig. 2D). The protein expression levels of the stem cell markers OCT-4, SOX2, CD44 and CD133 were investigated via western blotting. OCT-4, SOX2, CD44 and CD 133 were significantly increased after exposure to CIH for 24 h and greatly increased after exposure for 48 h, in both the PC-9 and A549 groups (Fig. 2E). Transwell migration assays indicated that both cell migration and invasion were significantly increased in a time-dependent manner after CIH exposure (Fig. 2F).

Epithelial-mesenchymal transition (EMT) is a key factor in cell migration and invasion (29-31). We analyzed the expression of EMT-associated proteins including E-cadherin, N-cadherin, Snail and vimentin. Western blotting results of these EMT markers showed that CIH enhanced the expression of N-cadherin, Snail and vimentin, and inhibited the expression of E-cadherin in a time-dependent manner (Fig. 2G). These results suggested that CIH contributed to overexpression of EMT proteins and led to cell migration and invasion. Immunofluorescence results showed that the ESM1 intensity markedly increased after CIH exposure in both PC-9 and A549 LCSCs (Fig. 2H). Western blot analysis confirmed the overexpression of ESM1 protein and further indicated that HIF-1α expression was significantly upregulated by CIH exposure (Fig. 2I). The results together suggested that CIH not only led to drug resistance but also enhanced cell proliferation, migration, invasion and EMT *in vitro* in a time-dependent manner.

Inhibition of ESM1 suppresses the effects of CIH exposure in promoting drug resistance, cell proliferation, migration and invasion in vitro. Our preceding experiments confirmed the upregulation of ESM1 during CIH exposure. To investigate the biological role of ESM1 in CIH-associated NSCLC progression and the underlying cellular mechanism, we constructed si-ESM1 plasmids and transfected them into PC-9 or A549 LCSCs to suppress the expression of ESM1. The RT-PCR and western blotting results verified the efficiency of si-ESM1 transfection in the PC-9 and A549 groups (Figs. 3A and S1A).

CCK-8 assays showed that, although CIH increased the cell viability in both the si-control and si-ESM1 groups, in the 48-h CIH exposure groups, si-ESM1 transfection, compared with si-control transfection, inhibited the increase in cell viability (Fig. 3B). Similar results were obtained in drug-resistance analysis of PC-9 and A549; cell viability decreased in a dose-dependent manner, but when the same dose was used,

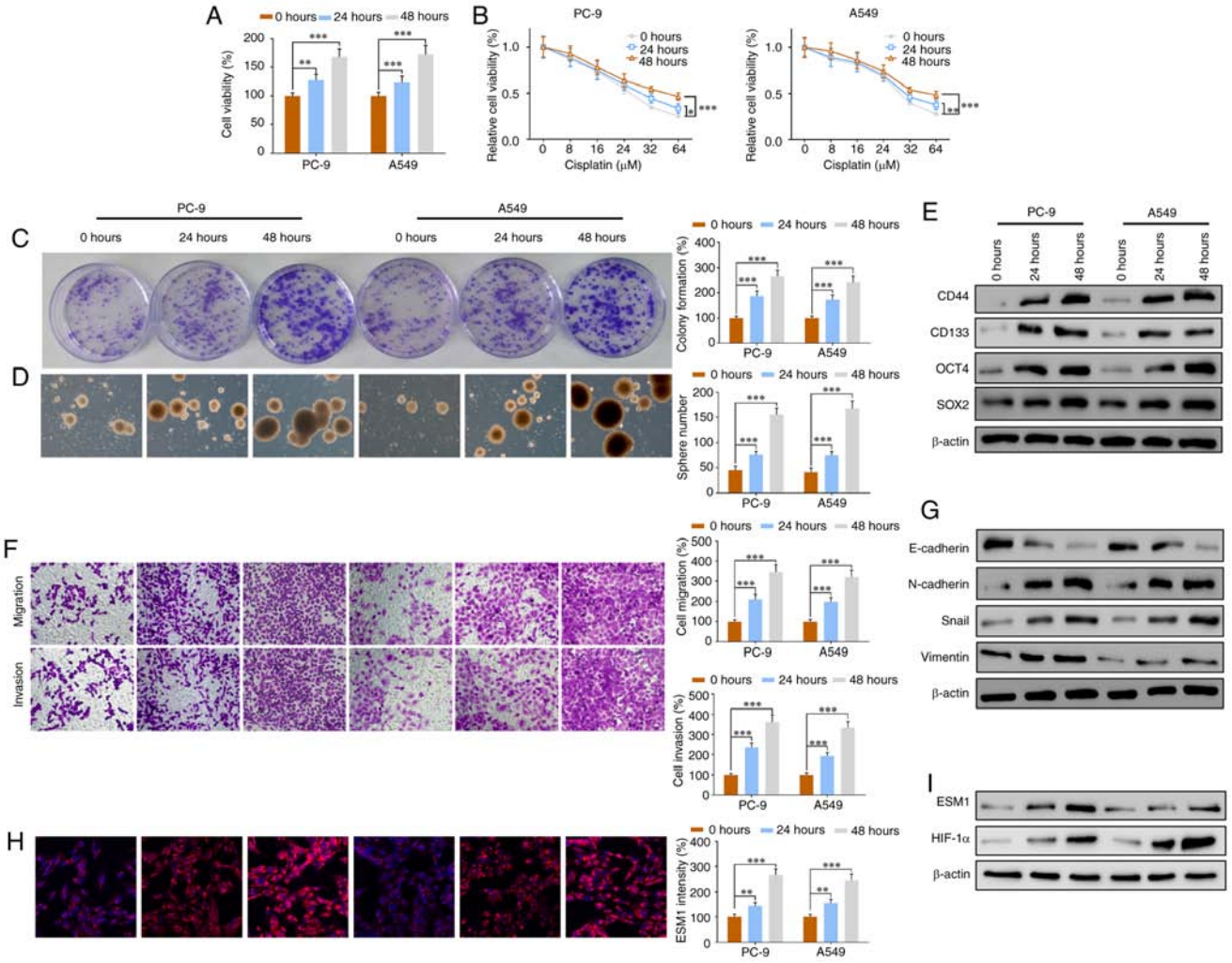

Figure 2. CIH exposure leads to drug resistance and promotes cell proliferation, migration and invasion *in vitro*. The cells were exposed to 5 sec of 14 to 15% O_2 in every 60 sec cycle for 24 or 48 h. (A) CCK-8 assay of cell viability in the PC-9 and A549 groups with 0, 24 or 48 h CIH exposure. (B) Changes in cell viability with increasing doses of cisplatin (0, 4, 1, 32 and 64 μM) in all groups. (C) Colony formation assays of LCSCs from two cell lines before and after 24 or 48 h CIH exposure. (D) Images of PC-9 and A549 tumor spheres. (E) Western blotting results of the CSC markers CD44, CD133, OCT4 and SOX2 in the PC-9 and A549 groups exposed to CIH for 0, 24 or 48 h. (F) Transwell assay results of cell migration and invasion in two cell lines with or without CIH. (G) Western blotting results of the EMT-associated proteins E-cadherin, N-cadherin, Snail and vimentin in all groups. (H) Immunofluorescence staining results of ESM1 and quantitative intensity in the PC-9 and A549 groups exposed to CIH for 0, 24 or 48 h. (I) ESM1 and HIF-1α western blotting results in all groups. Data are expressed as the means ± SEM (n=3); *P<0.05, **P<0.01, ***P<0.001. CIH, chronic intermittent hypoxia; LCSCs, lung cancer stem cells; ESM1, endothelial cell-specific molecule-1; HIF-1α, hypoxia inducible factor-1α.

si-ESM1 transfection significantly inhibited cell viability in both the 0 and 48 h CIH groups (Figs. 3C and S1B). Cell proliferation was assessed via colony formation assays. si-ESM1 did not prevent or eliminate the CIH-induced effects on promoting proliferation, but compared with the control group exposed to CIH for the same 48 h, the si-ESM1 group presented much lower colony formation (Fig. 3D).

Images and quantitative analysis of tumorspheres showed that the sphere numbers were markedly lower in the si-ESM1 group than the control groups (Fig. 3E). To further investigate the changes in cell stemness, we analyzed the expression of the CSC markers OCT-4, SOX2, CD44 and CD133. In both the control and si-ESM1 groups, CIH led to overexpression of OCT-4, SOX2, CD44 and CD 133 (Fig. 3F). However, si-ESM1 significantly suppressed the expression of OCT-4, SOX2, CD44 and CD 133 at the protein level, as compared with that in the si-control group, after exposure to CIH for 48 h (Fig. 3F).

Transwell chamber assays showed that the 48-h CIH exposure promoted cell migration and invasion in the control and si-ESM1 groups, but the increase in the si-ESM1 groups was significantly lower than that in the si-control groups (Fig. 3G). Western blot analysis of EMT-associated proteins showed that CIH enhanced N-cadherin, Snail and vimentin expression, whereas si-ESM1, compared with si-Control, effectively inhibited the CIH-induced increase (Fig. 3H). According to immunofluorescence assays, the ESM1 intensity increased in both the control and si-ESM1 groups after CIH exposure, but the increase in si-ESM1 was significantly lower than that in the control group (Fig. 3I). Western blotting results confirmed the same ESM1 expression changes and showed that CIH-induced overexpression of HIF-1α was inhibited by si-ESM1 transfection (Fig. 3J). These results together suggested that si-ESM1 significantly suppresses the CIH-mediated promotion of drug resistance, cell proliferation, migration, invasion and EMT in PC-9 and A549 cells.

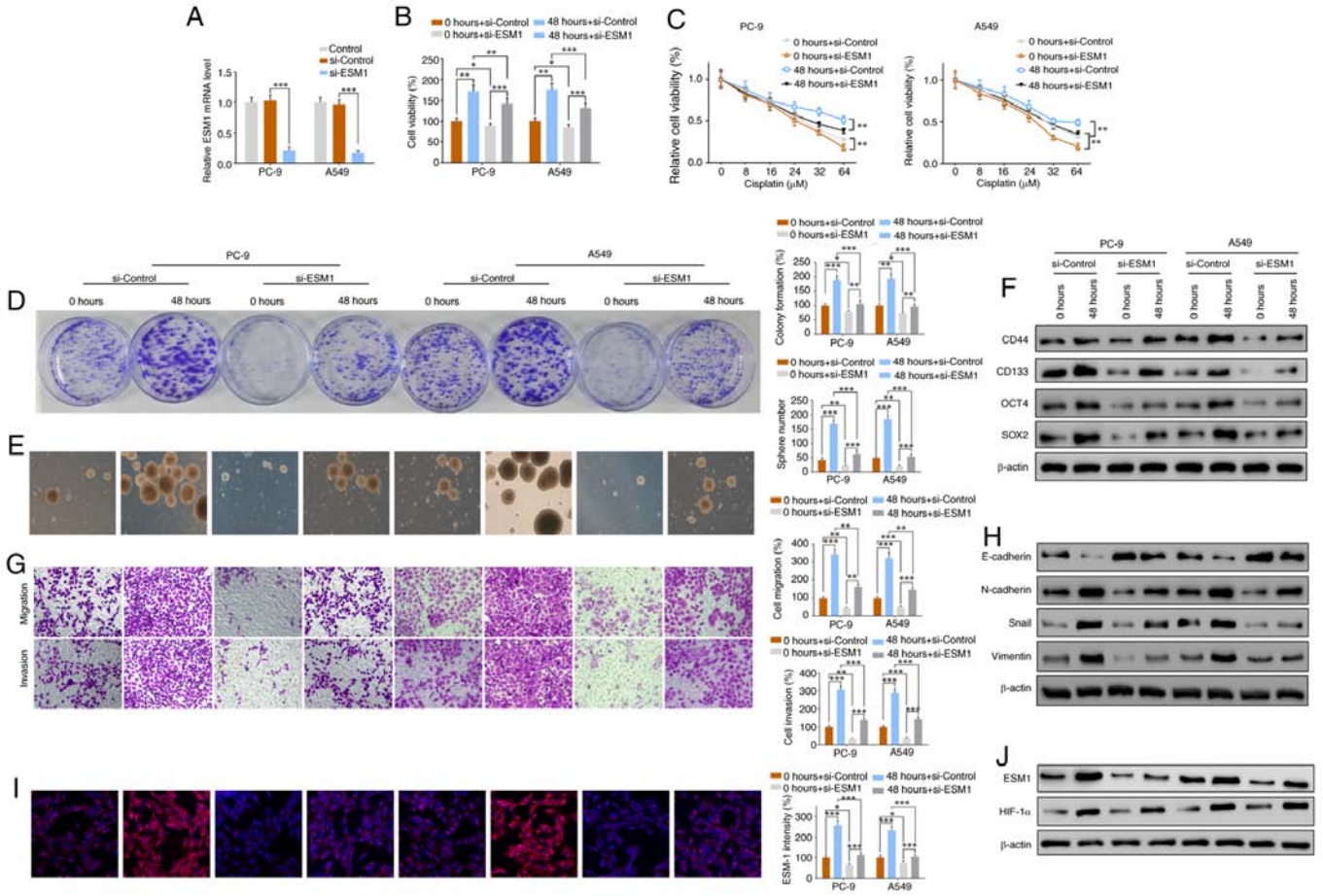

Figure 3. si-ESM1 plasmid transfection suppresses the effects of CIH exposure in promoting drug resistance, cell proliferation, migration and invasion. si-ESM1 plasmids were constructed and transfected into PC-9 or A549 LCSCs to suppress the expression of ESM1, and then exposed to a CIH environment for 0 or 48 h. (A) RT-qPCR results verified the mRNA level of ESM1 in the si-control and si-ESM1 groups. (B) CCK-8 assays of cell viability in the si-control and si-ESM1 groups with 0 or 48 h CIH. (C) PC-9 and A549 LCSC viability changes with increasing doses of cisplatin (0, 4, 16, 32 and 64 μM) in the si-control and si-ESM1 groups with 0 or 48 h CIH. (D) Colony formation assays of LCSCs with or without si-ESM1 transfection. (E) Images and quantitative sphere numbers. (F) Western blotting results of the CSC markers CD44, CD133, OCT4 and SOX2 in the si-control and si-ESM1 groups with 0 or 48 h CIH exposure. (G) Transwell assay results of cell migration and invasion in the si-control and si-ESM1 groups with 0 or 48 h CIH exposure. (H) Western blotting results of the EMT-associated proteins E-cadherin, N-cadherin, Snail and vimentin in all groups. (I) Immunofluorescence staining results of ESM1 and quantitative intensity in the si-control and si-ESM1 groups with 0 or 48 h CIH exposure. (J) ESM1 and HIF-1α western blotting results in all groups. Data are expressed as the means ± SEM (n=3); *P<0.05, **P<0.01, ***P<0.001. CIH, chronic intermittent hypoxia; LCSCs, lung cancer stem cells; ESM1, endothelial cell-specific molecule-1; HIF-1α, hypoxia inducible factor-1α.

CIH promotes tumor growth, proliferation and EMT, whereas ESM1 knockdown reverses the CIH-induced effects in a mouse model. Mice were injected with a PC-9 cell suspension into the left lung through the chest wall, and tumor generation was confirmed by magnetic resonance imaging. To further investigate the effects of CIH on histopathologic changes and tumor growth in lung tissue in a mouse model, we infected the mice with si-ESM1 lentivirus and/or exposed them to CIH for 48 h. Gross findings showed that CIH enhanced the tumor volumes and numbers in both the si-control and si-ESM1 groups (Fig. 4A). However, the CIH-induced increase in the control group was much greater than that in si-ESM1 group, thus indicating that si-ESM1 effectively suppresses tumor growth (Fig. 4A). H&E staining, Ki-67 immunohistochemistry and TUNEL assay results together showed that CIH promoted tumor growth and cell proliferation, whereas si-ESM1 reversed these effects (Fig. 4B-D).

Western blot analysis showed that the CSC markers CD44, CD133, OCT4 and SOX2 were highly overexpressed after CIH exposure, whereas si-ESM1 lentivirus infection suppressed the overexpression at the protein level (Fig. 4E). Overexpression of the EMT-related proteins N-cadherin, Snail and vimentin was detected after CIH for 48 h, whereas si-ESM1 effectively inhibited the expression of these proteins (Fig. 4F). ELISA analysis indicated that ESM1 expression in mouse serum increased significantly after CIH exposure, whereas si-ESM1 effectively suppressed the CIH-enhanced ESM1 expression (Fig. 4I). Western blotting results confirmed the function of si-ESM1 in suppressing ESM1 levels and indicated that CIH-induced HIF-1α overexpression was also inhibited via si-ESM1 transfection (Fig. 4H). Similarly, the immunofluorescence results showed that the level of ESM1 in tumor tissues was inhibited by si-ESM1 and was increased after CIH exposure (Fig. 4G). These results together suggested that si-ESM lentivirus transfection effectively suppresses CIH-induced overexpression of cell proliferation, tumor growth, EMT and HIF-1α.

Discussion

In the present study, our findings indicate a direct correlation between chronic intermittent hypoxia (CIH) and the

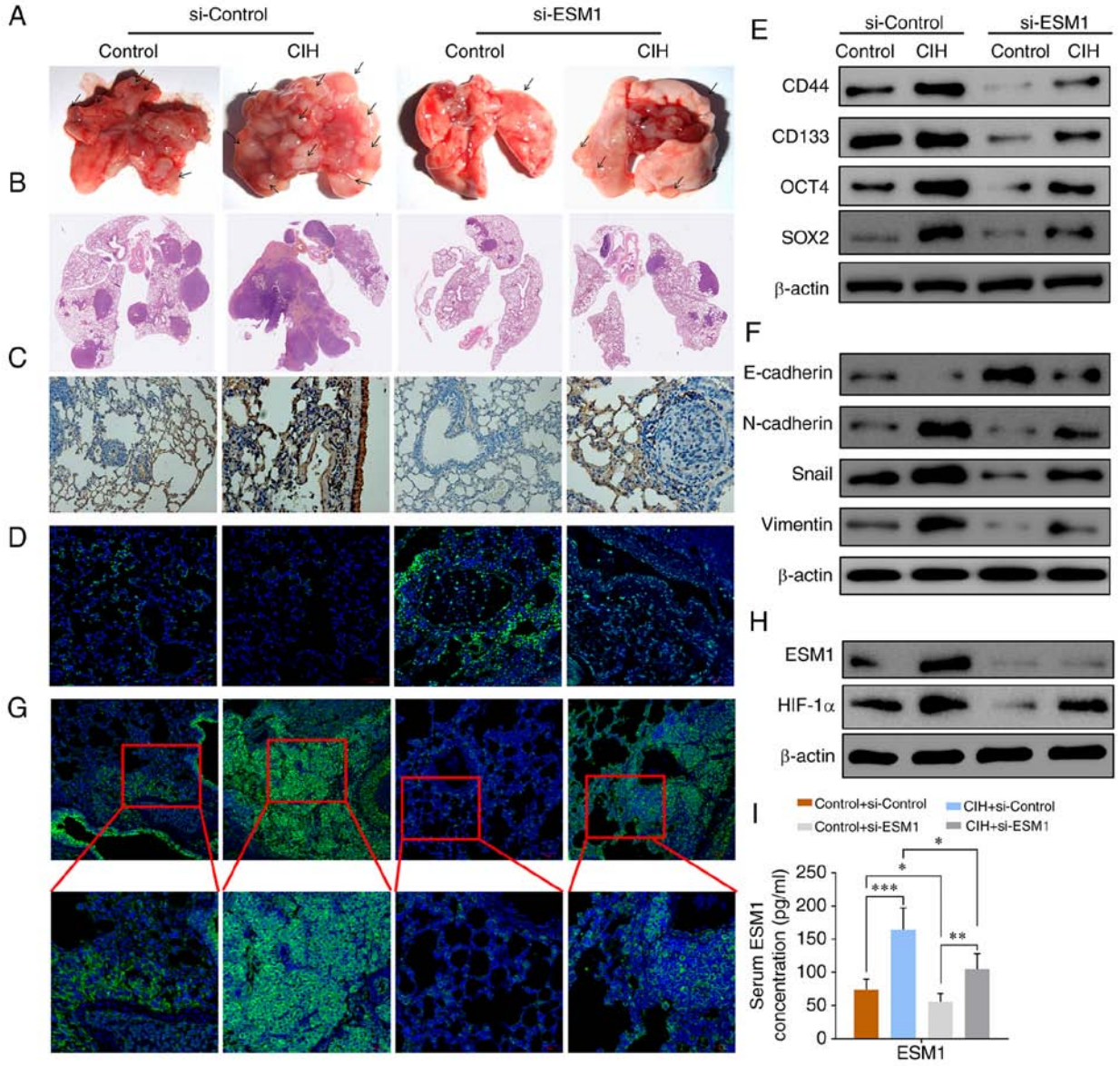

Figure 4. CIH promotes tumor growth, proliferation and EMT, whereas ESM1 knockout reverses the CIH-induced effects in a mouse model. The mice were injected with a PC-9 cell suspension into the left lung through the chest wall, and tumor generation was confirmed by magnetic resonance imaging examination. (A) Tumor volumes and numbers in lungs from both the si-control and si-ESM1 groups with or without CIH. (B) H&E staining results of lung sections from both the si-control and si-ESM1 groups with or without CIH. (C) Immunohistochemistry results for Ki-67, indicating cell proliferation and migration. (D) TUNEL assays of the si-control and si-ESM1 groups. (E) Western blotting results of the CSC markers CD44, CD133, OCT4 and SOX2 in the si-control and si-ESM1 groups with or without CIH exposure. (F) Western blotting results of the EMT-associated proteins E-cadherin, N-cadherin, Snail and vimentin in all groups. (G) Immunofluorescence assay of ESM1 intensity in all groups. (H) ESM1 and HIF-1α western blotting results in all groups. (I) ELISA results of ESM1 in the serum from the si-control and si-ESM1 groups with or without CIH exposure. Data are expressed as the means ± SEM (n=6); *P<0.05, **P<0.01, ***P<0.001. CIH, chronic intermittent hypoxia; ESM1, endothelial cell-specific molecule-1; EMT, epithelial-mesenchymal transition; HIF-1α, hypoxia inducible factor-1α.

enhancement of lung cancer stem cell (LCSC) NSCLC progression. Intermittent hypoxia was previously found to enhance melanoma metastasis to the lung in a mouse model of sleep apnea (15). In addition, accumulating evidence shows that hypoxia is a key regulator of angiogenesis in cancer (32). We found that CIH exposure markedly promoted tumor growth, cell spheres, EMT-associated proteins, and cell proliferation, migration and invasion, thereby promoting carcinogenesis in PC-9 and A549 LCSC cell lines and in CIH-exposed mice with lung tumors. Song and colleagues identified that hypoxia directly induces resistance to cisplatin and doxorubicin in NSCLC (33). We confirmed that obstructive sleep apnea (OSA)-associated CIH promoted drug resistance to cisplatin *in vitro* in a time-dependent manner. Furthermore, our findings suggest that endothelial cell-specific molecule-1 (ESM1) plays an important role in CIH-mediated drug resistance, and cell proliferation, migration and invasion *in vitro*, as well as in NSCLC mouse models. In addition, si-ESM1 effectively suppressed the CIH-induced aggravation of tumor growth, drug resistance and metastasis.

Cell proliferation and migration comprise the core mechanism of tumor metastasis. Highly proliferative tumors are often highly invasive (34). Previous studies have found that hypoxia stimulates the proliferation and migration of endothelial cells

and enhances the proliferation and tissue formation of human mesenchymal stem cells (35,36). In this study, animals and cells were exposed to 5 sec of 14 to 15% O_2 in every 60 sec cycle to simulate CIH exposure. CIH substantially stimulated cell proliferation, migration and invasion, whereas si-ESM1 reversed the effects of CIH in promoting tumor growth and metastasis. EMT plays a key part in the progression of cell migration and invasion. We identified that levels of N-cadherin, Snail and vimentin, which are EMT-associated proteins, were significantly increased during CIH exposure. Although EMT might still have occurred under CIH conditions with si-ESM1 treatment, si-ESM1 functioned as an EMT-suppressor and attenuated the increases in N-cadherin, Snail and vimentin in this study. However, the specific signaling pathways linking ESM-1 and EMT require further investigation.

According to a previous study, hypoxia can lead to therapeutic resistance through direct effects, owing to a lack of oxygen, which some drugs and radiation therapies require for maximal cytotoxicity, as well as indirect effects through altered cellular metabolism, which decreases drug cytotoxicity (37). Accumulating evidence indicates the major contribution of hypoxia and HIF-1 to drug resistance in a wide spectrum of neoplastic cells (38). A serious and primary problem in lung cancer chemotherapy is the emergence of inherent and acquired drug resistance in cancer cells (39). In this study, PC-9 and A549 cisplatin-resistant cells were obtained by repeated cisplatin treatment with increasing doses (1 to 10 μmol/l). Tumorsphere formation was used for enriching LCSC cells. CIH enhanced cisplatin resistance in a CIH environment, whereas si-ESM1 transfection effectively prevented the increase in drug resistance. Given that OSA complications in lung cancer are common, ESM1 might serve as a therapeutic target for inhibiting the damage due to CIH in LCSC.

ESM1, a molecule regulated by inflammatory cytokines and associated with endothelial dysfunction, is markedly overexpressed in many types of cancer (19-21,23,24,28). The expression of ESM1 is regulated by HIF-1α, which has a key role in adaptation to hypoxic environments (40,41). Hypoxia inducible factor-1α (HIF-1α) was highly upregulated by CIH exposure, whereas si-ESM1 inhibited the overexpression of HIF-1α. In some studies, HIF-1α has been reported to be an important mediator of apoptosis and proliferation (25,42). Accumulating evidence suggests that HIF-1α may be an upstream regulator of ESM1; in some studies ESM1 was found to regulate HIF-1α (43,44). Our study implies that HIF-1α and ESM1 are both important factors in the downstream signaling pathways involved in CIH-induced tumor progression. Targeting ESM1 inhibited CIH-induced and HIF-1α-mediated tumor growth, as well as cell proliferation, migration and invasion. However, the specific molecular interaction between ESM1 and HIF-1α still requires further investigation, as does the mechanism linking CIH, ESM1 and HIF-1α. Our research still has some limitations for many reasons. Previous studies have shown the effect of CIH on lung cancer (13,45,46). Hence, our article only focused on the mechanism of CIH on LCSCs. Whether the research results can be more widely applicable to NSCLC still needs further research.

Collectively, our findings suggest that CIH enhances tumorsphere formation, drug resistance, cell proliferation, migration and invasion in LCSC progression via an ESM1 and HIF-1α signaling pathway. ESM1-knockout sufficiently suppressed the effects of CIH in promoting tumor growth and metastasis. Together, our results indicate that ESM1 may serve as a new therapeutic target in the treatment of OSA-associated NSCLC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

XG and LL designed the experiments. XG and JZ performed most of the experiments with the assistance of YS and HS. XG and YC collected and analyzed the data. YL validated the data analysis. XG drafted the manuscript and LL revised the draft. All authors approved the final manuscript before submission.

Ethics approval and consent to participate

The present study was performed in strict accordance with Guide for the Care and Use of Laboratory Animals (8th edition, 2011, published by The National Academies Press (https://www.nap.edu/catalog/12910/guide-for-the-care-and-use-of-laboratory-animals-eighth). The protocol was reviewed and approved by the Shanghai Ninth People's Hospital Institutional Review Board (permit no. HKDL2013001b). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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