SLUG is required for SOX9 stabilization and functions to promote cancer stem cells and metastasis in human lung carcinoma

Sudjit Luanpitpong[1,2,3,*], Jingting Li[4], Amruta Manke[2], Kathleen Brundage[5], Emily Ellis[6], Sarah L. Mclaughlin[6], Paweorn Angsutararux[1], Nawin Chantra[1], Maria Voronkova[3], Yi Charlie Chen[7], Liying Wang[8], Pithi Chanvorachote[9], Ming Pei[4], Surapol Issaragrisil[1], and Yon Rojanasakul[2,3,*]

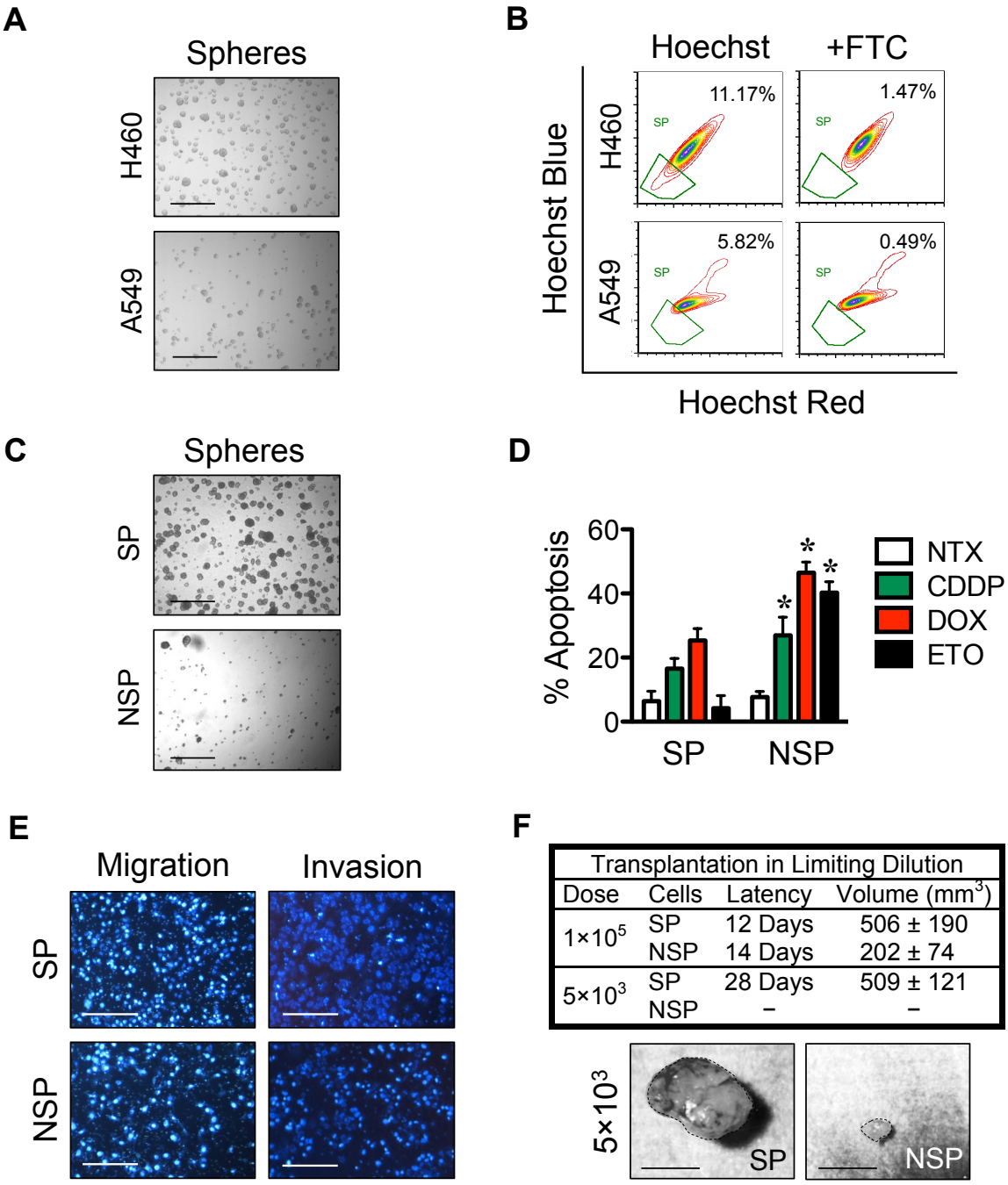
[1]Siriraj Center of Excellence for Stem Cell Research, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand; [2]Pharmaceutical and Pharmacological Sciences Program, [3]Mary Babb Randolph Cancer Center, [4]Stem Cell and Tissue Engineering Laboratory, [5]Flow Cytometry Core Facility, [6]Animal Models and Imaging Facility, West Virginia University, Morgantown, WV 26506, USA; [7]Natural Science Division, Alderson Broaddus University, Philippi, WV 26416, USA, [8]Allergy and Clinical Immunology Branch, National Institute for Occupational Safety and Health, Morgantown, WV 26505, USA; [8]Cell-Based Drug and Health Product Development Research Unit, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand.

Running title: SLUG regulates SOX9 stability in lung carcinoma

***Correspondence:** Sudjit Luanpitpong, Siriraj Center of Excellence for Stem Cell Research, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. Phone: +662 419 2907. Email: suidjit@gmail.com; Yon Rojanasakul, Mary Babb Randolph Cancer Center, West Virginia University, WV 26506, USA. Phone: +1 304 293 1476. Email: yrojan@hsc.wvu.edu

SUPPLEMENTARY INFORMATION

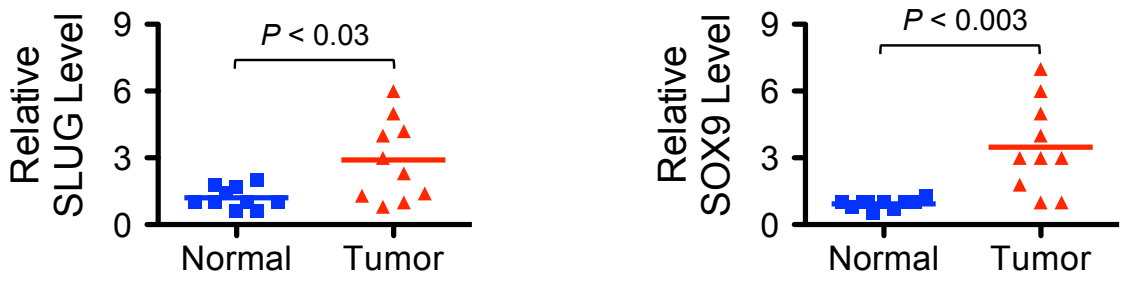
Supplementary Information includes Supplementary Figure S1−S4 and Supplementary Table S1.



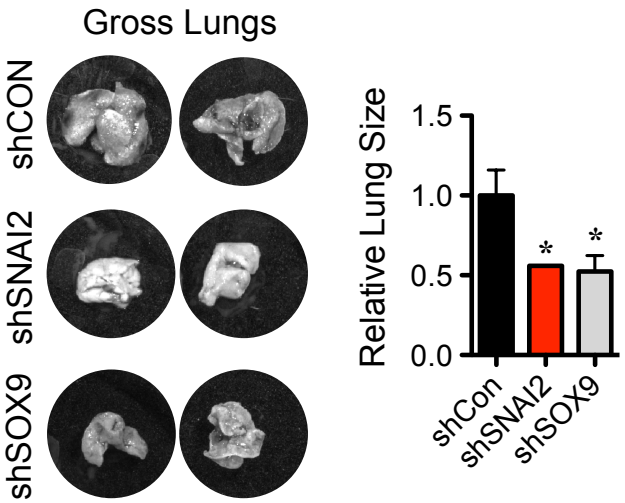
Transplantation in Limiting Dilution			
Dose	Cells	Latency	Volume (mm^3)
1×10^5	SP	12 Days	506 ± 190
	NSP	14 Days	202 ± 74
5×10^3	SP	28 Days	509 ± 121
	NSP	–	–

Supplementary Figure S1. Lung CSCs display aggressive cancer phenotypes. (**A**) Human NSCLC H460 and A549 cells were analyzed for tumor sphere formation. CSCs were isolated from NSCLC H460 cells based on SP phenotype under CSC-selective conditions. (**B**)

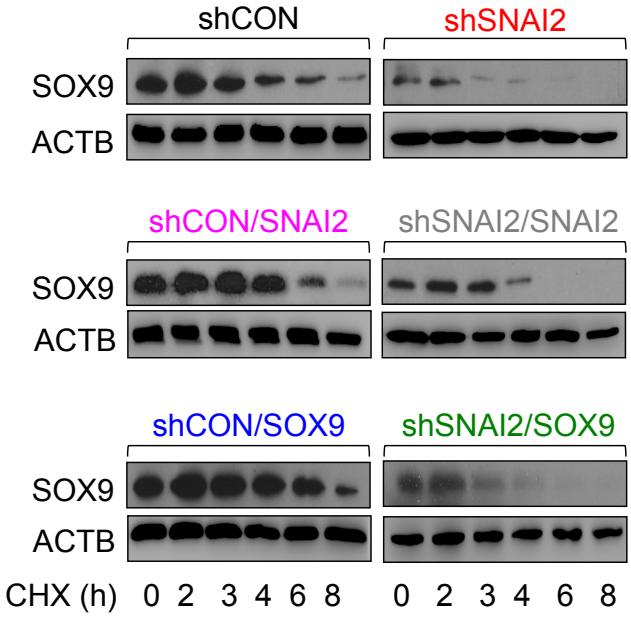
Analysis of side population (SP) in H460 and A549 cells in the presence or absence of fumitremorgin C (FTC) using FACS. SP cells (*box)* were determined by their disappearance in the presence of FTC and were shown as percentage of the pool population. (**C-F**) CSCs and their non-CSC counterpart were isolated from H460 cells based on SP phenotype using FACS and designated as SP and NSP cells, respectively. Analysis of (**C**) tumor sphere formation, (**D**) apoptosis after exposure to various chemotherapeutic drugs, including cisplatin (CDDP; 100 μM), doxorubicin (DOX; 1 μM) and etoposide (ETO; 500 nM) and (**E**) cell migration and invasion in SP and NSP cells. (**F**) Tumorigenicity of SP and NSP cells when subcutaneously injected into NSG mice in limiting dilution (5×10^3–1×10^5 cells/flank).



Supplementary Figure S2. SLUG and SOX9 protein expression in human lung cancer and matched normal lung tissues were analyzed by Western blotting and immunoblot signals were quantified by densitometry and normalized to β-actin and the first band of each blot. SLUG and SOX9 levels of normal and tumor tissues were compared.



Supplementary Figure S3. Inhibition of SLUG and SOX9 suppresses experimental lung cancer metastasis *in vivo*. LUC2-labeled shSNAI2, shSOX9 or shCON H460 cells were injected into NSG mice via tail vein at the dose of 1×10^6 cells/mouse. Gross appearance of the lungs of mice bearing shSNAI2, shSOX9 or shCON H460 cells. Smaller lungs were observed in mice bearing shSNAI2 and shSOX9 cells when compared with shCON cells. Data are mean ± S.D (n = 3). *$P < 0.05$ *vs*. shCON cells; two-sided Student's *t*-test.



Supplementary Figure S4. Cycloheximide-chase assay. Various clones of human lung carcinoma H460 cells, including shCON, shSNAI2, shCON/SNAI2, shSNAI2/SNAI2,

shCON/SOX9 and shSNAI2/SOX9 cells were treated with protein translation inhibitor cycloheximide (CHX; 10 μg/mL) for various times, and SOX9 level was analyzed by Western blotting. Representative immunoblots of SOX9 and loading control β-actin (ACTB) are shown here.

Supplementary Table S1. Primer Sequences for ChIP assay.

Gene	Region	Primer	Sequence 5' to 3'	Length (bp)
Human *GAPDH*	Promoter	Forward	CTCAAGACCTTGGGCTGGG	132
		Reverse	TCGAACAGGAGGAGCAGAGA	
Human *SOX9*	Promoter	Forward	ACCCTACCGTCCGCCCTTTG	171
		Reverse	CCGCCTCACCTTAGAGCCAC	