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RESEARCH PAPER

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Jmjd2c maintains the ALDH^{bri+} cancer stemness with transcription factor SOX2 in lung squamous cell carcinoma

Min Wang[a], Yuling Hu[a], Feng Cai[b], Lili Guo[c], Yimin Mao[a], and Yingmin Zhang[a]

[a]Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, Luoyang, Henan Province, China; [b]Department of Respiratory and Critical Care Medicine, Affiliated Hospital of Nantong University, Nantong, Jiangsu Province, China; [c]Department of Pathology, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, Luoyang, Henan Province, China

ABSTRACT

Lung squamous cell carcinoma (LSCC) is a deadly cancer in the world. Histone demethylase Jmjd2c is a key epigenetic regulator in various tumors, while the molecular mechanism underlying Jmjd2c regulatory in LSCC is still unclear. We used the aldehyde dehydrogenasebright (ALDH^{bri+}) subtype as a research model for cancer stem cells (CSCs) in LSCC and detected the sphere formation ability and the proportion of ALDH^{bri+} CSCs with Jmjd2c interference and caffeic acid (CA) treatment. Additionally, we carried out bioinformatic analysis on the expression file of Jmjd2c RNAi mice and performed western blotting, qRT-PCR, Co-IP and GST pull-down assays to confirm the bioinformatic findings. Moreover, we generated Jmjd2c-silenced and Jmjd2c-SOX2-silenced ALDH^{bri+} tumor-bearing BALB/c nude mice to detect the effects on tumor progression. The results showed that Jmjd2c downregulation inhibited the sphere formation and the proportion of ALDH^{bri+} CSCs. The SOX2 decreased expression significantly in Jmjd2c RNAi mice, and they were positively co-expressed according to the bioinformatic analysis. In addition, SOX2 expression decreased in Jmjd2c shRNA ALDH^{bri+} CSCs, Jmjd2c and SOX2 proteins interacted with each other. Furthermore, Jmjd2c interference revealed significant blocking effect, and Jmjd2c-SOX2 interference contributed even stronger inhibition on ALDH^{bri+} tumor progression. The Jmjd2c and SOX2 levels were closely related to the development and prognosis of LSCC patients. This study indicated that Jmjd2c played key roles on maintaining ALDH^{bri+} CSC activity in LSCC by interacting with transcription factor SOX2. Jmjd2c might be a novel molecule for therapeutic targets and biomarkers in the diagnosis and clinical treatment of lung cancer.

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Lung squamous cell carcinoma; cancer stem cells; Jmjd2c; SOX2

Introduction

Lung cancer is one of the most common malignant tumors in humans, and its morbidity and mortality have been on the rise in recent years.[1] LSCC is one of the common subtypes that associates with poor clinical prognosis and lacks available targeted therapy.[2,3] It is inevitable that drug resistance developed by LSCC patients to platinum-based chemotherapy leads to tumor recurrence.[4] The 5-y survival rate of patients has been hovering at 15%.[5] Thus, it is of urgent requirement to explore novel molecules for therapeutic targets and biomarkers in LSCC.

Epigenetic aberrations have been described in the etiology of cancer that chromatin modifiers and remodelers such as DNA methylation, histone modification or microRNA, which disrupt gene expressions, are believed to be strongly related to the development and progression of solid malignancies.[6–8] In contrast to genetic changes, epigenetic alterations are pharmaceutically reversible.[9,10] Reliable epigenetic markers are targeted for clinical diagnosis and treatment. Jmjd2c, originally cloned from LSCC in 2000, is a member of Jumonji C domains containing proteins (JmjD family), which are a major class of histone lysine demethylases.[11–14] Jmjd2c is capable of demethylating tri- or di-methylated histone 3/2 in either K9 or K36 residuals, balancing the methylation state of histones and affecting heterochromatin formation, genomic imprinting and gene expression regulation.[15] Jmjd2c was involved in embryonic and stem cell regulation,[16] and defects from Jmjd2c function were associated with the genesis and progression of various tumors, such as osteosarcoma, breast cancer, blood neoplasms, prostate carcinomas, and so on.[17–20] Furthermore, Jmjd2c was observed overexpressed in lung tumors, and revealed even higher expressions in metastatic lung tissues than that in non-metastatic ones.[21] Knockdown of Jmjd2c blocked the migration and invasion of lung cancer cells,[21] which indicated that Jmjd2c was a promising anti-lung-cancer target. However, the molecular mechanism underlying Jmjd2c regulation in lung cancers has not yet been clarified.

CSCs are a subpopulation of tumor cells, possessing high self-renewal and differentiation properties, which promote tumor initiation or cause relapses.[22,23] CSCs are thought to be the source of all tumor cells in malignant tumors and the reason for the resistance to

CONTACT Yingmin Zhang zhangym0379@126.com Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital, and College of Clinical Medicine, Henan University of Science and Technology, No. 24, Jinghua Road, Jianxi District, Luoyang, Henan 471003, China
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chemotherapy.[24] Thus, reducing CSCs in residual tumors and effectively maintaining chemotherapy sensitivity may become a breakthrough in solving drug resistance, reducing recurrence rate, and improving prognosis for patients.[5] Transcription factor sex-determining region Y-box2 (SOX2) is a key regulator in stem cell biology,[25] and dysregulation of SOX2 expression induces pluripotent stem cells, drives CSC properties, fuels tumorigenesis, and promotes tumor aggressiveness.[26] SOX2 is closely correlated with self-renewal and chemoresistance of CSCs.[27]

In this study, we used the ALD^{bri+} subtype as a research model for CSCs in LSCC, and confirmed that Jmjd2c interference suppressed the self-renewal and pluripotent CSC generation. In addition, we elucidated that SOX2 expression was regulated by Jmjd2c, meanwhile they positively co-expressed with each other at protein levels with bioinformatic analysis on the expression profiling of the Jmjd2c RNAi mice, and confirmed these findings in ALDH^{bri+} CSCs. Moreover, we established Jmjd2c-silenced and Jmjd2c-SOX2-silenced ALDH^{bri+} tumor-bearing BALB/c nude mice and explained that Jmjd2c silence retarded tumor growth and Jmjd2c-SOX2 silence contributed stronger prevention on tumor progression. Thus, this study demonstrated that Jmjd2c maintained the ALDH^{bri+} CSC activity with transcription factor SOX2 and acted as a mark in LSCC. Inhibitors targeting Jmjd2c are expected to become anti-tumor drugs for clinical treatment of lung cancer.

Results

Jmjd2c was necessary for the self-renewal and pluripotent ALDH^{bri+} CSC generation

To investigate the function of Jmjd2c on CSC regulation and tumor development, we detected the self-renewal potential of CSCs by the sphere formation assay in Jmjd2c-silenced and CA treated ALDH^{bri+} CSCs. The results showed that both constructions of Jmjd2c interference significantly reduced the CSC sphere numbers compared to the control group of ALDH^{bri+} CSCs (**$p < 0.01$) (Figure 1(a,b)), and the following shRNA jmjd2c interference refers to shRNA-2 jmjd2c (Figure S1). In addition, the inhibitor of the Jmjd2c CA treatment significantly reduced the value of spheres/5000 cells, and higher concentration (10 μM, refer to the following CA treatment) revealed stronger inhibition of CSC sphere formation capacity (**$p < 0.01$) (Figure 1(a,c)). Results from aldefluor assay showed that both the Jmjd2c silencing and CA treatment dramatically reduced the ratio of ALDH^{bri+} CSCs, the values were 5.54% and 4.14%, respectively, compared to the 16.5% in the control (Figure 2). These results indicated that Jmjd2c was a key regulator of ALDH^{bri+} CSC activity in lung squamous cell carcinoma.

SOX2 was involved in Jmjd2c signaling regulating stem cell differentiation

To figure out the molecular mechanism underlying Jmjd2c signaling regulating lung cancer progression, we carried out

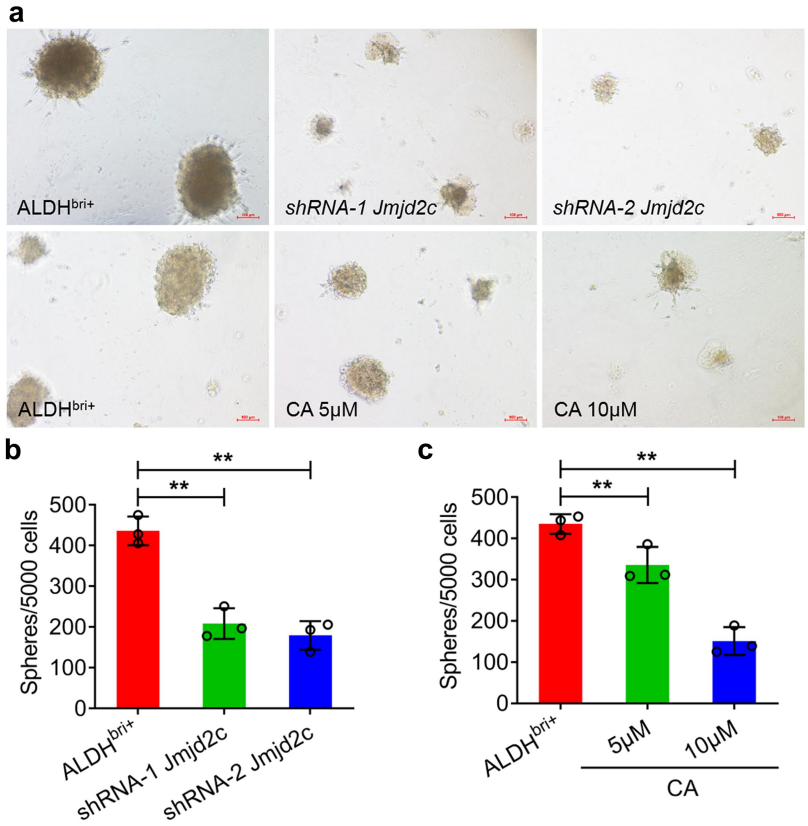

Figure 1. The clonal sphere-forming ability with Jmjd2c silencing and CA treatment in ALDH^{bri+} CSCs. (a) The images of the formed spheres. (b) and (c) the quantification of the sphere sizes in a with Jmjd2c silencing (b) and CA treatment (c) in ALDH^{bri+} CSCs. Data were represented as means±SD, $n \geq 3$; **$p < 0.01$. Bars = 100 μm.

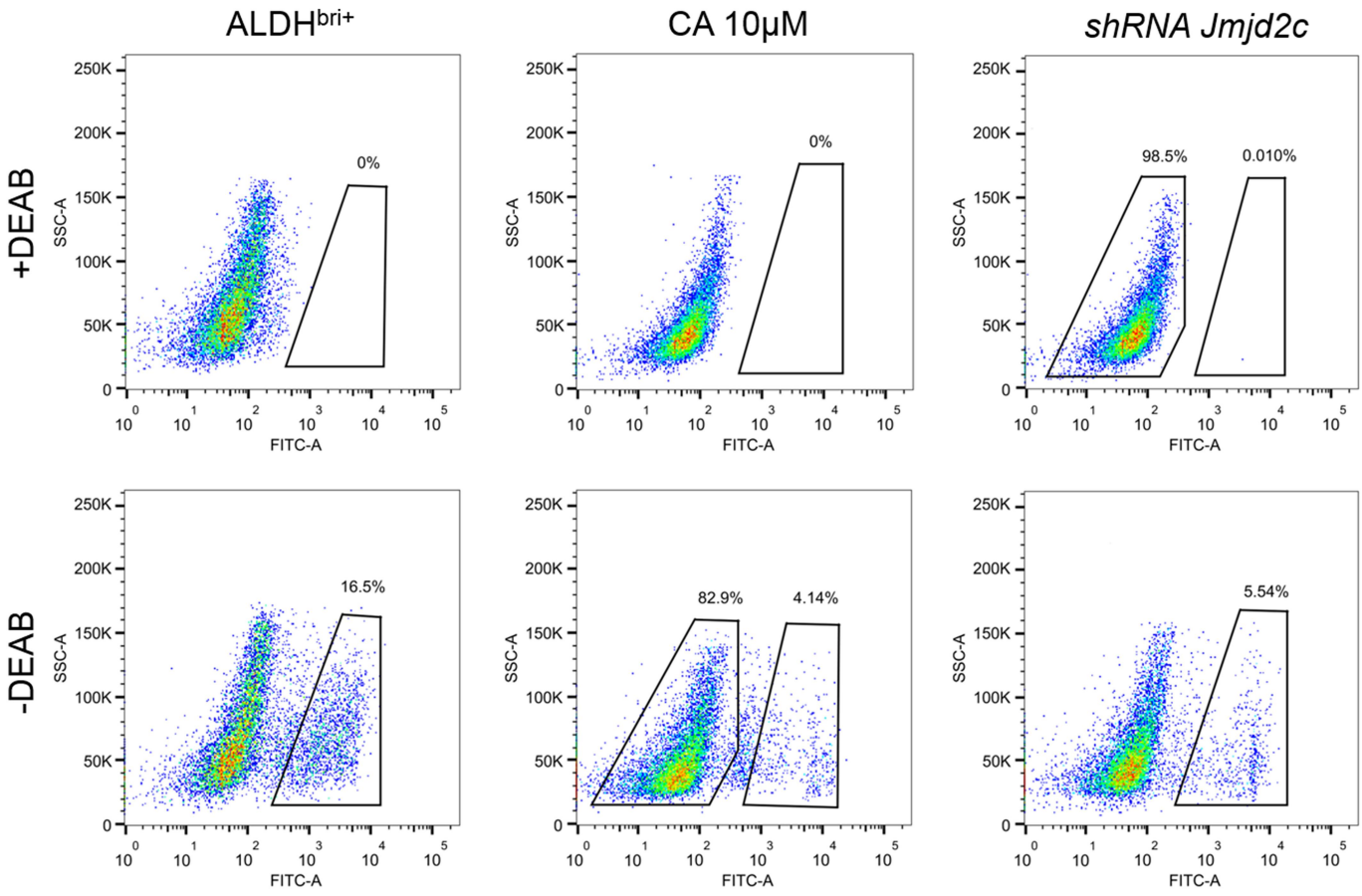

Figure 2. The aldefluor assay showed the ratio of ALDH^{bri+} CSCs with 10 μM CA treatment and Jmjd2c silence.

a bioinformatics analysis on the expression profiling using an array of the Jmjd2c RNAi mice. The volcano plot analysis showed 977 DEGs upregulated and 731 DEGs downregulated, which were depicted in a heatmap (Figure S3). Further GO analysis of the DEGs revealed that 31 genes classified as stem cell differentiation in BP category were enriched significantly (Figure 3(a)). Among these 31 genes, the heatmap analysis showed 15 genes were downregulated and 16 genes were upregulated, and both SOX2 and Jmjd2c (Kdm4c) were deregulated (Figure 3(b)). Correlation analysis indicated that SOX2 and Jmjd2c were absolutely positive (Figure 3(c)) and protein–protein interaction network analysis by STRING showed SOX2 and Jmjd2c could interact with each other (Figure 3(d)). Thus, these results suggested that SOX2 was a crucial regulator involved in Jmjd2c signaling regulating stem cell activity.

To further confirm these findings, we performed western blotting and quantitative real-time PCR (qRT-PCR) experiments to detect the SOX2 expressions in Jmjd2c silenced ALDH^{bri+} CSCs (Figure 4(a–e)). The protein expressions of Jmjd2c and SOX2 were both dramatically increased in ALDH^{bri+} CSCs compared to the CSC controls (**$p < 0.01$, ***$p < 0.001$) (Figure 4(a–c)). However, they both substantially reduced in Jmjd2c-silenced or CA-treated ALDH^{bri+} CSCs (**$p < 0.01$) (Figure 4(a–c)). The mRNA expressions from qRT-PCR were consistent with the western blotting results (*$p < 0.05$, **$p < 0.01$) (Figure 4(d,e)). These results suggested that Jmjd2c regulated the expression of SOX2.

Moreover, the Co-immunoprecipitation (Co-IP) assay indicated that the Myc-Jmjd2c immunoprecipitated the SOX2 proteins (Figure 4(f)), which was confirmed by the GST pull-down assay that GST-SOX2 fused proteins pulled down the Myc-Jmjd2c proteins (Figure 4(g)). These results indicated that SOX2 and Jmjd2c interacted with each other at protein levels.

Jmjd2c-SOX2-silenced ALDH^{bri+} CSCs retarded the tumor progression potently in tumor-bearing BALB/c nude mice

To clarify the biological impact of Jmjd2c-SOX2 signaling on tumor progression in mice, we generated Jmjd2c-silenced and Jmjd2c-SOX2-silenced ALDH^{bri+} tumor-bearing BALB/c nude mice (Figure 5(a), Figure S1 and S2). The observation after 17-d ALDH^{bri+} CSC injection, the tumor size from Jmjd2c-SOX2-silenced mice was obviously smaller than that of the control group (Figure 5(b)). The statistical analysis showed that tumor size from Jmjd2c-silenced mice was significantly reduced compared to the ALDH^{bri+} controls, and the Jmjd2c-SOX2-silenced mice generated even more smaller tumors (**$p < 0.01$) (Figure 5(c)). The hematoxylin–eosin (HE) staining of the mouse lung tissues showed that clonogenicity of the

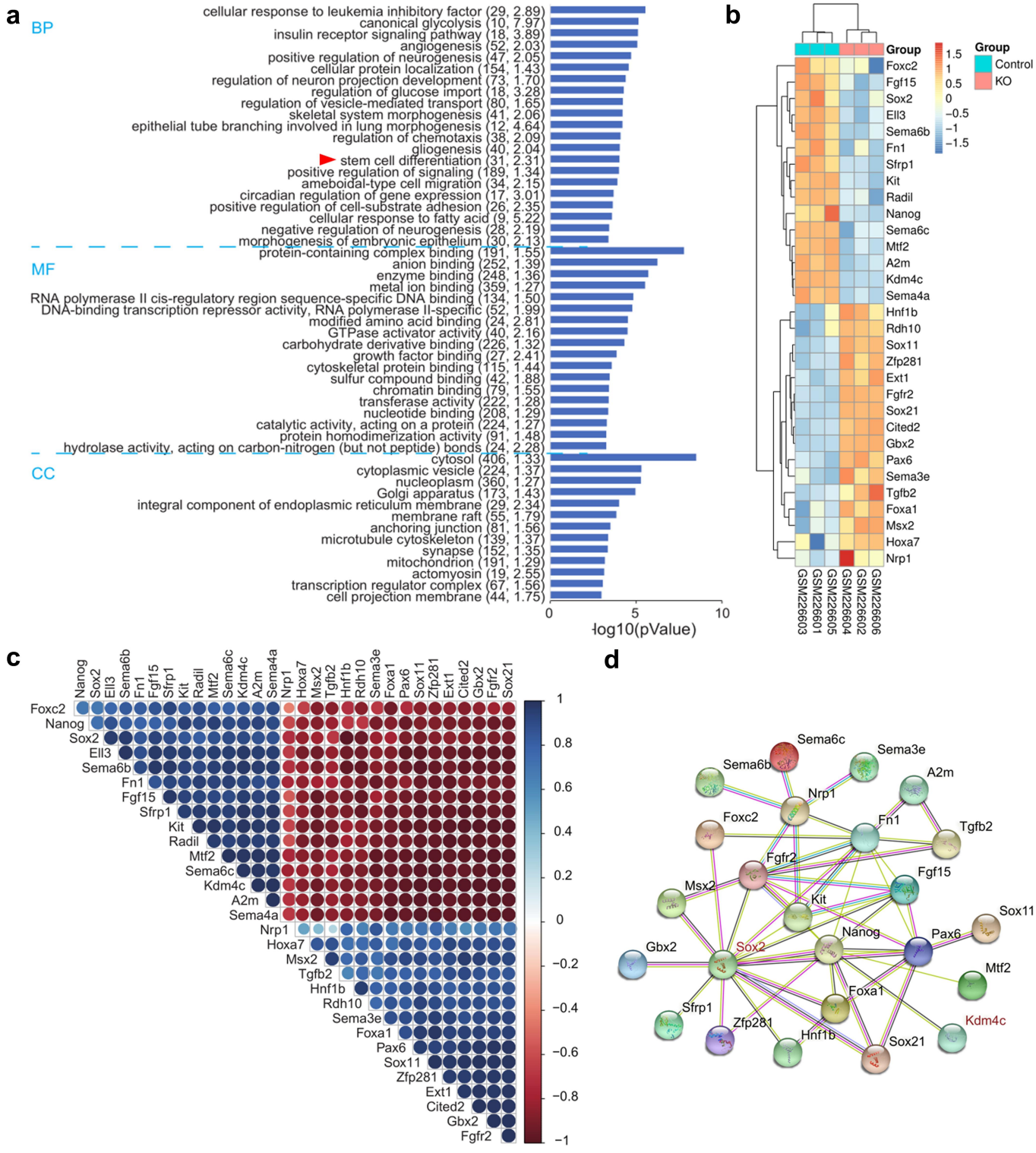

Figure 3. The GO and protein-protein association analysis of the Jmjd2c RNAi mice. (a) The GO analysis of BP (biological process), MF (molecular function), and CC (cellular component) for the genes. X-axis: The minus logarithm of the *P*-value. Y-axis: the GO category. (b) A heatmap showed the 31 DEGs classified to stem cell differentiation in GO category. X axis: sample name; Y axis: gene name. (c) Correlation of the DEGs classified to stem cell differentiation was analyzed. The correlation coefficient ranges from – 1 (red color) to 1 (blue color). The red represented absolute negative correlations, and blue represented absolute positive correlations. (d) The protein–protein interaction network of the DEGs classified to stem cell differentiation was analyzed by STRING.

Jmjd2c-SOX2-silenced mice was obviously reduced (Figure 6(a)), which suggested that the micrometastases of the ALDH^{bri+} CSCs were inhibited by Jmjd2c-SOX2 silencing. In addition, the immunochemistry results showed that the expressions of Jmjd2c and SOX2 decreased in lung tissues of the Jmjd2c-silenced mice (**$p < 0.01$) (Figure 6(b–d)), which was consistent with the western blotting analysis in ALDH^{bri+} CSCs (Figure 4(a–c)). SOX2 expression further declined to even

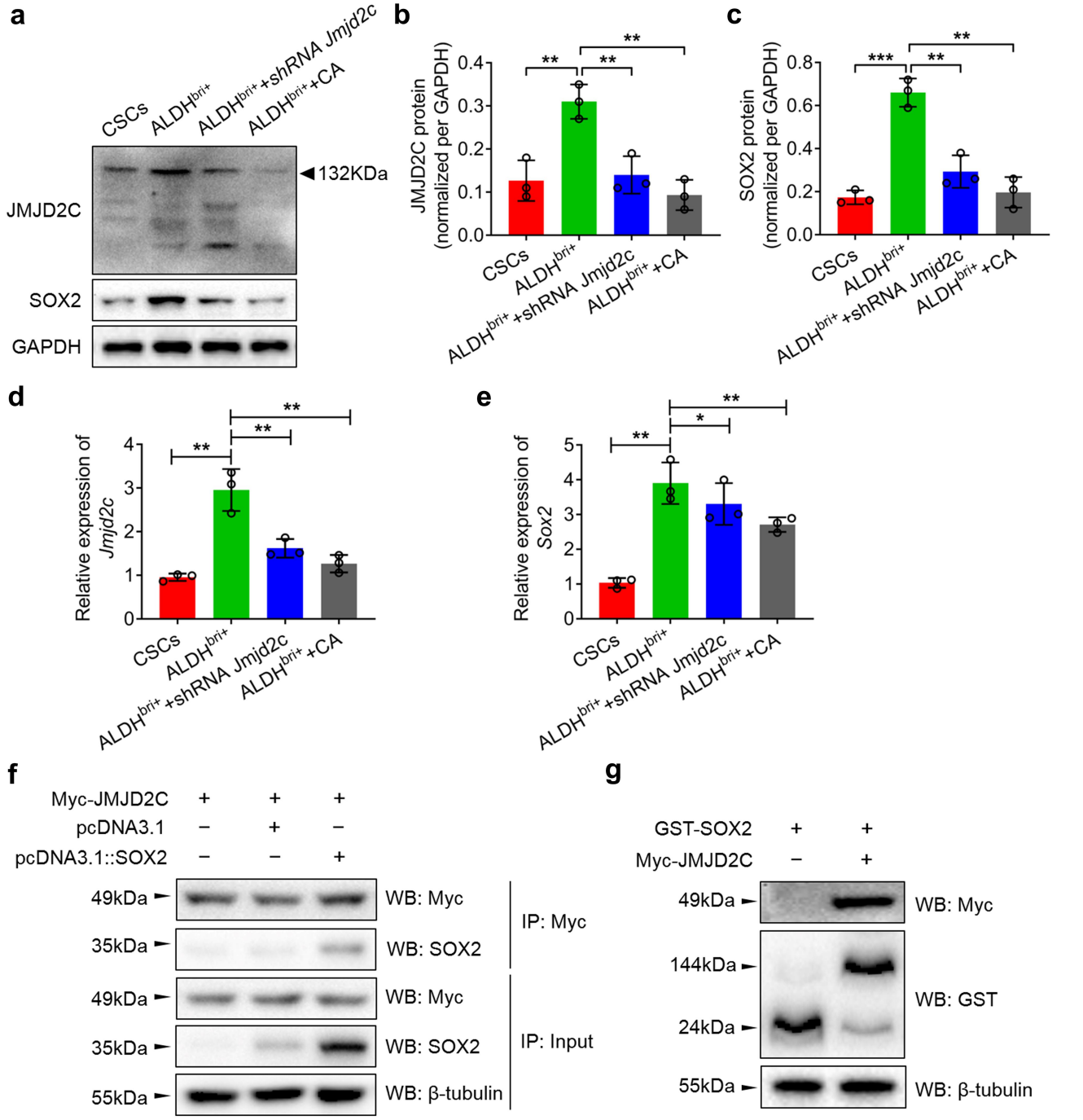

Figure 4. Jmjd2c regulated the expression of SOX2 and they interacted with each other at protein level. (a) The western blotting analysis showed the protein expressions of Jmjd2c and SOX2 with Jmjd2c silencing and CA treatment in ALDH^{bri+} CSCs. The red triangle indicated the bands of Jmjd2c. GAPDH was applied as internal control. (B) and (C) the quantitative analysis of Jmjd2c (b) and SOX2 (c) protein expressions normalized to GAPDH. (d) and (e) the mRNA expressions of Jmjd2c and SOX2 with Jmjd2c silencing and CA treatment in ALDH^{bri+} CSCs. (f) and (g) the Co-IP assay (f) and GST-pull down assay (g) showed the interaction of Jmjd2c with SOX2 in ALDH^{bri+} CSCs. β-tubulin was applied as the internal control. Data were represented as means±SD, $n \geq 3$; *$p < 0.05$, **$p < 0.01$.

lower levels in Jmjd2c-SOX2-silenced mouse lungs, but not Jmjd2c (**$p < 0.01$) (Figure 6b–d).

Jmjd2c and SOX2 expression are closely related to the development and prognosis of LSCC patients

To further determine the role of Jmjd2c and SOX2 in LSCC, we collected patient tissues from the Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology; 20 samples were from patients were diagnosed with LSCC tissues, and 20 were from paracancerous tissue. Immunohistochemistry showed that in normal and LSCC tissues, the Jmjd2c and SOX2 proteins were mainly located in the cytoplasm (Figure 7(a,b)). Furthermore, survival analysis of Jmjd2c and SOX2 was performed using Kaplan Meier-plotter. The results showed that Jmjd2c and SOX2 were negatively associated with the overall survival (Figure 7(c)).

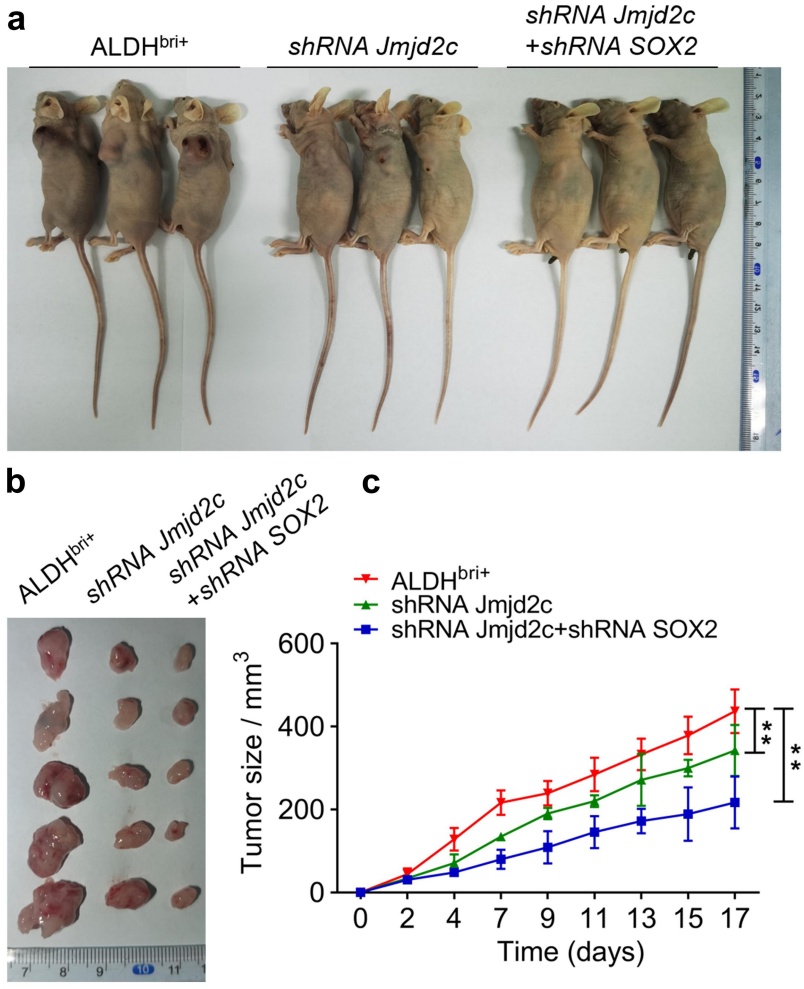

Figure 5. The tumorigenic capacity of Jmjd2c-silenced and Jmjd2c-SOX2-silenced ALDH^{bri+} CSCs in BALB/c nude mice. (a) The image of the BALB/c nude mice bearing tumors. (b) The tumors isolated from the groups of a were arranged and displayed. (c) The statistical analysis of the tumor sizes of the three groups within the 17 d. Data were represented as means±SD, $n \geq 3$; $^{**}p < 0.01$.

Discussion

Lung cancer is emerging as the leading cause of death and has become a public health issue worldwide.[28,29] LSCC is a non-small cell lung cancer (NSCLC), accounting for nearly 40% of lung cancer, but lacks targeted therapy and poor clinical diagnosis.[30,31] Jmjd2c was suggested to epigenetically regulate cancer cells and altered Jmjd2c histone methylation regulation led to various tumor initiation and progression.[17,31] In this study, Jmjd2c interference or CA treatment significantly inhibited ALDH^{bri+} CSC sphere formation and reduced the proportion of ALDH^{bri+} CSCs. These results indicated that Jmjd2c was a key regulator maintaining ALDH^{bri+} CSC stemness and that the self-renewal ability of ALDH^{bri+} CSCs was sensitive to the down-regulation of Jmjd2c. As CSCs are a population of self-renewal cells with high tumorigenic potency and cancer is viewed as stem cell diseases,[32,33] Jmjd2c should be a promising target and inhibition of Jmjd2c might be a choice for treatment of LSCC patients, which was confirmed by the previous uncovers that knockdown of Jmjd2c suppressed the migration and invasion of lung cancer cells.[21]

Emerging evidence revealed that histone methylation played a crucial role in regulating gene expression and genome stability.[34] The JmJC domain was identified as a novel demethylase signature motif, and demethylation conducted by JmjC domain containing protein was conserved from yeast to human.[35] In this study, 31 genes classified as stem cell differentiation were enriched in the microarray data of Jmjd2c RNAi mice. Thus, Jmjd2c epigenetically regulated expressions of genes mediating stem cell activity. SOX2 was significantly decreased in Jmjd2c RNAi mice and Jmjd2c silenced ALDH^{bri+} CSCs. SOX2 was a key player mediating the stemness of various stem cells. It expressed that CSCs and abnormal SOX2 expressions were associated with kinds of cancer types and promoted resistance to chemotherapies.[36] Thus, Jmjd2c might maintain the ALDH^{bri+} CSC stemness through epigenetically regulating SOX2 expressions.

In addition, SOX2 and Jmjd2c were positively co-expressed according to the correlation analysis, and they interacted with each other based on the results from Co-IP assay and GST pull-down assay in ALDH^{bri+} CSCs. Loose chromatin structures facilitate transcription factors accessing their target DNA sequences, during which epigenetic factors play key roles.[37]

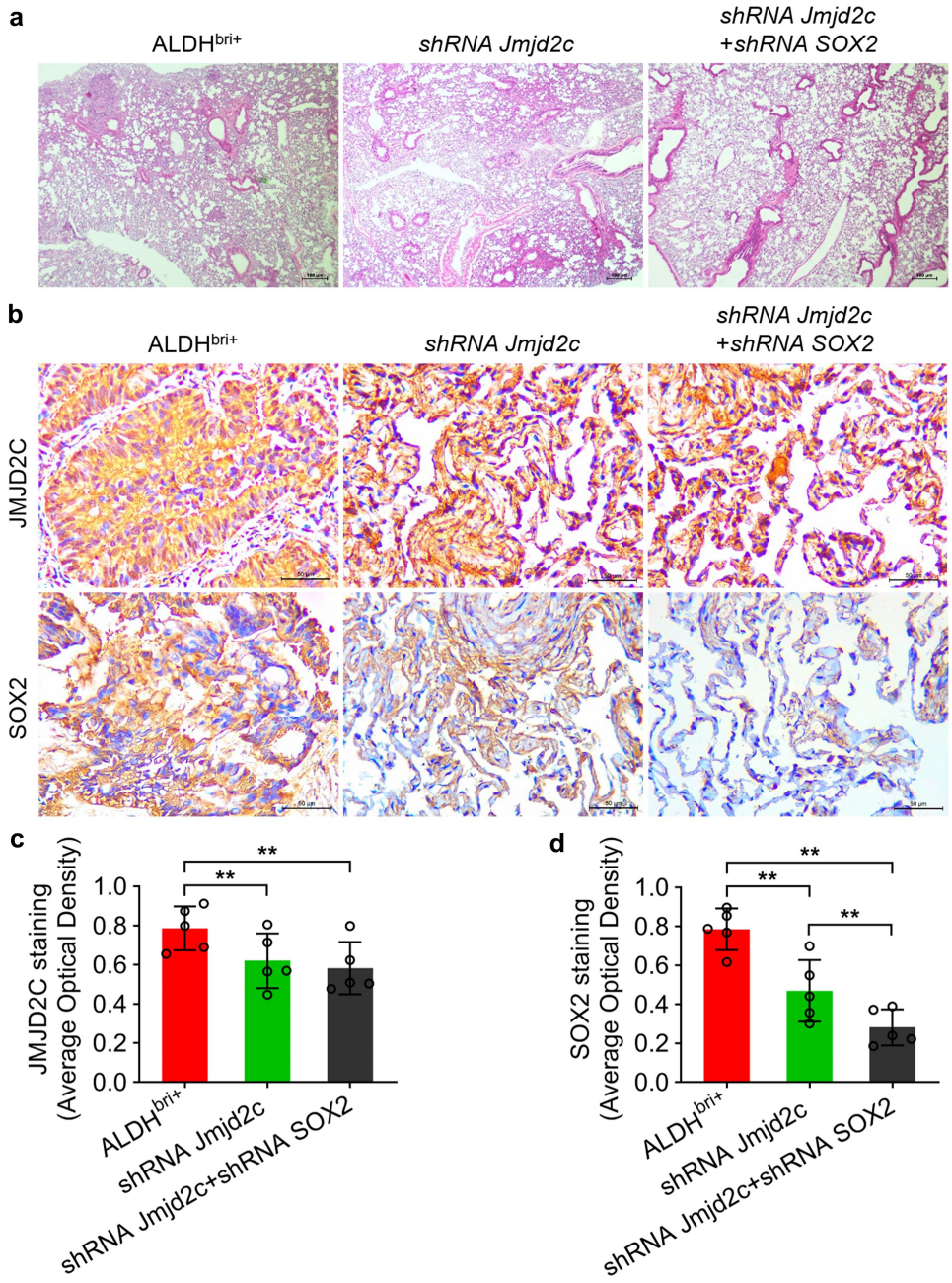

Figure 6. The micrometastases was reduced in mouse lung tissues injected with Jmjd2c-silenced and Jmjd2c-SOX2-silenced ALDH^{bri+} CSCs. (a) The HE staining results revealed the micrometastases in different groups. (b) The protein expressions of Jmjd2c and SOX2 with Jmjd2c silence or Jmjd2c-SOX2 silence from immunochemistry in mouse lung tissues. (c) and (d) quantification of the results in B. Bars = 100 μm.In A, 50 μm in B. Data were represented as means±SD, $n \geq 3$; $^{**}p < 0.01$.

Jmjc2d was reported to demethylate H3K9me2/3 and H3K36me2/3, two histone modifications involving transcriptional repression.[38] Thus, the interaction of Jmjd2c and transcription factor SOX2 might indicate that Jmjd2c relaxed chromatins by balancing the methylation state of histones and facilitated the recruitment of SOX2 to the transcription start site (TSS), affecting a series of downstream gene expressions and maintaining cancer cell stemness in LSCC. In this study, Jmjd2c-SOX2 interfered ALDH^{bri+} tumor bearing mice revealed weak clonogenicity, inhibiting the micrometastases of the ALDH^{bri+} CSCs and reduced tumor size. Additionally, Jmjd2c and SOX2 expressions are closely related to the development and prognosis of LSCC according to the results of immunochemistry and overall survival analysis. Therefore, blockade of Jmjd2c-SOX2 signaling might be efficient to control LSCC tumor progression.

In summary, this study suggested that Jmjd2c maintained the ALDH^{bri+} cancer stemness with transcription factor SOX2 in lung cancers. Inhibitors targeting Jmjd2c is promising to be an anti-tumor drugs for clinical treatment for LSCC patients. Due to the complexity of epigenetic factor regulatory on chromatin structure and gene expression, more investigations are needed in the future.

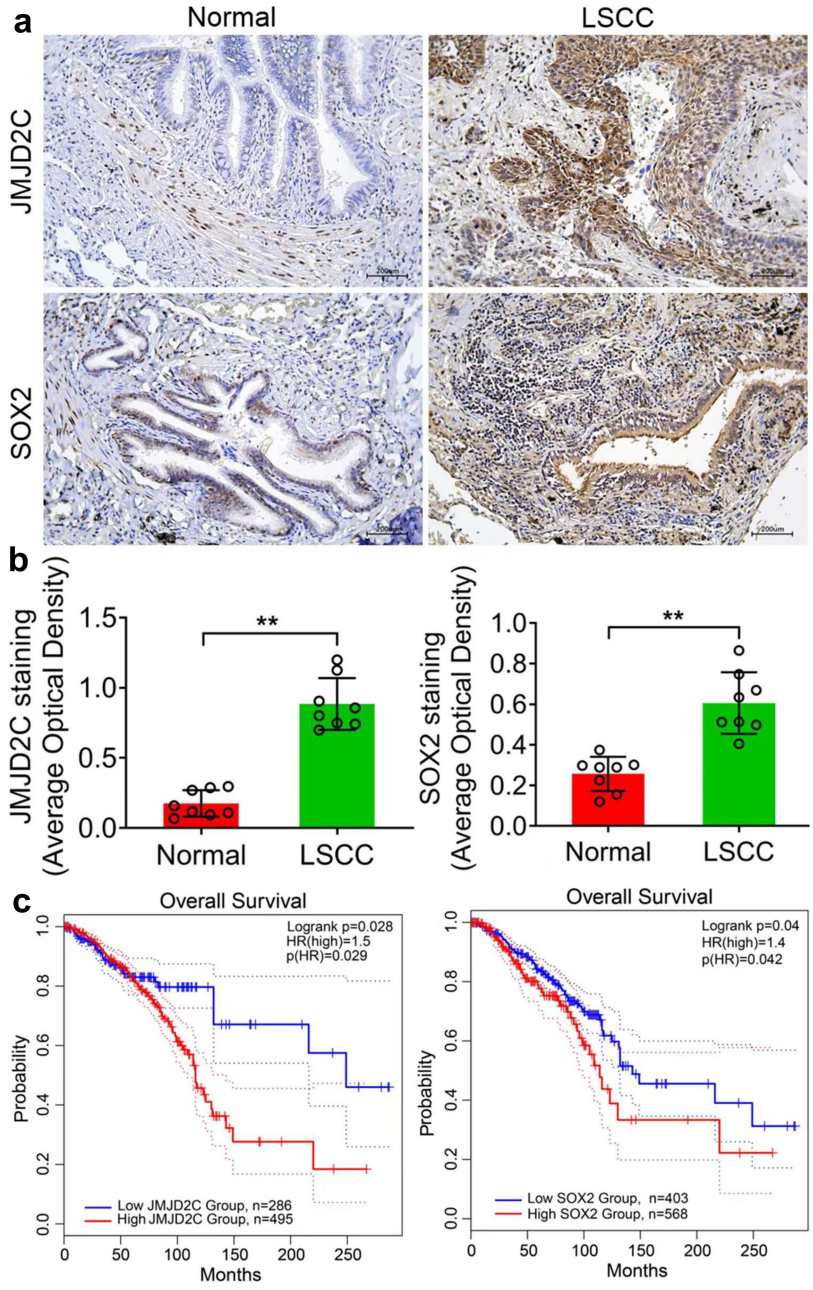

Figure 7. Jmjd2c and SOX2 expression are closely related to the development and prognosis of LSCC. (a) Immunochemistry results showed the protein expressions of Jmjd2c and SOX2 in normal and LSCC tissues. (b) The quantitative analysis of Jmjd2c and SOX2 expressions in A. (c) Overall survival of Jmjd2c and SOX2 in LSCC based on Kaplan Meier-plotter. The patients were stratified into high-level group and low-level group according to different expression ratio. The data are represented as means ± SD, $n \geq 100$, ***$p < 0.001$.

Materials and methods

Cell culture

The primary ALDH^{bri+} CSCs of LSCC was obtained as described previously,[39] and were inoculated in petri dishes. When the cells proliferated to 80% of the dish, trypsinize them and then cultured in Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F12) (Thermo Fisher Scientific, US) with 10% fetal bovine serum (Zhenjiang, China) under 37°C and 5% CO2 conditions. The harvested cells were prepared for the following experiments.

The lentiviral-mediated Jmjd2c-silenced ALDH^{bri+} CSCs

The PLL3.7-Jmjd2c/SOX2 vectors were transformed to the 293T cells and cultured under 37°C and 5% CO_2 conditions for 10 h. Then, add Titer Boost to the mixtures. The harvested viruses were then transformed into the ALDH^{bri+} CSCs to obtain the Jmjd2c-silenced and Jmjd2c-SOX2-silenced ALDH^{bri+} CSCs.

Sphere formation assay

Resuspended the ALDH^{bri+} CSCs, adjusted the cell concentration to 5000 cells/ml, cultured with 24-well plates, and

obtained the primary spheres 7 d later. Collected these primary spheres and resuspend the cells in DMEM/F12 and adjusted the cell concentration to 5000 cells/ml again. Cultured these cells in 24-well plates and obtained the secondary spheres another 7 d later. Observed and photographed the spheres under a microscope. Those larger than 40 μm in diameter were counted.

Aldefluor assay

The $ALDH^{bri+}$ CSCs were prepared same as the above method. The ALDH activity was detected with the ALDEFLOUR^{TM} assay kit (NWBiotech, Beijing) following the manufacturer's instruction on a flow cytometer (BD FACSCanto II, BD Biosciences, USA).

Western blotting analysis

The proteins were extracted using RIPA protein extraction reagent (Solarbio, Beijing). Approximately, 30 μg lysis products were subjected to SDS – PAGE, transferred to polyvinylidene fluoride (PVDF) membranes and blocked in 5% milk. The rabbit anti-Jmjd2c and rabbit anti-SOX2 (Abcam, US) were applied in dilution 1:1000. After washed, the membrane was incubated with goat anti-rabbit IgG (Abcam, UK) in dilution 1:5000. The protein bands were visualized with the enhanced chemiluminescence detection method controlled by GAPDH.

qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, US) following the manufacturer's instructions. The PrimeScript® RT Master Mix Perfect Real Time kit (TAKARA, Japan) and the SYBR Green Master Mix (Applied Biosystems, US) were employed in RNA reverse transcription and qPCR on an instrument (Applied Biosystems 7900HT Real-Time System, US). $2^{-\Delta\Delta Ct}$ method was applied to calculate the relative gene levels normalized to actin.

Co-IP assay

The Jmjd2c and SOX2 proteins were enriched with the Protein A/G Immunoprecipitation Kit (Invitrogen, US). Five milligrams of Myc-Jmjd2c and SOX2 was lysed and used for IP with Myc antibody (Abcam, China). The IP of the interacting protein SOX2 was controlled by Myc-Jmjd2c with empty pcDNA3.1. The input was applied to determine the initial protein levels of Jmjd2c and SOX2 in each group.

GST pull-down assay

Approximately 100 μg of GST-SOX2 fusion protein was incubated with 50 μl of glutathione agarose (Yeasen, China) at 4°C for 1 h. Then, added approximately 100 μg of Myc-Jmjd2c fusion protein to the immobilized GST-SOX2 solutions and incubated at 4°C overnight. Finally, eluted the bound protein with 10 mM glutathione (pH 8.0) and analyzed it by western blotting analysis. Beta-tubulin was used as a loading control.

The construction of subcutaneous tumor-bearing BALB/c nude mice

Eighteen female BALB/c nude mice (16–18 g) were randomly divided into three groups. Mice of one group were injected with 1×10^7 Jmjd2c-silenced $ALDH^{bri+}$ CSCs each, and another group was injected with 1×10^7 Jmjd2c-SOX2-silenced $ALDH^{bri+}$ CSCs, controlled by one group injected with equal amount of primary $ALDH^{bri+}$ CSCs, subcutaneously on the right thigh. Observe the tumor growth and measure tumor volume every 2 d. All the mice were sacrificed with pentobarbital (100 mg/kg) after 17 d and stripped of the subcutaneous tumors in nude mice.

HE staining

The lungs of the tumor-bearing BALB/c nude mice were isolated and fixed with paraformaldehyde (4%), embedded in paraffin, sectioned to 5 μm in thickness and stained with HE solution (Solarbo, Beijing).

Immunochemistry

The LSCC tissues and mouse lung tissues were fixed in 10% formalin and stored in 70% ethanol. Tissues were sectioned to 5 μm in thickness and were deparaffinized and rehydrated in order. Slides were incubated in 3% hydrogen peroxide for 30 min and blocked in serum for 1 h, then incubated with the primary antibodies with 1:1000 in dilution overnight at 4°C. After washed with PBS solutions, the slides were incubated with the secondary antibodies with 1:5000 in dilution for 1 h. The protein expressions were then visualized using 3,3-diaminobenzidine (DAB) chromogen (Sigma-Aldrich, US).

Statistical analysis

GraphPad Prism 5.0 (GraphPad Inc., US) and SPSS 22.0 (IBM Corporation, US) were applied to statistical analysis. Independent Student's t test or one-way ANOVA were performed to analyze the differences between distinctive groups. All the data were presented as means ± SD, and $p < .05$ was considered statistically significant.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Yingmin Zhang http://orcid.org/0000-0001-5991-6900

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Authors' contributions

Conception and design: M.W.; analysis and interpretation of data: Y.H, F.C., L.G. and Y.M.; drafting of the article: M.W. and Y.Z.; revision of the article for important intellectual content and final approval: all authors.

Availability of data and materials

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

Consent for publication

The authors of this study have read and approved the submitted form of this manuscript and declare that it is not under consideration for publication elsewhere.

Ethics approval and consent to participate

All experimental protocols were approved by the Ethics Committee of the First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology. The study is reported in accordance with ARRIVE guidelines. All methods were performed in accordance with the relevant guidelines and regulations.

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