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Yishen Shen April 10, 2023

The Role of Sox7 on the WNT Signaling Pathway in Lung Adenocarcinoma

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Abstract

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Lung cancer is the most common form and a major cause of cancer death worldwide. Lung adenocarcinoma (LUAD) is the primary subtype of non-small cell lung cancer (NSCLC) which accounts for 80% to 85% of lung cancers. Sox7 is a transcriptional regulator, and it is a tumor suppressor in prostate, colon, and breast cancers. It is consistently downregulated or silenced in advanced tumors, including NSCLC. The canonical WNT signaling pathway is implicated in metastasis and relapse during LUAD progression. Sox7 suppresses β-catenin–mediated transcription by depleting active β-catenin (ABC) in the canonical WNT signaling pathway in prostate and colorectal cancer. After analyzing human specimens and cell line data, Sox7 was found to have a low mutation frequency, and its expression positively correlated with survival rate. Eight LUAD cell lines were identified and confirmed with low or high Sox7 expression. In both H358 and H522 LUAD cells, Sox7 ectopic expression led to lower proliferation, colony formation, and migration. In H358-Sox7, a G1 to S phase shift and decrease in ABC were observed. However, in H522-Sox7, no significant changes in cell cycle progression or β-catenin and ABC productions were observed. Upon introduction of β-catenin into H522 cells, WNT activity was elevated. Understanding the mechanisms behind H522 and H358 cells' behavioral changes would require further exploration. To expand our findings into more cell lines, Sox7 knock down was performed in HOP-62, PC-9, and H1792, and increased migration abilities were exhibited in all three cell lines. Results from this study indicated that Sox7 inhibits LUAD's proliferation and migration, but only alters WNT signaling in some LUAD cells.

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Table and Figures

Introduction

Lung cancer continues to be a significant problem worldwide, accounting for 1.8 million deaths in 2020 [1]. Despite recent advances, it stays the most common form of cancer and a major cause of deaths globally. Lung cancer incidence is expected to increase in less developed regions of the world due to the rising prevalence of smoking [2]. It has one of the lowest 5-year relative survival rates (21%), and 57% of the patients are diagnosed with metastatic disease [3]. LUAD is the primary subtype of NSCLC for which the mortality rate is rising especially in women, non-smokers, and young adults [4, 5]. Therefore, it is urgent to develop effective targeted treatments for metastatic LUADs.

SRY-Box Transcription Factor 7 (Sox7) belongs to the SOX gene family characterized by the 79-amino acid DNA-binding domain known as the HMG box. The Sox family has 20 members that are grouped into eight (A-H) subfamilies based on protein similarities [6]. Sox7, along with Sox17 and Sox18, makes up the SoxF subfamily. It acts as a transcriptional regulator and plays a role in modulating diverse developmental processes [7]. Sox7 acts as a tumor suppressor in prostate, colon, and breast cancers. Consistently, Sox7 is downregulated or not expressed in advanced tumors and correlates with poor prognostics. [8, 9, 10]

Wingless-related integration site (WNT) signaling pathway has been prevalent in cancer biology. The central player of WNT is a cytoplasmic protein termed β-catenin. The stability of β-catenin is regulated by a cytoplasmic destruction complex which is composed of axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK-3β) [9]. CK1 and GSK-3β phosphorylate β-catenin on its N-terminus, preparing β-catenin for ubiquitination and subsequent proteasomal degradation. Upon deactivation of the destruction complex, cytoplasmic β-catenin becomes stabilized and

translocates to the nucleus. Inside the nucleus, β-catenin interacts with T-cell factor/ lymphoid enhancer factor (TCF/LEF) transcription factors and activates β-catenin/T-cell factor – regulated transcription (CRT) [11]. During embryonic development, the activity of the WNT signaling pathway is tightly regulated to ensure proper cell proliferation, survival, and differentiation. However, in cancer, WNT can escape the control system [11]. During LUAD progression, aberrant activation of the canonical WNT signaling pathway promotes metastasis to the brain and bone regions. WNT activation also leads to relapse in multiple organs in primary LUAD. [12] With the aid of statistical analysis, Hayano and his team suggested that Sox7 is a tumor suppressor downregulated or silenced in the majority of NSCLC [13]. In prostate and colorectal cancer, Sox7 suppresses β-catenin–mediated transcription by binding to β-catenin and depleting ABC in the canonical WNT signaling pathway [14]. ABC is an active form of β-catenin that is dephosphorylated on Ser^{37} or Thr^{41}. Consequently, an important question about Sox7's function is whether and how β-catenin is negatively regulated by Sox7, which in turn deactivates the WNT signaling pathway in LUAD. The hypothesis for this study is that Sox7 negatively regulates β-catenin and thus deactivates the WNT signaling pathway in LUAD.

Yishen Shen performed all experiments except for flow cytometry presented in Figure 8. Specifically, Yishen Shen prepared the samples for flow cytometry and Yijian Fan ran the samples. Flow cytometry data was analyzed by Yishen Shen.

Hypothesis

We postulate that down-regulated expression of Sox7 leads to more ABC and thus activates the WNT signaling pathway in LUAD.

Research Aims

Aim 1 - To determine the basal level expressions of Sox7 and WNT signaling status in human LUAD cell lines.

Sox7 mediates the degradation of ABC and acts as a tumor suppressor by inhibiting the WNT signaling pathway in prostate and colorectal cancers. However, this pathway has not been investigated in LUAD cells; therefore, we plan to analyze the expression of Sox7 and WNT signaling status in LUAD cell lines.

Aim 2 - To determine the effect of overexpressing and downregulating Sox7 on WNT signaling in LUAD cells.

Sox7 expresses at low levels in the majority of NSCLCs (92% of 62 NSCLC tissue samples), but no functional studies have been conducted in LUAD [13]. We plan to analyze the effects of Sox7 overexpression and knockdown on LUAD cells' proliferative, migratory, and colony-formation abilities.

Materials and Methods

Materials – Rabbit polyclonal anti-Sox7 antibody (cat# AF2766) was purchased from R&D Systems. Rabbit polyclonal anti-β-catenin (cat# 9587) antibody, mouse monoclonal anti-actin (cat# 3700), rabbit polyclonal anti-Caspase-3 antibody (cat# 9662S), rabbit monoclonal anti-Cleaved-Caspase-3 antibody (cat# 9664S), horseradish peroxidase (HRP) linked anti-mouse antibody (cat# 7076S), and HRP-linked anti-rabbit antibody (cat# 7074S) were purchased from Cell Signaling Technology (Microarray). Mouse monoclonal anti-ABC (clone 8E7) antibody (cat# 05-665) was from Sigma-Aldrich Co. LLC. HRP-linked anti-goat antibody was purchased from Santa Cruz Biotechnology, Inc. (cat# sc-2020). The pCMV-

Tag2/Sox7 expression plasmid was a gift from Dr. Akira Murakami (Laboratory of Molecular and Cellular Biology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Japan) [15], whereas the pOT-Flash and pOF-Flash expression plasmids were provided by Dr. Bert Vogelstein (Ludwig Center at Johns Hopkins and Howard Hughes Medical Institute, Baltimore, MD) [16, 17]. Sox7 small interfering RNA (siRNA) was purchased from Sigma-Aldrich Co. LLC (MISSION® esiRNA**,** cat# EHU122021). To control for any nonspecific off-target effects of the siRNA transfection, a negative control siRNA from the same company was also used (cat# SIC001).

Kaplan-Meier analysis – On the Kaplan-Meier Plotter home page (website: https://kmplot.com/analysis/index.php?p=background), under "mRNA gene chip", the tab "Start KM Plotter for Lung Cancer" was chosen. The Affy id/ Gene symbol for Sox7 was 228698_at. "Adenocarcinoma" was selected for Histology. After selecting "Draw Kaplan-Meier plot", Kaplan-Meier meta-analysis survival plot was generated. P values were calculated using the log rank test.

Cell Lines and Cell Culture – Eight LUAD (H522, H358, H1650, A549, HCC827, HOP-62, PC-9, and H1792) cell lines were purchased from American Type Culture Collection (ATCC) and were grown and maintained according to ATCC specifications. The identities of these LUAD cell lines were authenticated by STR genotyping service at Emory University (Atlanta, GA).

Restriction Enzyme Digestion – Restriction enzyme digestion was performed with 1.5 μL 1x rCutSmart™ Buffer (cat# B6004S, NEB) and 0.3 μL EcoRI-HF (cat# R3101, NEB) per 0.3 μg plasmid (pCMV-Tag2B/Sox7 and pCMV-Tag2B). Total volume was brought up to 20 μL with nuclease free water. Digestion mixtures were incubated at 37°C for 15 min and

0.15-0.3 μg plasmid (10 μL digestion mixture) per lane were loaded on 1% agarose gel. Tris-borate-EDTA (TBE) agarose gel (1%) was ran for 135 min at 120 V. Images were captured at 75 min, 105 min, and 135 min.

Transient Plasmid Transfection – H358 and H522 cells were plated at 5 x 10^5 and 8.5 x 10^5 per well on 6 well plates 18-24 h prior to transfection. Transfections were carried out with 12.5 μL Lipofectamine 2000 (cat# 11668, Invitrogen) and 2.5 μg pCMV-Tag2B/Sox7 or pCMV-Tag2B (vector control) plasmids in 800 μL Opti-MEM™ I reduced serum medium (cat# 31985, Gibco™) per well. The transfection mixture was replaced with complete RPMI 1640 medium (cat# 10-040-CV, Corning™) with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep) post 6 h of incubation. This medium was replaced again with complete RPMI 1640 medium after 24 h. Cells were harvested 48 h post-transfection.

Generation of two Stable Cell Lines (H522-Sox7, H522-CON; H358-Sox7, H358-CON) – H522 and H358 cells were transfected as described above. Instead of harvesting the cells 48 h post transfection, cells were selected with 500 (H522) and 1000 (H358) μg/mL G418 Sulfate (cat# 10131035, Gibco™) in complete RPMI 1640 medium for 3 to 4 weeks.

Western Blot Analysis – Cells were lysed with 2.5 mL of 1x lysis buffer (cat# 9803, Cell Signaling Technology, Inc.), 25 μL PMSF (cat# 10837091001, Roche), and 0.25 Protease Cocktail Inhibitor tablet (cat# 11836153001, Roche). Protein concentrations were measured, and 50 μg total protein per lane was loaded into 10% SDS-PAGE gels. Gels were run for 0.5 h at 80 V and 1.5 h at 123 V and transferred to PVDF membranes for 1.3 h at 25 V. After blocking in 10% milk for 0.5 h, membranes were probed with antibodies against Sox7, β-catenin, and ABC. Actin was used as the loading control.

Cell Proliferation Assay – Two hundred to 5000 parental and/or stable H522 and H358 LUAD cells were seeded per well into 96-well plates. The numbers of cells in each well were analyzed 24, 48, 72, and 96 h post-seeding using the sulforhodamine B (SRB) assay [18]. The culture medium was removed, and the cells were fixed by incubating in 100 µL of cold 10% (w/v) trichloroacetic acid (cat# AAA1115630, Thermo Scientific Chemicals) at room temperature for 45 min. The plates were washed with deionized water 6 times, 10 sec each, and air dried. Sixty to 70 µL of SRB solution (0.4% w/v in 1% acetic acid) were added to each well and then incubated for 30 min at room temperature. Unbound SRB was removed by washing 5 times, 10 sec each, in 1% acetic acid. After air-drying the plates, 100 µL of 10 mM unbuffered Tris base (pH 10.5) was added to each well. Plates were shaken for 15 min at room temperature to completely solubilize the SRB stain bound to the cells. Absorbance was measured at 570 nm with application Gen5. (version 3.11, BioTek) and Synergy H1 Multimode Reader (BioTek). Six replicate wells were analyzed for each experimental group and error bars represent one standard deviation. The average absorbance of the six replicate wells was calculated and plotted at the respective time points. Error bars represent 1 standard deviation.

Colony Formation Assay – A549, H358, and H522 cells were plated at 10^5, 8.5 x 10^5, and 2.5 x 10^5 cells respectively on 6 well plates 18-24 h prior to transfection. Transfections were carried out with 12.5 µL Lipofectamine 2000 (cat#11668, Invitrogen) and 2.5 µg pCMV-Tag2B/Sox7 or pCMV-Tag2B plasmids in 800 µL Opti-MEM™ I reduced serum medium (cat#31985, Gibco™) per well in triplicate. The transfection mixture was replaced with complete RPMI 1640 medium (cat# 10-040-CV, Corning™) after 6 h of incubation. Selections with 5 x 10^2 (H522), 10^3 (H358), or 2 x 10^3 (A549) µg/mL G418 Sulfate (cat#

10131035, Gibco™) in complete RPMI 1640 medium for 14 to 18 days were carried out 48 h post-transfection. Cells were visualized with crystal violet staining.

Cell Cycle Analysis – H522 and H358 parental and stable cells were seeded in 10 mm dishes. Post trypsinizing, 10^6 cells were collected in 15 mL tubes, resuspended in PBS, and fixed in cold 70% ethanol for a minimum of 30 min at 4 °C. After removing ethanol, cells were suspended in and stained by a PI/RNase staining buffer (cat# 550825, BD Biosciences) for 15 – 30 min at room temperature in the dark. Flow cytometry was run with the BD FACSymphony™ A3 cell analyzer (BD Biosciences). A total of 10,000 gated cells were analyzed with FlowJo™ v10.8 Software (BD Biosciences) using the Watson (Pragmatic) model. Cisplatin treatment was used as a positive control.

Wound Healing Assay in Stable Sox7 Over-expressing Cells – Stable H522 and H358 cells were seeded in 24 well plates and incubated until reaching 95% - 100% confluency in monolayers. Cross scratching with a 1000-µL pipette tip was applied to each well followed by rinsing with PBS. Images were taken at 3 different time points post-scratching with the software INFINITY ANALYZE (version 7, Teledyne Lumenera). The % wound closure was analyzed with the application, ImageJ (version 1.53a, National Institutes of Health). The edge of the wound was traced manually with the "freehand selections" feature and the wound (cell-free opening in the middle) area was measured in pixels. The percent wound closure was calculated using the formula: percent wound closure = (wound area at 0 h post scratching – wound area at a specific time point)/ wound area at 0 h post scratching * 100%. Error bars represent 1 standard deviation.

Luciferase Reporter Assay – H522 and H358 parental and stable cells were seeded at 0.7 x 10^5 or 10^6 cells per well, respectively, in 24 well plates 18-24 h before transfection.

All transfections were carried out with 2 µL Lipofectamine 2000 in 60 µL Opti-MEM™ I reduced serum medium per well. The *Firefly* luciferase activity was measured with the "Luminescence" detection method on application Gen5. (version 3.11, BioTek) and Synergy H1 Multimode Reader (BioTek) 48 h post-transfection and normalized with the background *Renilla* luciferase activity using the Dual-Luciferase Reporter Assay System from Promega (cat# E1910). For the CRT assay, each well was transfected with 100 ng of pOT-Flash, which encoded T-cell factor/lymphoid enhancer factor – responsive reporter, or 100 ng of pOF-Flash, which acts as a negative control with a mutated T-cell factor/lymphoid enhancer factor binding site, with or without 100 ng of β- catenin plasmid, per well. Reactions were carried out in triplicates and error bars represent one standard deviation. The relative CRT levels were calculated via *Firefly* luminescence*/ Renilla* luminescence which allows normalization to *Renilla* as a luciferase internal control.

Wound Healing Assay in Sox7 siRNA Knock Down Cells – PC-9, H1792, and HOP-62 cells were plated at 60%-70% confluency in 6-well plates and 24-well plates 18-24 h before transfection. In 24-well plates, 1.2 µL lipofectamine 2000 was incubated with 50 µL Opti-MEM™ I reduced serum medium and 15 pmol Sox7 siRNA or control siRNA for 15 min at room temperature. Mixtures were added to the respective wells, and an additional 140 µL Opti-MEM™ I reduced serum medium was added per well. After 6 h of incubation, the media was replaced with 500 µL of complete media per well. Cells were harvested 48-90 h post-transfection. Wound healing assay was carried out on PC-9, H1792, and HOP-62 cells as described above.

Results

Sox7 expression positively correlates with LUAD patients' survival rate.

The data used to construct Figure 1 was collected from Cancer Biomedical Informatics Grid (caBIG, http://cabig.cancer.gov/, microarray samples are published in the caArray project), the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), and The Cancer Genome Atlas (TCGA, http://cancergenome. nih.gov). Meta-analysis of 865 LUAD patients' microarray data (n = 2437) indicated a clear trend toward a decreased overall survival rate for patients with lower Sox7 expression (expression was split by median) (Figure 1). The median survival period for the low-expression cohort is 76 months and for the high-expression cohort is 119.87 months.

Sox7 mutation frequency is low in LUAD patients.

Transcription factor Sox7 is encoded by the Sox7 gene in the human genome. Sox7 mutation frequency was evaluated using 4 LUAD studies from cBioPortal (Figure 2a). The mutation data was annotated and standardized using Genome Nexus (https://www. genomenexus.org) which utilizes the Ensembl Variant Effect Predictor (VEP) (https://useast.ensembl.org/info/docs/tools/vep/index.html) with the canonical transcript (https://github.com/mskcc/vcf2maf/blob/main/data/isoform_overrides_uniprot) from UniProt.

We queried 2598 samples collected from 2503 patients in 9 studies on LUAD (Broad, Cell 2012; CPTAC, Cell 2020; MSKCC, 2020; MSKCC, 2021; MSKCC, Science 2015; NPJ Precision Oncology, MSK 2021; OncoSG, Nat Genet 2020; TCGA, Firehose Legacy; TSP, Nature 2008). Out of 9 studies, 1562 out of 2598 samples did not report Sox7 mutation data, and 4 studies were found to contain samples with Sox7 mutations (Figure 2a). The 9 studies were

processed and filtered by cBioPortal based on the mutation calls as provided by each publication.

In the TCGA study (Firehose Legacy), the Sox7 gene is altered in 7.17% of 516 cases. In this study, the frequency of Sox7 missense mutation is 0.97%, amplification is 0.39%, deep deletion is 5.62%, and multiple alterations (missense mutation and deep deletion) is 0.19%. In the Broad study (Cell 2012), Sox7 gene is altered in 7.1% of 183 cases. In this study, the frequency of Sox7 missense mutation is 1.09%, truncating mutation is 0.55%, and deep deletion is 5.46%. In the study from OncoSG (Nat Genet 2020), Sox7 gene is altered in 5.3% of 302 cases. The frequency of Sox7 missense mutation is 0.33%, amplification is 0.33%, and deep deletion is 4.64%. In the MSKCC study (Science 2015), Sox7 gene is altered in 2.86% of 35 cases due to missense mutation. In those 9 studies, in-frame, structural variation/ fusion, and splice mutations are not detected. For the Sox7 copy numbers in the 10 missense and truncating mutation cases, 1 deep deletion, 1 shallow deletion, and 2 gains are identified. These observations suggested that the mutation or alteration frequency of Sox7 is low, 0.33% to 2.86%, in LUAD.

The expression of Sox7 does not negatively correlate with methylation at a statistically significant level in CCLE LUAD cell lines.

We evaluated the correlation between Sox7 expression and methylation in 57 cell lines from depmap portal, Cancer Cell Line Encyclopedia (CCLE). The correlation coefficient (r) was -0.15510 (P = 0.24931). For each unit increase in the normalized log2(TPM+1) expression of Sox7, the methylation was expected to decrease 0.15510. The p-value is insignificant. Thus, there is no overall correlation between the expression of Sox7 and methylation (Figure 2b).

Selection of high and low Sox7 expressing lung cancer cell lines, and validation of Sox7 level expression in these LUAD cell lines.

Four LUAD cell lines with low Sox7 log2(TPM+1) expression levels (H522, H358, H1650, A549) and four with high Sox7 log2(TPM+1) expression levels (HCC827, HOP-62, PC-9, and H1792) were cultured (Figure 3a) and their corresponding methylation fractions were presented in Table 1.

To confirm eight LUAD cell lines' Sox7 basal level expression, western blot analysis was performed as shown in Figure 3b. One band was observed in H522, H358, H1650, and HCC827 cells, and two bands were observed in A549, HOP-62, PC-9, and H1792 cells with anti-Sox7 antibody. PC-9 and H1792 were shown to possess significantly higher Sox7 expression levels than other LUAD cell lines, specifically higher Sox7 isoform 2 (Q9BT81-2) expression at 48.5 KDa represented by the thicker band (Figure 3b). The presence of 2 bands was consistent with Peng's paper in 2018 [19]. The lower thinner band at 42 KDa corresponded to the canonical Sox7 (isoform 1, Q9BT81-1) and its isoform 2 may be a result of alternative splicing as predicted by UniProt (https://www.uniprot.org/uniprot/Q9BT81 #Q9BT81-1) (Figure 3b). As shown in the Supplemental Figure 4 in Peng's paper, Sox7 isoform 1 and isoform 2 differ in their N-terminal end. Isoform 2 lacks the bipartite nuclear localization signal (NLS) [19]. Isoforms 1 and 2 contain identical sequences for basic cluster NLS and β-catenin interacting motifs.

Verification of pCMV-Tag 2B/Sox7 plasmid.

The identities of the pCMV-Tag 2B/Sox7 and pCMV-Tag 2B expression plasmid were verified with restriction enzyme digestion (Figure 4). pCMV-Tag 2B is 4.324 kbp long. With the incorporation of a 1.2 kbp Sox7 to the MCS site, pCMV-Tag 2B/Sox7 is expected to be

around 5.5 kbp. To visualize the size of the plasmid, the EcoRI recognition site was chosen to be cut (Palindromic Recognition Sequence: 5'-GAATTC-3', cut between G and A). As shown in Figure 3a, after running the gel for 75 min, uncut plasmid without Sox7 insert had 2 bands, 11.99 kbp and 3.03 kbp (representing the open-circular and supercoiled plasmid), while cut plasmid without Sox7 insert was 4.33 kbp. Uncut plasmid with Sox7 insert had bands of 13.51 kbp and 4.29 kbp while the cut plasmid with Sox7 insert was 5.55 kbp. The sizes of the plasmids were calculated using a standard log curve. Cut plasmids (linear DNA fragments) travel slower than uncut nicked and supercoiled plasmids due to their larger size and more frictional resistance from the gel. The cut plasmids' location corresponded with the expected sizes respectively and therefore the identity of pCMV-Tag 2B/Sox7 plasmid was verified.

The relationship between transiently upregulating Sox7 in Sox7 low-expression cell line, H358, and apoptosis is indeterminate.

To investigate the effect of Sox7 overexpression on cellular apoptosis in H358 cells, pCMV-Tag 2B/Sox7 expression or pCMV-Tag 2B plasmid was transiently transfected into H358 cells (Figure 5a, b). Stronger bands relative to the control plasmid pCMV-Tag2B treatment group were observed at 48-49 KDa which potentially correspond to Sox7 isoform 2. The changes at the 42 KDa band were not significant and so Sox7 isoform 1 was not notably overexpressed. 90 h post plasmid transfection, adherent and floating cells were collected separately (Figure 5b). Expression of Sox7 in both adherent and floating (unattached) H358 cells declined (fading bands) and thus validating the transfection was transient. Sox7 expression in floating H358 cells decreased to a greater extent since Sox7 in presumably dead floaters denatured as indicated by the lower bands (~ 30 KDa).

To explore the relationship between Sox7 upregulation and apoptosis, membranes were probed with total and cleaved caspase-3. Cleaved caspase-3 level was highly elevated in both Sox7 overexpression and control groups compared to untreated and mock treatment groups in Figure 5a. In Figure 5b, total caspase-3 level was higher in the Sox7 overexpression groups than that of control groups for lanes corresponding to 48 h post transfection and adherent cells 90 h post transfection. Cleaved caspase-3 was not observed in the 4 lanes at far left, CON and Sox7 groups at 48 h and 90 h (adherent cells only) post transfection. For the 2 lanes in 90 h Fl, only cleaved caspase-3 was present since floating cells are generally dead and have already gone through apoptosis. The results from Figure 5a, b indicated that the effect of Sox7 overexpression (mainly Sox7 isoform 2 up-regulation) on apoptosis is indeterminate.

Stable Sox7 overexpression suppresses cell proliferation in H522 and H358.

The transfection procedure potentially led to lots of programmed cell death and the Sox7 overexpression was temporary. To maintain Sox7 upregulation and avoid excessive cell death during future experiments, two stable cell lines overexpressing Sox7, H358-Sox7 and H522-Sox7, were established and validated with western blots (Figure 6). Stronger bands relative to the stable vector control groups, H358-CON and H522-CON, were observed at 48-49 KDa which potentially correspond to Sox7 isoform 2. Sox7 isoform 1 expression was only observed in H358-Sox7 and H358-CON cells.

SRB analysis was performed to analyze the effects of Sox7 upregulation on cell proliferation. Stable H522 and H358 cells were analyzed at 24, 48, 72, and 96 h post seeding. As shown in Figure 7, both H522-Sox7 and H358-Sox7 exhibited lower cell proliferation compared to their corresponding vector control groups. From day 1 to 4, the average

proliferation rate (represented by absorbance at 570 nm) of H522-Sox7 is 0.302, and for H522-CON is 0.435 with a 44.04% difference. The average proliferation rate of H358-Sox7 is 0.616, and for H358-CON is 0.696 with a difference of 12.99%. Compared to H358, stable overexpression of Sox7 induced more pronounced proliferation inhibition in H522. This data led us to wonder about the effect of stable Sox7 overexpression on LUAD cells' colony formation abilities.

Stable Sox7 overexpression suppresses colony formation in H522 and H358.

To further explore the effects of Sox7 upregulation in low Sox7 expressing LUAD cells, colony formation assays were carried out where pCMV-Tag 2B/Sox7 expression plasmid or control vector was stably transfected into H358 and H522 LUAD cells. Post selection with G418, reduction in colony formation was observed in the Sox7 overexpression groups compared to the empty vector control groups in both H522 and H358 cells, with H522-Sox7 exhibiting more significant reduction than H358-Sox7 (Figure 8). Thus, Sox7 ectopic overexpression from stable transfection greatly reduced single cells' ability to grow into colonies via colony expansion. To elucidate the mechanism(s) that caused decrease in cell proliferation and colony formation abilities at differential levels in H522-Sox7 and H358-Sox7 cells, cell cycle analysis was performed.

Stable transfection leads to increased G1 arrest in both H358 and H522. H358-Sox7 shifts more from S to G1 phase compared to H522-Sox7.

Cell cycle progression was analyzed in H358 and H522 parental and stable cells (Figure 10). In H358-Sox7, 78.8% cells were at G1 phase and 4.97% were at S phase; in H358-CON, 53.7% were at G1 phase and 22.3% were at S phase (Figure 10a). In H522-Sox7, 55.1% of cells were at G1 phase and 24.3% were at S phase; in H522-CON, 56.4% were at G1 phase

and 22.2% were at S phase (Figure 10b). Compared to parental untreated H358 (G1: 33.9%, S: 38.0%) and H522 (G1: 44.9%, S:28.6%), stable H358 and H522 both exhibited increased G1 phase arrest, indicating the incorporation of Sox7 overexpression or vector control plasmids led to more apoptosis. This data corresponds to Figure 5a where both Sox7 and CON groups had thick bands for cleaved caspase-3, indicating higher levels of apoptosis compared to the UNT and MOCK groups. The cell cycle analysis also revealed that, compared to the stable controls (H358-CON and H522-CON), H358-Sox7 cells showed an increased G1 phase arrest by ~25.1% while H522-Sox7 showed a slight decrease in G1 arrest by ~1.3%. Therefore, stable Sox7 upregulation led to more G1 phase arrest in H358 than H522 cells.

Stable Sox7 overexpression suppresses cell migration in H522 and in H358.

To evaluate the impact of stable Sox7 overexpression on cell migration *in vitro*, wound healing assays were performed. H522-Sox7 and H358-Sox7 showed statistically significant lower percent wound closure compared to the vector control groups (Figure 10). H358 exhibited a more drastic decrease in migratory ability post Sox7 overexpression with one-tailed P-values of 0.0085 (24 h) and 0.0021 (91 h), while analysis of stable H522 yielded one-tailed P-values of 0.1017 (14 h) and 0.0040 (45 h).

H358-Sox7 has lower ABC production than H358-CON. Stable H522 cells shows no total β-Catenin or ABC production.

From previous literature, researchers found that WNT activity specifically elevates the level of a β-catenin subpopulation dephosphorylated at residues Thr^{41} and Ser^{37}. Thus, dephosphorylated β-catenin was defined as an active form of β-catenin, ABC, that activates CRT [20]. We next analyzed the amount of total β-catenin and ABC expression levels in H358 and H522 post stable Sox7 upregulation. As shown in Figure 11, the expression level of total

β-catenin was the same in H358-Sox7 and H358-CON. However, H358-Sox7 had a significantly lower level of ABC than H358-CON, suggesting that the stable ectopic overexpression of Sox7 depletes ABC. On the other hand, no bands corresponding to total β-catenin and ABC were observed in H522-Sox7 and H522-CON, suggesting they expressed zero to extremely low levels of total β-catenin and ABC.

Stable and parental H522 cells show low CRT activity. Upon β-catenin introduction, parental H522 cells show significantly elevated CRT.

To investigate if and how the presence of β-catenin and CRT activity potentially affect cells' migration ability, dual luciferase assay was carried out. As shown in Figure 12a and b, H522-Sox7, H522-CON, and H522 cells were transfected with either pOT-Flash or pOF-Flash plasmids. Low levels of CRT activity (< 0.004) were observed in all three cell lines. Inside the nucleus, β-catenin binds to T-cell factor/lymphoid enhancer factor and activates CRT [9]. To further confirm our findings in Figure 11, H522 cells were transfected with β-catenin plasmids (Figure 12 c). CRT level in pOT-Flash and pOF-Flash groups increased drastically by 315.19% and 237.46%. In conjunction with our discovery that parental and stable H522 expresses zero to extremely low levels of β-catenin, restoration of β-catenin in H522 promoted CRT activity. Luciferase assay with H358 cells is still under optimization and thus no data has been collected.

Sox7 siRNA treatment causes partial knockdown in Sox7 high-expression cell lines, H1792, PC-9, HOP-62.

To address the dichotomy between stable H358 and H522 cells' migratory abilities and CRT activity levels, cell lines with high Sox7 expression were incorporated to expand the representative cell line samples. We carried out RNAi analysis with Sox7 siRNA in H1792,

PC-9, and HOP-62 cells. Sox7 siRNA treatment resulted in slight suppression of Sox7 isoform 2 expression and no suppression of Sox7 isoform 1 expression in all 3 LUAD cells (Figure 13).

Transient Sox7 knockdown promotes cell migration.

To evaluate the impact of Sox7 knockdown on cell migration, wound healing assays were performed. High Sox7 expressing LUAD cells (PC-9, H1702, and HOP-62) were treated with Sox7 siRNA knockdown. All three cell lines showed statistically significant lower percent wound closure compared to the vector control group (Figure 13). Thus, Sox7 expression level affects cellular migratory ability.

Discussion

Existing literature demonstrated that Sox7 is downregulated or silenced in LUAD. Low expression or loss of Sox7 is associated with higher cell survival and proliferation, decreased apoptosis, and increased formation of drug-resistant phenotype against various chemotherapy agents. [13, 21, 22] Our statistical analysis from Figures 1 and 2 corresponds with previous studies and further supports Sox7's low expression in LUAD. Multiple mechanisms could promote repression of gene expression, including alternations in DNA methylation levels, chromatin compaction due to modifications in histone proteins, mRNA degradation, and/or suppression of its translation. It has been found that Sox7 downregulation is mainly ascribed to promoter hypermethylation in prostate cancer, acute myeloid leukemia, and myelodysplastic syndrome [9, 23, 24]. We found the expression of Sox7 does not negatively correlate with methylation at a statistically significant level in CCLE LUAD cell lines. This discrepancy could be potentially due to differences in cancer tissue types. Furthermore, Sox7 downregulation in LUAD could be induced via other mechanisms.

For example, several microRNAs (miR-24-3p, Mir-9, miR-935, miR-21-5p, miR-616) and lncRNA (MEG3) have been shown to inhibit Sox7 expression in lung cancer [25]. It would be interesting to investigate if there is an association between those upstream regulators of Sox7 and cellular apoptosis.

To explore the effects of Sox7 expression on cellular behaviors, lung cancer cell lines with either high Sox7 or low Sox7 expression were identified as potential experimental models. Four LUAD cell lines with low Sox7 log2(TPM+1) expression levels (H522, H358, H1650, A549) and four with high Sox7 log2(TPM+1) expression levels (HCC827, HOP-62, PC-9, and H1792) were selected, and their Sox7 protein expression levels were validated by western blot analysis. Two protein bands at 42 KDa and 48.5 KDa were found, corresponding to two alternatively spliced isoforms. This study focused on the 48.5 KDa isoform since it showed more variation in expression level compared to the other isoform.

The effects of transient expression of Sox7 isoform 2 in LUAD were first evaluated. Transient transfection procedures induced high levels of apoptosis in H358. We also observed that Sox7 upregulation could not be maintained 48 h post transient transfection. Thus, two stable cell lines with high Sox7 expression, H358 and H522, were established. We found that stable Sox7 upregulation suppresses cell proliferation and colony formation in both H358-Sox7 and H522-Sox7. In conjugation with our discovery that H358-Sox7 shifts from S to G1 phase, the data from these results suggests that Sox7 overexpression reduces H358 cells' proliferative and colony formation abilities via inducing S to G1 phase arrest. No significant disturbance was observed in stable H522, and thus further research is required to explain the mechanism(s) behind significant reduction in H522-Sox7 proliferation and colony expansion compared to H522-CON.

We also assessed whether stable Sox7 upregulation affects LUAD cells' migratory ability, and found it caused decreased migration in both stable H522 and H358 cell lines with H358 exhibiting a more pronounced level of decrease. Ectopic expression of β-catenin cooperates with growth factors to induce cell motility in bladder and kidney carcinomas, and thus β-catenin is a critical modulator for epithelial cell migration [26, 27]. To further validate this discovery in LUAD, western membranes were probed with total β-catenin and ABC. We found that the overexpression of Sox7 in H358-Sox7 suppresses ABC expression level. Therefore, the restoration of Sox7 expression in H358 cells are likely to suppress cell migration through the inhibition of WNT signaling.

We also attempted to validate this mechanism in a second LUAD cancer cell line, H522. H522 shows no total β-catenin or ABC production. This contradicts with data from depmap portal, CCLE, that β-catenin log2(TPM+1) expression is 7.100872552 in H522 with less than 0.02 methylation fractions, zero hotspot mutations, and zero damaging mutations. To confirm our results that H522 produces zero to low levels of β-catenin and ABC, luciferase assay was performed in both parental and stable H522 cells. We observed low relative CRT activity (< 0.004) in both stable and parental H522 cells. Specific CRT activity is defined as Relative CRT^{pOT}/ Relative CRT^{pOF}. The specific CRT levels for H522-Sox7 and H522-CON are 116.00% and 115.15%, indicating the lack of β-catenin mediated transcription. In contrast, post ectopic β-catenin expression in H522, the specific CRT activity elevated substantially by 162.58%, indicating that WNT signaling can be induced in H522 cells by ectopic β-catenin expression. Nevertheless, even though β-catenin mRNA might be present in H522, it was not efficiently translated into β-catenin, or this protein was actively degraded. More experiments are needed to elucidate the discrepancy between β-catenin mRNA and protein levels in H522.

Nevertheless, the decreased migration in H522 cells is not due to the regulation of β-catenin activity by Sox7.

To further explore the role of Sox7 on cell migration, we knocked down Sox7 expression in three high-Sox7 expressing cell lines (1792, PC-9, and HOP-62) by transient RNAi. Transient Sox7 knockdown via siRNA induced increased migration in all three cell lines, indicating Sox7 downregulation led to higher migratory abilities. This agrees with the wound healing data from the stable Sox7 upregulation cell lines that Sox7 regulates cell migration.

These preliminary findings reveal several future research directions. Previous studies have shown that Sox7 could bind to β-catenin and suppress β-catenin/T cell factor (TCF)-mediated transcription [9, 28]. Moreover, they discovered that Sox7 inhibits β-catenin-mediated transcription by competing with BCL9 to bind to β-catenin, disrupting the β-catenin/BCL9 interaction in human embryonic kidney 293T cells [28]. It is also found that Sox7 negatively regulates the Wnt/β-catenin signaling pathway by impeding the transcription machinery of β-catenin/ TCF/ LEF-1 transcription complex in endometrial cancer [29]. The application of the above mechanisms in LUAD would require future exploration.

LUAD is the leading cause of cancer death and the most common primary lung cancer in the United States. The treatment of LUAD is stage-dependent. For early stages, surgical resection is usually performed combined with adjuvant chemotherapy due to a substantial risk of relapse. For advanced and metastatic disease, a combination of surgery, chemotherapy, and radiation is carried out for pain management and other complications. The majority of LUAD are either locally advanced or metastatic by the time of diagnosis. For

locally advanced cases, the survival rate is less than 30% while for distant metastasis cases the 5-year survival rate is less than 5% [30]. Sox7 downregulation or depletion has been found in the majority of NSCLC [13]. Our study revealed that Sox7 inhibits LUAD's proliferation and migration, and it alters WNT signaling in some LUAD cell lines. The downregulation of Sox7 could stem from mRNA and/or protein expression, stability, and potential degradation. As shown in Figure 2b, LUAD cells' with and without hypermethylation both can have low mRNA expression (log2(TPM+1) expression < 0.5) and there is no correlation between Sox7 expression and methylation. Thus, for LUAD cells with low methylation, Sox7 downregulation could be caused by other mechanisms such as Sox7 protein degradation. Further studies targeting Sox7 restoration on the protein level will be needed. For LUAD cells with high methylation, Sox7 expression could be restored via promoter demethylation reagents. Our study could potentially lead to a novel therapeutic treatment for advanced and metastatic LUAD.

Table

LUAD Cell Line	Sox7 log2(TPM+1) Expression	Sox7 Methylation Fraction (1kb upstream from transcription start site, TSS)	Metastatic Potential
H522	0.137503523749935	0.19083	High
H358	0.2016338611696504	0.78138	Medium
H1650	0.2750070474998698	0.31916	Medium
A549	0.2986583155645151	0.59024	Low
HCC827	1.097610796626422	0.54769	High
HOP-62	2.5360529002402097	N/A	N/A
PC-9	3.372952097911829	N/A	N/A
H1792	3.814550423461808	0.34557	High

Table 1 Sox7 log2(TPM+1) expression, methylation fraction, and metastatic potentials of 8 LUAD cell lines used in this study. The Sox7 log2(TPM+1) expression and methylation data were collected from depmap portal, Cancer Cell Line Encyclopedia (CCLE). Four LUAD cell lines with low Sox7 log2(TPM+1) expression level (H522, H358, H1650, A549) and four with high Sox7 log2(TPM+1) expression levels (HCC827, HOP-62, PC-9, and H1792) were analyzed with their corresponding methylation fractions.

Figures

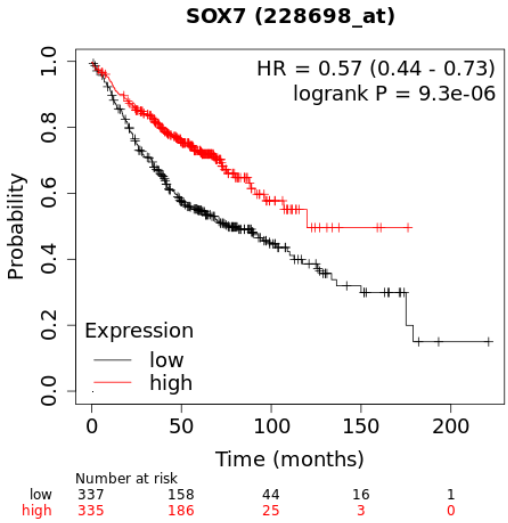

Figure 1 Sox7 expression is positively correlated with patients' overall survival rate in LUAD. Kaplan-Meier analysis of the overall survival rate of patients with high Sox7 expression vs low Sox7 expression. P values were calculated using log rank test; meta-analysis survival plots were generated using KM plotter [https://kmplot.com/analysis/index.php?p=service&cancer=lung]. Low Sox7 expression is associated with lower overall survival in LUAD patients.

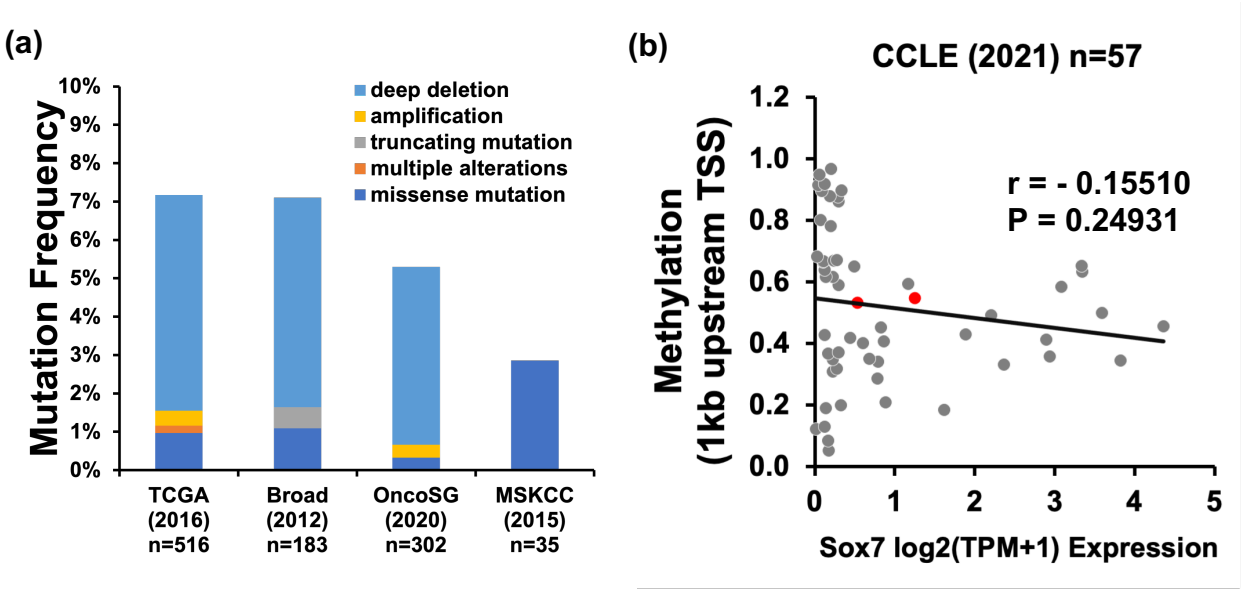

Figure 2 Sox7 mutation frequency is low and its expression has insignificant negative correlation with methylation in LUAD. (a) Sox7 mutation frequency ranges from 2.86% to 7.17% in LUAD studies. Sox7 mutation data was retrieved from cBioPortal. (b) The log2(TPM+1) expression of Sox7 negatively correlates with methylation in LUAD cell lines. Sox7 expression and methylation data was retrieved from depmap portal, CCLE. The 2 red dots represent LUAD cell lines containing Sox7 mutation, and the grey dots represent nonmutant cell lines. TPM stands for transcripts per million.

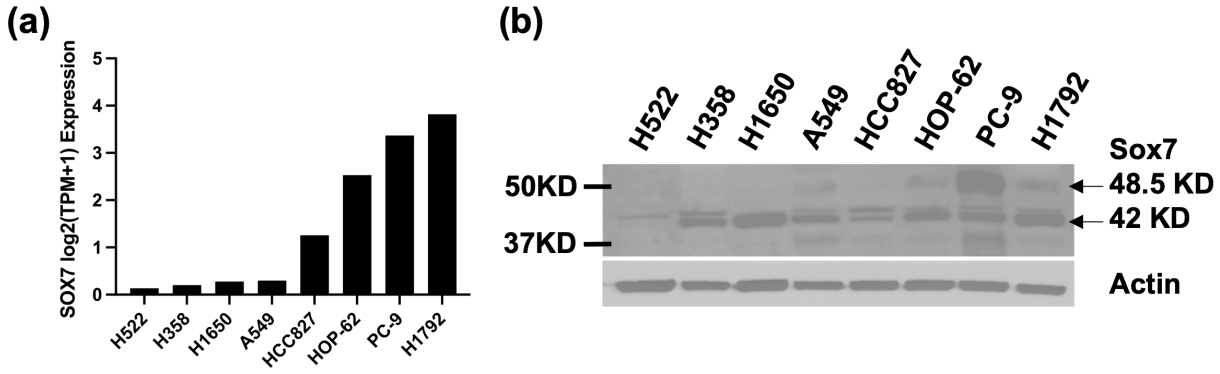

Figure 3 Basal level expressions of Sox7 in 8 LUAD cell lines and the presence of 2 Sox7 isoforms. (a) Sox7 log2(TPM+1) expressions in H522, H358, H1650, A549, HCC827, HOP-62, PC-9, and H1792. Data was retrieved from depmap portal, CCLE. (b) Sox7 protein levels in H522, H358, H1650, A549, HCC827, HOP-62, PC-9, and H1792 were determined by western blots.

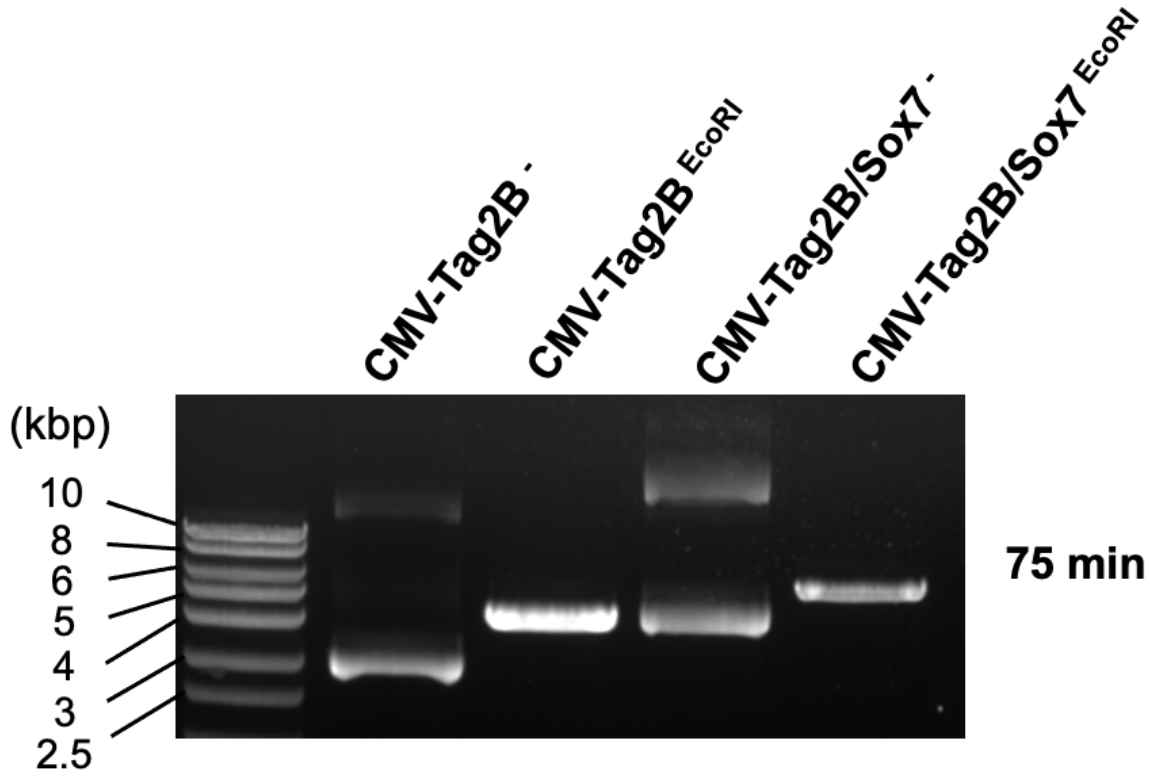

Figure 4 pCMV-Tag 2B/Sox7 and pCMV-Tag 2B plasmids' identities were verified with gel electrophoresis. Uncut plasmids and plasmids digested with restrictive enzyme EcoRI were run on 1% agarose gel. Image was captured at 75 min.

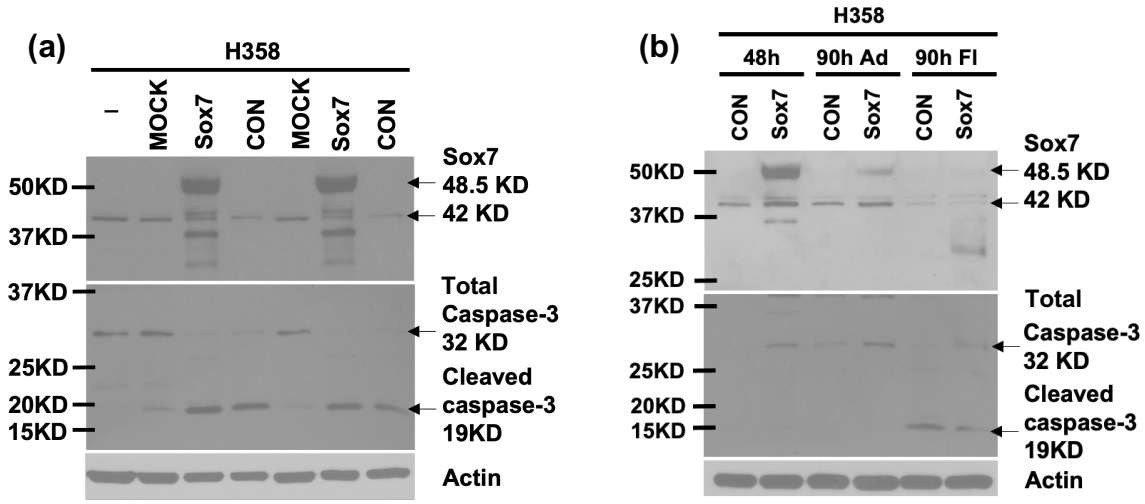

Figure 5 The relationship between transient Sox7 overexpression and cell apoptosis is inconclusive. Transient transfection leads to high level of apoptosis. (a) H358 cells were seeded at 5 x 10^5 per well in 6-well plates. At 70-80% confluency, H358 cells were transfected with pCMV-Tag2B/Sox7 plasmid (Sox7) or pCMV-Tag2B plasmid (CON). 48 h post transfection, cells were collected and lysed. Prepared lysate samples were run on western blots and probed with Sox7, total caspase-3, cleaved caspase-3, and actin antibodies. (b) 90 h post transfecting H358 cells with pCMV-Tag2B/Sox7 plasmid, Sox7 overexpression declines. Ad stands for H358 adherent cells and Fl stands for floaters.

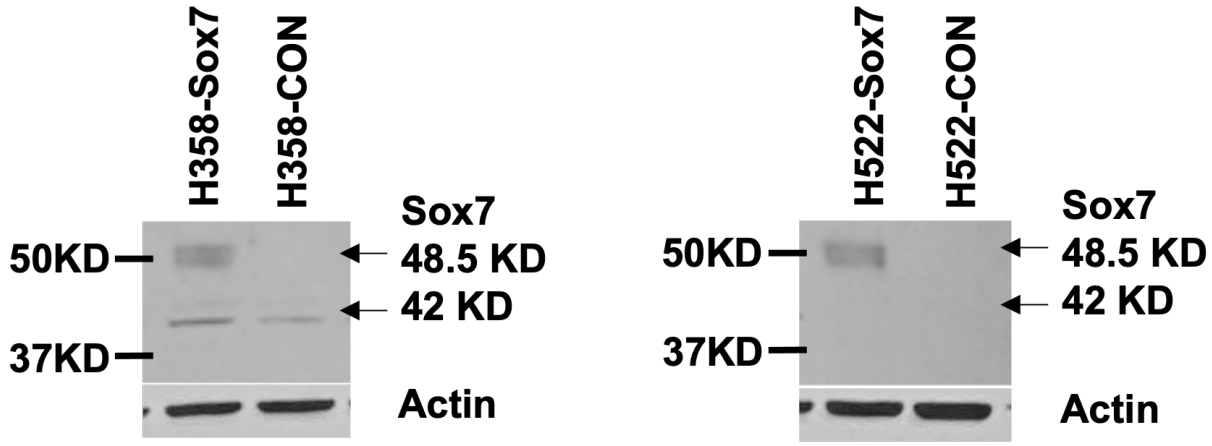

Figure 6 Validation of Sox7 expression in H358-Sox7 and H522- Sox7. H522 and H358 cells were transfected according as described in "**Transient Plasmid Transfection**" in this paper. 48 h post transfection, cells were selected with G418 in complete RPMI 1640 medium for 3 to 4 weeks.

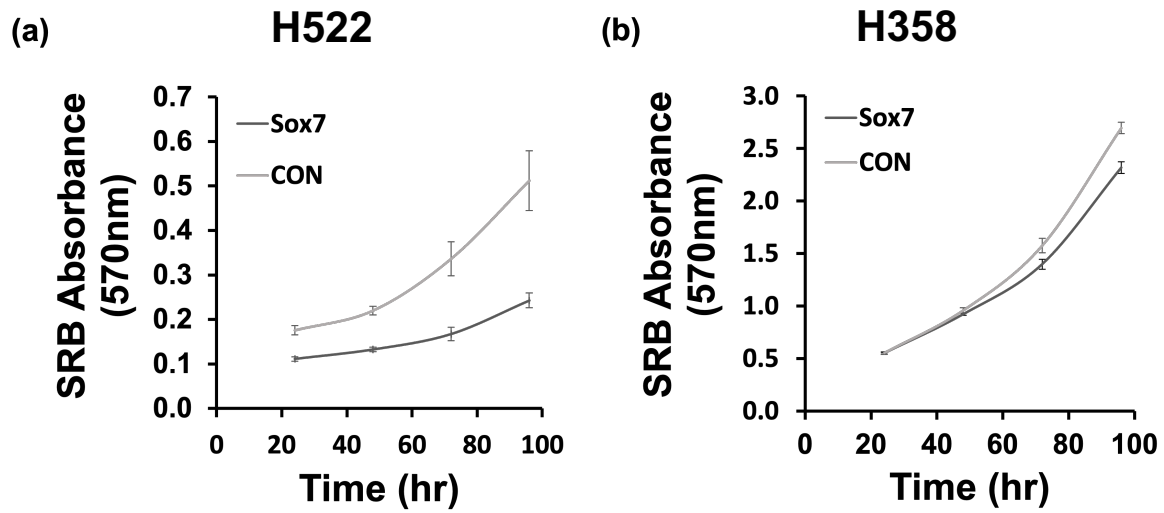

Figure 7 Stable overexpression of Sox7 in H522 and H358 suppresses cell proliferation. (a) SRB analysis of stable H522 with an initial seeding of 1000 cells, and (b) stable H358 with an initial seeding of 5000 cells per well from day 1 to 4. Two hundred, 1000, and 5000 stable H522 and H358 cells were seeded per well into 96-well plates. The numbers of cells in each well were analyzed 24, 48, 72, and 96 h post seeding. Absorbance was measured at 570 nm. Six replicate wells were analyzed for each experimental group.

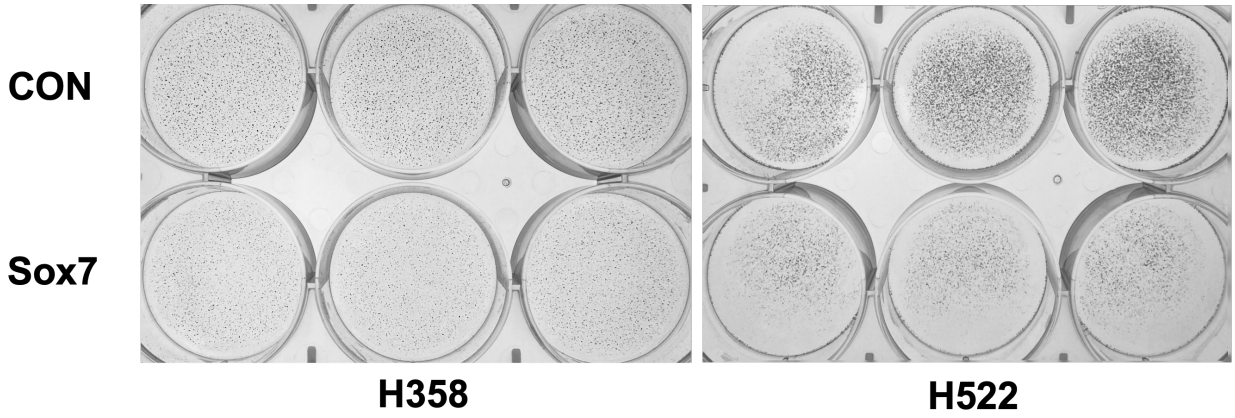

Figure 8 Stable overexpression of Sox7 suppresses colony formation in H358 and H522. H358 and H522 cells were plated at 8.5 x 10^5 and 2.5 x 10^5 cells respectively on 6 well plates 18-24 h prior to transfection. Transfections were carried out with either pCMV-Tag2B/Sox7 or pCMV-Tag2B (vector control) plasmids in triplicates. Cells were selected with G418 for 14 to 18 days and stained with crystal violet.

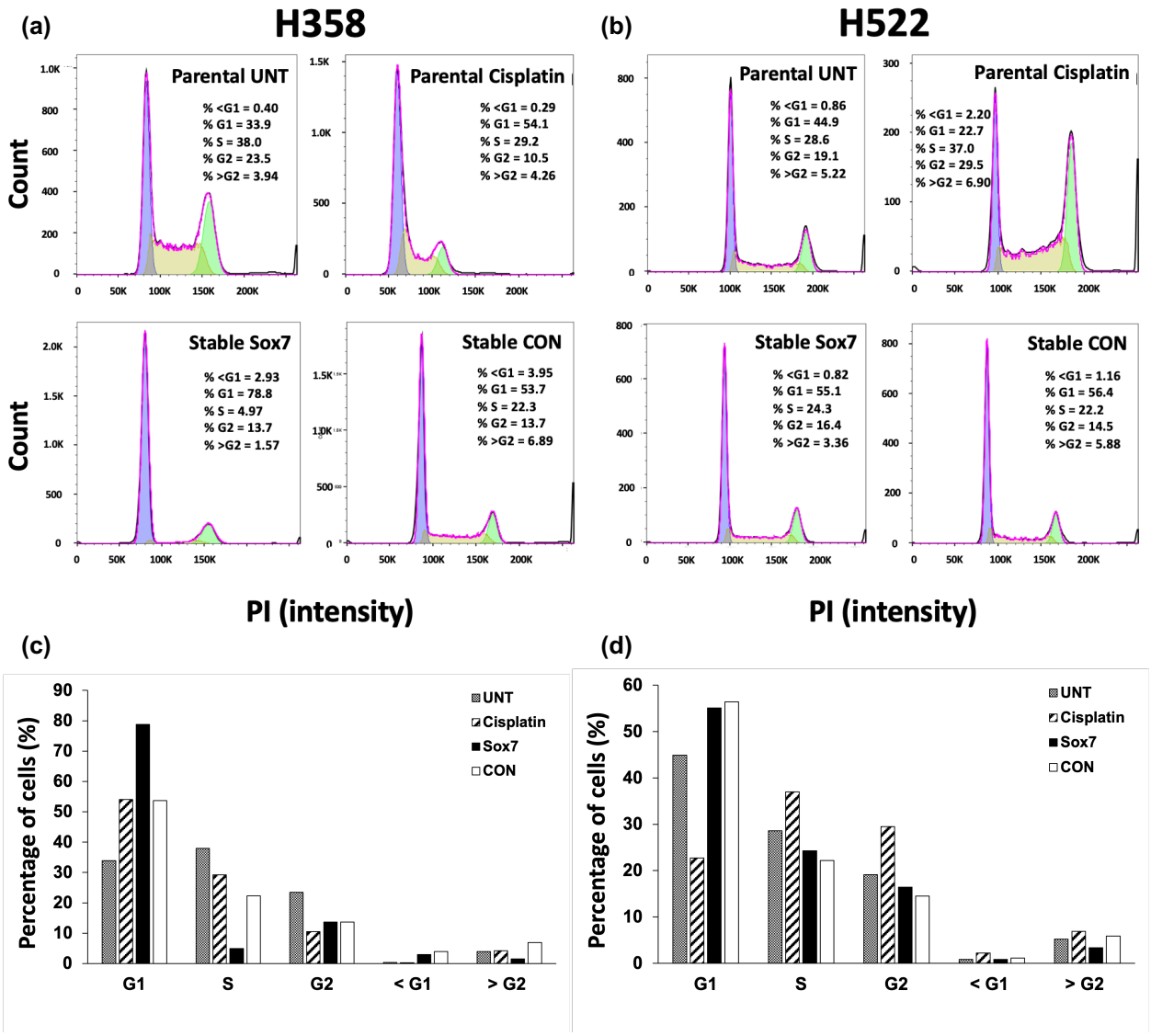

Figure 9 Stable Sox7 overexpression in H358 causes a shift from S to G1 phase (G1-phase arrest); stable Sox7 overexpression in H522 shows no distinct change in cell cycle. (a, c) H358 and (b, d) H522 parental and stable cells were seeded in 10 mm dishes. Post trypsinizing, 10^6 cells were collected in 15 mL tubes, resuspended in PBS, and fixed in cold 70% ethanol. After removing ethanol, cells were suspended in and stained with PI/RNase staining buffer. A total of 10,000 gated cells were analyzed with FlowJo™ v10.8 Software. Cisplatin treatment was used as a positive control.

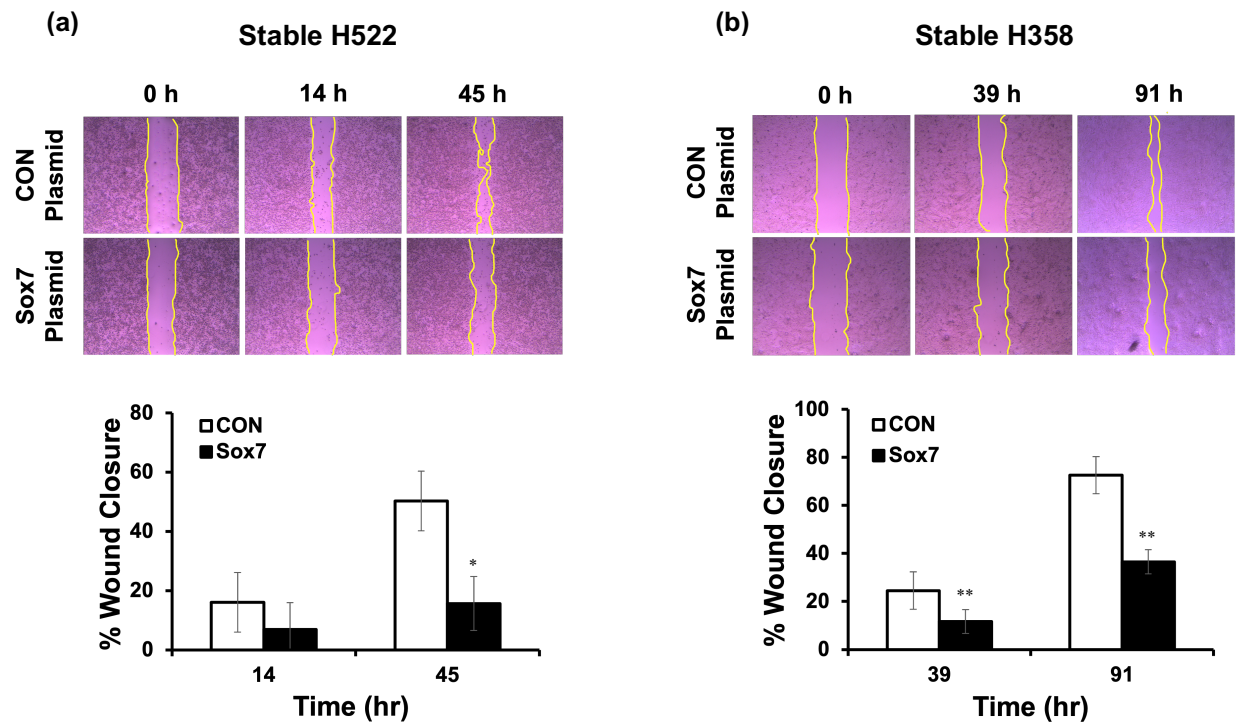

Figure 10 Stable Sox7 overexpression suppresses cell migration in H522 and in H358. (a) Stable H522, and (b) stable H358 cells were seeded into 24-well plates. LUAD cells were incubated until reaching 95% - 100% confluency in monolayers. Cross scratching with a 1000- µL pipette tip was applied to each well. Images were taken at 3 different time points post scratching (*P < 0.05 versus control; **P < 0.01 versus control).

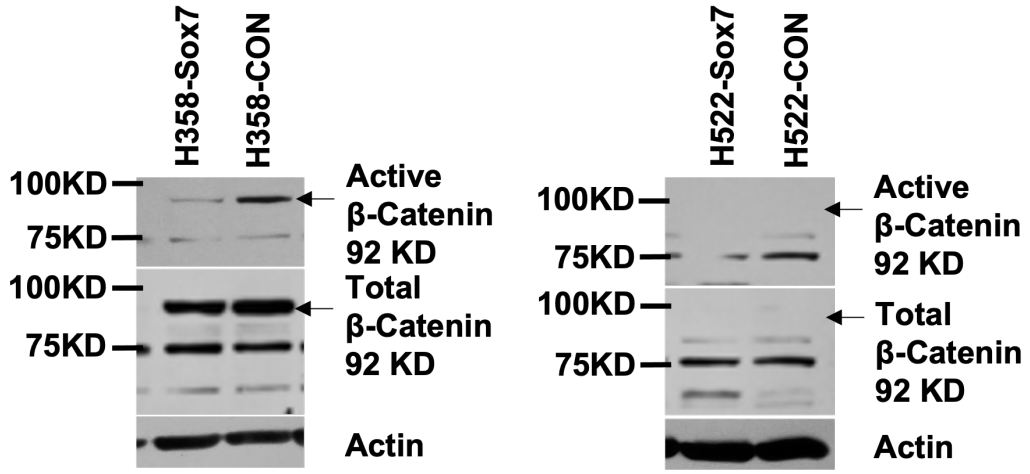

Figure 11 H358 cells with stable Sox7 overexpression shows lowered ABC production. H522 cells with stable Sox7 overexpression shows no total β-Catenin or ABC production. H358 and H522 cells were seeded at 60-70% confluency in 6-well plates. They were transfected with pCMV-Tag2B/Sox7 or pCMV-Tag2B plasmids. 48 h post transfection, cells were selected for 3 to 4 weeks. Post establishing stable cell lines, cells were collected and lysed. Prepared lysate samples were run on western blots and probed with Sox7, total β-Catenin, ABC, and actin antibodies.

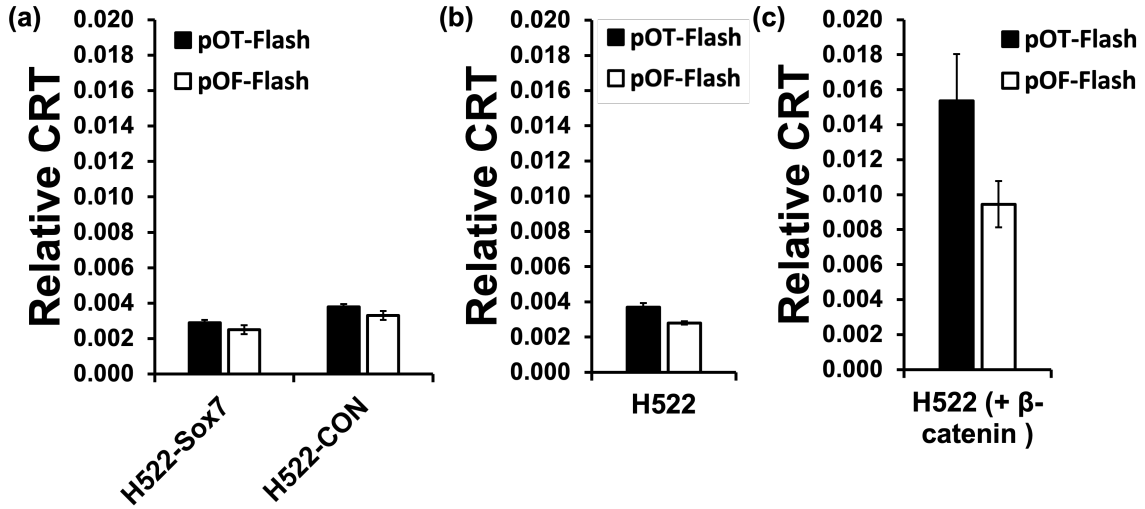

Figure 12 Stable and parental H522 cells show low β-catenin/T-cell factor – regulated transcription (CRT); upon β-catenin introduction, H522 cells show significantly elevated CRT. H522 parental and stable cells were seeded at 0.7 x 10^5 or 10^6 cells per well in 24 well plates 18-24 h before transfection. For CRT assay, each well was transfected with 100 ng of pOT-Flash, which encoded T-cell factor/lymphoid enhancer factor – responsive reporter, or 100 ng of pOF-Flash, which acts as a negative control with a mutated T-cell factor/lymphoid enhancer factor binding site. Reactions were carried out in triplicates and error bars represent one standard deviation. The relative CRT levels were calculated via *Renilla* luminescence/ *Firefly* luminescence, which allows normalization to *Renilla* luciferase internal control. (a) Both H522-Sox7 and H522-CON cells show low CRT activity (CRT < 0.004). (b) Parental H522 cells show low CRT activity (CRT < 0.004). (c) Post introduction of 100 ng of β-catenin plasmid, H522 cells show elevated CRT activity (CRT with pOT-Flash: 0.015362, CRT with pOF-Flash: 0.009449).

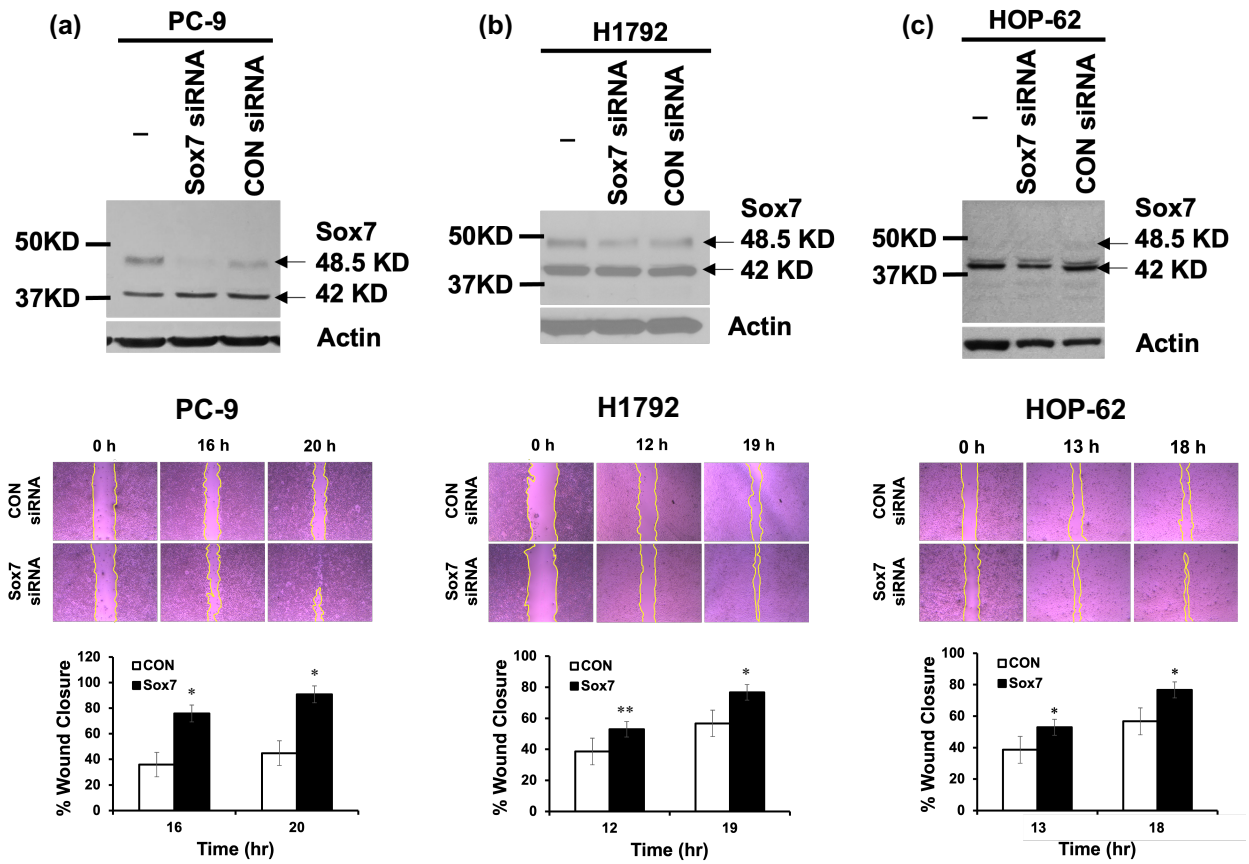

Figure 13 Sox7 siRNA knockdown enhances cell migratory ability. (a) PC-9, (b) H1792, and (c) HOP-62 cells were seeded at 60%-70% confluency in 24-well plates 18-24 h prior to transfection. Sox7 siRNA and control siRNA were transiently transfected into these cells. 6 h post transfection, transfection medium mix was replaced with complete medium. All 3 LUAD cells were incubated until reaching 95% - 100% confluency in monolayers. Cross scratching with a 1000- µL pipette tip was applied to each well. Images were taken at 3 different time points post scratching (*P < 0.05 versus control; **P < 0.01 versus control).

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