Pluripotent Stem Cell Protein Sox2 Confers Sensitivity towards LSD1 Inhibition in Cancer Cells

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Supplementary Information

Supplementary Figure Legends:

Figure S1. Sox2 and LSD1 Expression in lung squamous carcinoma and adenocarcinoma and differential inhibitory effects of LSD1 inhibitors on the growth of ovarian and breast cancer cells.

A and B. A: Sox2 and LSD1 expression in human lung squamous cell carcinoma (left panels, N=13) and lung adenocarcinoma (right panels, N=17) tissues. Serial tissue sections from clinical patient samples were immunostained with anti-Sox2 or LSD1 antibodies. LSD1 and Sox2 were elevated in squamous cell carcinoma pathological tissues. Sox2 is non-detectable in all 17 adenocarcinoma cases and LSD1 is lower in lung adenocarcinoma cases. Scale bar, 100 microns. Lower panels represent magnified images (4x). B: Statistical analysis of the correlation (Pearson's) between Sox2 and LSD1 expression in lung squamous cell carcinomas in A. Related to Figure 1.

C. Ovarian and breast cancer cells were treated with control (DMSO) or 50 μM LSD1 inhibitors CBB1003 and CBB1007 for 30 hours as indicated. Cell growth was monitored by microscopy. Scale bar, 100 microns. While ovarian carcinoma Hs38.T and ES-2 and breast carcinoma BT549 and SK-BR-3 cells were not sensitive to LSD1 inhibitors, breast carcinoma MCF7 and ovarian carcinoma IGROV-1 and SKOV-3 cells, as well as ovarian teratocarcinoma PA-1 cells were sensitive to LSD1 inhibition.

D. Indicated ovarian and breast cancer cells were transfected 50 nM luciferase (Luc) or LSD1 specific siRNAs for 60 hours and cell growth was monitored by microscopy and ablation efficiency by Western blotting. Figure S1C and S1D are related to Figure 2A.

Figure S2. Loss of LSD1 activity causes growth inhibition in ovarian A2780, breast T47D and other carcinoma cells.

A and B**.** Ovarian A2780 and breast T47D carcinoma cells were treated with various concentrations of LSD1 inhibitors CBB1003 and CBB1007 for 30 hours as indicated. Cell growth was monitored (A) and quantified by the MTT assay (B). All error bars indicate mean ± SD. *: $p < 0.05$ and **: $p < 0.01$.

C. A2780 and T47D cells were transfected with 50 nM luciferase or LSD1 specific siRNAs for 60 hours. Cell growth inhibition was analyzed by MTT. The effects of LSD1 siRNAs on LSD1 and methylations on histone H3K4 proteins were monitored by Western blotting using specific antibodies (right). **: $p < 0.01$.

D. LSD1 and methylations of H3K4 in A and B were examined by Western blotting with anti-LSD1 and histone H3K4 methylation antibodies as indicated.

E. Sox2-expressing myelogenous leukemia and rhabdoid tumor cells were also sensitive to LSD1 inhibitors. The growth of human Sox2-expressing rhabdoid tumor G401 cells was inhibited after treatment of 50 μM CBB1007 or CBB1003 for 30 hours but not that of Sox2-negative melanoma MDA-MB-435s cells. The statistical differences between compound treated and control groups were analyzed by One-way ANOVA. *: $p < 0.05$ and **: $p < 0.01$.

F. Human myelogenous leukemia K562 cells, which express Sox2, were treated with increasing concentrations of LSD1 inhibitor CBB1007 for 30 hours and relative cell viability of compound-treated and control groups was analyzed. Figure S2A-C and S2E and F are related to Figure 2A.

Figure S3. **Ablation of LSD1 by specific siRNAs selectively induced growth inhibition in ovarian and breast cancer cells**

A. Sox2-expressing A549, IGROV1, T47D and Sox2-negative H1437 cells were transfected with 50 nM luciferase (Luc) or LSD1 specific siRNAs for 60 hours and the cell cycle was analyzed by flow-cytometry (FACS). The Sox2-expressing cells were G1 cell cycle arrested by LSD1 inactivation but not Sox2-negative H1437 cells. Related to Figure 2E.

B. ChIP assays for the presence of LSD1 and changes of H3K9me2, H3K4me1 /me2, and H3K27me3 along the regulatory regions of the *cyclin B and cyclin D* genes after LSD1 inactivation in Sox2-expressing lung carcinoma A549 cells. Data are presented as mean ± SD. Related to Figure 3F.

C. G1 cell cycle arrest does not cause changes in Sox2 expression and genes for differentiation in Sox2-expressing cancer cells. Left panels: Sox2-expressing lung carcinoma A549 cells were arrested in the G1/S border by the double thymidine block

method. The G1 arrest was analyzed by flow-cytometry (FACS). Middle panels: The G1 cell cycle arrest does not induce the expression of differentiation genes as indicated. Right panels: The G1 cell cycle arrest does not affect Sox2 and FOXA2 expression by Western blotting analyses. Related to Figure 2E and 3A.

Figure S4. Loss of Sox2 inhibited the growth of Sox2-expressing breast and ovarian carcinoma cells.

A. Sox2-expressing A549, IGROV1, and T47D and Sox2-negative H1437 cells were transfected with luciferase and Sox2 specific siRNAs for 48 hours. The cell cycles of these cells after Sox2 ablation were analyzed by flow-cytometry. Related to Figure 4B.

B. Ovarian teratocarcinoma PA-1 cells were either treated with 50 μM CBB1007 for 30 hours, or transfected with luciferase (Luc) or LSD1 specific siRNAs for 48 hours. The protein levels of Sox2, Oct4, Lin28 and control proteins CUL1 and tublin were monitored by Western blotting analysis with specific antibodies. Related to Figure 3A.

C. Ablation of Sox2 induced growth inhibition in cells that are sensitive to LSD1 inhibitors, but not in insensitive cells. Ovarian and breast cancer cells were transfected with luciferase (Luc) or Sox2 specific siRNAs for 60 hours as indicated. Cell growth was monitored by microscopy. Scale bar: 100 microns. The effects of Sox2 siRNA were analyzed by Western blotting as indicated. Related to Figure 4A.

Figure S5. Inactivation of LSD1 induces genes for differentiation in Sox2-expressing cells.

A. LSD1 inhibitor CBB1003 induced the expression of FOXA2, HNF4A, BMP2, EOMES, and Sox17 genes for differentiation in NCI-H520, A2780 and T47D cells. Related to Figure 7A-C.

B. LSD1 inhibitors CBB1003 and CBB1007 induced the expression of differentiation gene HNF4A in lung, breast, and ovarian carcinoma cells that express Sox2 but not in cancer cells that are Sox2-negative. Related to Figure 7D.

Figure S6. LSD1 inhibitors increase the methylated H3K4 but not methylated H3K9 or H3K27 on differentiation genes.

A-B. Sox2-expressing lung carcinoma A549 cells were treated with 50 μM CBB1007 for 30 hours and the association of LSD1 and changes of H3K4me1, H3K4me2, H3K9me2, and H3K27me3 with the BMP2 (A) and Sox17 (B) transcriptional regulatory regions after LSD1 inhibition were analyzed using the ChIP assays. Related to Figure 7E.

C. Sox2-expressing lung carcinoma A549 and ovarian carcinoma A2780 cells were treated with 50 μM CBB1007 for 30 hours or transfected with luciferase or LSD1 siRNAs for 48 hours as indicated. The changes of H3K27me3 along the transcriptional regulatory regions of *Sox2, FOXA2, and cyclin A* genes after LSD1 inactivation were analyzed using the ChIP assays. Related to Figure 7E and F.

D. LSD1 does not bind to the transcriptional regulatory regions of *Lin28* and *Klf4* genes. The ChIP assays were used to analyze the binding of LSD1 to *Lin28* and *Klf4* genes and no significant differences were found between anti-LSD1 antibodies and control IgG. Related to Figure 7G.

Figure S7. LSD1 binds to CoREST and loss of CoREST phenocopies the selective growth inhibition of LSD1 inactivation in Sox2-expressing cancer cells.

A. LSD1 binds to CoREST in NCI-H520, A549, and H1299 cells. The LSD1 protein complexes were immunoprecipitated from the lysates of H520, A549 and H1299 cells and the complexes were blotted with anti-LSD1 and CoREST antibodies.

B. Ablation of CoREST by specific siRNAs induced growth inhibition in Sox2-expressing H520 and A549 cells but not in Sox2-negative H1299 cells. Indicated cells were transfected with luciferase (Luc) or CoREST specific siRNAs for 48 hours and cell growth were monitored by microscopy. Related to Figure 1-3.

C. Examination of ablation efficiency of CoREST and the effects of CoREST deficiency on LSD1 and Sox2 in B.

D. Quantitative analysis of cell growth inhibition in B by the MTT assay.

E. Association of CoREST and the changes of LSD1 binding and H3K4me1, H3K4me2, H3K9me2, and H3K27me3 with the transcriptional regulatory region of *Sox* in control (Luc) and CoREST ablated A549 cells by specific siRNAs were analyzed using the ChIP assays.

F. CoREST inactivation phenocopied the effects of LSD1 inactivation on the methylations of H3K4, H3K9, and H3K27 on Sox2-expressing lung carcinoma NCI-H520 and A549 cells and Sox2-negative H1299 cells.

Supplementary Tables:

Table S1: Summary of Sox2 and LSD1 expression in human squamous lung carcinomas and adenocarcinomas.

The expression of Sox2 and LSD1 on serial tissue sections from 13 cases of human squamous cell lung carcinomas, which were pathologically classified into 5 poorly differentiated and 8 moderately differentiated cases, was compared with that of 17 cases of human adenocarcinomas, which have 2 poorly differentiated and 15 moderately differentiated cases, by immunohistological staining. The intensities of immunostaining of Sox2 and LSD1 were blindly scored by an independent pathologist. All lung adenocarcinoma cases and one case of squamous cell lung carcinomas were found negative for Sox2 staining. Strongest LSD1 staining was found to associate with poorly differentiated squamous cell lung carcinomas and the intensity of LSD1 was higher in Sox2-exprssing squamous cell lung carcinomas than that of lung adenocarcinomas. Statistical analysis revealed a significant correlation between Sox2 and LSD1 expression (Pearson's correlations: R^2=0.4372 and P =0.014) in lung carcinomas. Related to Figure 1A and B.

Table S2: Summary of carcinoma cells that are sensitive to LSD1 inactivation and Sox2 expression.

The expression of pluripotent stem cell proteins Oct4, Sox2, Lin 28, Nanog, and Sall4 in various lung, ovarian, breast, and other carcinoma cells and their sensitivities towards LSD1 inhibitors in Figure 2D are summarized. Related to Figure 2D.

Table S3: Oligonucleotide PCR primers used for real time quantitative RT-PCR/PCR and ChIP. Related to Figure 3B, 3E-F, 4E and F, 6, 7B-G, S3B and C, S5, S6, and S7E.

Extended Experimental Procedures:

Tissue culture and cell lines

Cell culture was conducted as described earlier (Wang et al., 2011). PA-1 and MCF7 were maintained in Eagle's Minimum Essential Medium; Hs38.T, F9, HeLa and 293 cells were in Dulbecco's Modified Eagle's Medium; IGROV-1 cells were in RPMI Medium 1640 without folic acid; SKOV-3, ES-2, SK-BR-3 and G401 cells were in McCoy's 5a Medium; A2780, OVCAR-3, OVCAR-8, T47D, BT-549, and H1299 cells were in RPMI-1640 Medium; MDA-MB-231, MDA-MB-468, MDA-MB-453, MDA-MB-435s and MDA-MB-361 cells were in Leibovitz's L-15 Medium; A549 cells were maintained in F-12K Medium and K562 cells were maintained in Iscove's Modified Dulbecco's Medium. All media were supplemented with 10% FBS, whereas OVCAR-3 cells were cultured with 20% FBS. The media for OVCAR-3 and MCF7 cells were supplemented with 0.01mg/ml bovine insulin, while the medium for BT-549 cells was with 0.023 IU/ml insulin.

Immunological procedures and Antibodies

Protein detection using Western blotting was conducted as previously described (Wang et al., 2011). The rabbit polyclonal anti-LSD1, Sall4, Nanog, H3K4Me1, H3K4Me2, H3K9me1, H3K9me2, H3K9me3, and Histone 3 antibodies were purchased from Abcam; anti-LIN28 and anti-Klf4 antibodies were from Proteintech Group; anti-H3K4Me3 antibody was from Millipore; anti-FOXA2 antibodies were from Sigma; and anti-Sox2 antibodies were from Bethyl Laboratory. The goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP and mouse monoclonal anti-Oct4 antibodies were purchased from Santa Cruz Biotechnologies.

RNA extraction, reverse transcription, and quantitative real-time RT-PCR/PCR

Total RNA was extracted using TRIZOL reagent (TaKaRa) and complementary DNA was generated according to instructions in the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa) as described previously (Wang et al., 2011). The cDNAs were diluted to 1/20 and 1μl of each diluted sample was used as template for each sample. Real-time quantitative PCR was performed on an ABI Prism 7300 sequence detection

system (Applied Biosystems) using SYBR Green (TaKaRa) according to the manufacturer's instructions. PCR amplification of the housekeeping gene *β-Actin* was performed as a control. Experiments for specific silencing or induction of gene expression were repeated at least three times. The real-time PCR primers of human genes are described in Supplementary Table 3.

Cell viability and cell cycle assays

Cell viability assays were conducted as previously described (Wang et al., 2011). For the MTT assay, cells were seeded onto 96 -well culture plates with a density of 2000-5000 cells/well, and incubated with CBB1003 or CBB1007 at various concentrations for 26-30 hours. Dimethylsulfoxide (DMSO) was used as a solvent control. MTT was added to a final concentration of 0.5 mg/ml and cells were re-incubated for 4 hours. After removing the medium, 200 μl DMSO were added to dissolve formazan, followed by incubation for 10 minutes, and the absorbance was measured at 490nm by a BioRed microplate reader. All assays were performed in triplicates for each concentration. The cell viability rate was calculated as the relative percentage of MTT absorption as follows: percentage (%) of cell viability = (mean experimental absorbance/mean control absorbance) × 100. For the cell cycle analysis, the cells were fixed with increasing percentages of ethanol. The fixed cells nuclei were washed with 1XPBS and then stained with 0.5 ml PBS containing 10 μg/ml RNase A and 20 μg/ml propidium iodide (PI) for Flow-cytrometry analysis as described previously (Higa et al., 2003). The double thymidine block was done as previously described (Higa et al., 2003). The active growing A549 cells were treated with 2 mM thymidine for 14 hours and then the thymidine block was released 10 hours. Thymidine was added again for 14 hours to arrest the cells in the G1/S border.

Chromatin immunoprecipitation (ChIP)

The ChIP assay was performed according to previously described protocols (Whyte et al., 2012). Briefly, 1×10^7 human A2780 cells were used for each immunoprecipitation. Cells were fixed in 0.75% formaldehyde, collected, resuspended in the FA lysis buffer (0.1% SDS, 0.1% sodium deoxycholate, 1mM EDTA, 1% Triton X-100, 140mM NaCl

and 50mM HEPES-KOH, pH 7.5) and sonicated to generate DNA fragments of 500 – 1000 base pairs (bp). The sonicated chromatin was cleared and incubated with specific primary antibodies or normal rabbit IgG overnight followed by incubation with protein A Sepharose beads (pre-adsorbed with sonicated single-stranded herring sperm DNA and BSA) for 1 hour. After incubation, the immunocomplexes were washed sequentially with wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, at pH 8.0 and 150mM NaCl), final wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, at pH 8.0, and 500mM NaCl). Immunocomplexes were eluted in elution buffer (0.1% SDS and 0.1M NaHCO3) and the crosslinking was reversed overnight at 65^0C. DNA was extracted with phenol/chloroform and precipitated with ethanol. Purified DNA was quantified by real-time PCR. Re-ChIP assays for the co-presence of H3K9Me2 and H3K4Me2 were conducted as described (Geisberg and Struhl, 2004). Chromatin fragments were first immunoprecipitated with anti- H3K9me2 antibodies or control IgG. The fragments were eluted with H3K9me2 peptide and re-immunoprecipitated with anti-H3K4me2 antibodies. Conversely, the chromatin fragments were first immunoprecipitated with anti-H3K4me2 and then anti-H3K9me2 antibodies. Real-time quantitative PCR ratios reflected the relative enrichment to the Input of the indicated histone methylations on specific genes after sequential immunoprecipitations.

Immunohistochemistry

Immunohistochemical staining was performed as described previously (Wang et al., 2011). Briefly, tumor tissues from clinic patients were fixed in 10% neutral buffered formalin and then embedded in paraffin. Serial section was carried out to produce 5 microns slices. Slides were deparaffinized, rehydrated, and immersed in 3% H_2O_2 to inactivate the endogenous peroxidase. Antigens were retrieved and immunostained with anti- LSD1 and anti-Sox2 antibodies. The slides were then incubated with the Rabbit-Probe Streptavidin-Peroxidase polymer detection system, developed with 3, 3'-diaminobenzidine (DAB) substrate, counterstained with hematoxylin, dehydrated, and then mounted with Neutral balsam. Images were captured on a Zeiss fluorescence

microscope (Axio Observer) coupled with a cooled charge-coupled device camera (AxioCam MRM, Zeiss) and processed by using AxioVision program.

Supplementary References:

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