

LKB1 inhibits lung cancer progression through lysyl oxidase and extracellular matrix remodeling

Yijun Gao^{a,1}, Qian Xiao^{b,1}, HuiMin Ma^{b,1}, Li Li^a, Jun Liu^b, Yan Feng^a, Zhaoyuan Fang^a, Jing Wu^b, Xiangkun Han^a, Junhua Zhang^c, Yihua Sun^c, Gongwei Wu^a, Robert Padera^d, Haiquan Chen^c, Kwok-kin Wong^e, Gaoxiang Ge^{b,2,3}, and Hongbin Ji^{a,2,3}

^aLaboratory of Molecular Cell Biology and ^bState Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China; ^cDepartment of Thoracic Surgery, Shanghai Cancer Center and Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China; ^dDepartment of Pathology, Brigham and Women's Hospital, Boston, MA 02115; and ^eDepartment of Medical Oncology, The Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115

Edited by Bruce E. Johnson, The Dana-Farber Cancer Institute, Boston, MA, and accepted by the Editorial Board September 27, 2010 (received for review April 13, 2010)

LKB1 loss-of-function mutations, observed in ~30% of human lung adenocarcinomas, contribute significantly to lung cancer malignancy progression. We show that lysyl oxidase (LOX), negatively regulated by LKB1 through mTOR-HIF-1 α signaling axis, mediates lung cancer progression. Inhibition of LOX activity dramatically alleviates lung cancer malignancy progression. Up-regulated LOX expression triggers excess collagen deposition in *Lkb1*-deficient lung tumors, and thereafter results in enhanced cancer cell proliferation and invasiveness through activation of β 1 integrin signaling. High LOX level and activity correlate with poor prognosis and metastasis. Our findings provide evidence of how LKB1 loss of function promotes lung cancer malignancy through remodeling of extracellular matrix microenvironment, and identify LOX as a potential target for disease treatment in lung cancer patients.

The high mortality of lung cancer (1) is largely attributable to failure of early diagnosis and metastasis frequently observed at the time of diagnosis. Tumor suppressor loss of function is widely adopted in tumor initiation and progression. The roles of LKB1 as a tumor suppressor have emerged from the observation of increased risk of malignancy in gastrointestinal tract in Peutz-Jeghers syndrome (PJS) patients harboring germ-line *LKB1* mutations (2, 3). Although rare in most types of human cancers (4, 5), *LKB1* loss-of-function somatic mutations are frequently observed in human non-small-cell lung cancer (NSCLC) (6–10). Mice with oncogenic *Kras*^{G12D} mutant develop lung tumors with long latency and low aggressiveness. Concomitant loss of *Lkb1* significantly shortened the latency, increased tumor burden, and promoted lung cancer invasion and distant metastasis, comparable to that from *p53* loss (6). Yet, the molecular mechanisms involved remain largely unknown.

Cancer progression is a reciprocal process involving intimate interaction between tumor cells and tumor stroma, including extracellular matrix (ECM). ECM alteration and remodeling is one of the most frequently observed and most important events during malignancy progression, which subsequently modulates cell-matrix and cell-cell interaction and results in altered cell behavior (11). Increasing interests and efforts have been paid to those enzymes involved in ECM remodeling, among which lysyl oxidase (LOX) is of particular interest. LOX oxidizes lysine residues in collagen and elastin, resulting in covalent cross-linking and stabilization of these ECM structural components (12). Aberrant LOX expression or enzymatic activity has been linked to a variety of pathological conditions, including breast cancer and lung cancer (13–16). LOX is associated with hypoxia in human breast cancer and head and neck tumors, and is responsible for hypoxia-induced tumor metastasis (13). Although studies have implicated deregulated LOX mRNA and protein levels in lung adenocarcinomas (17, 18), the roles and molecular mechanisms of LOX involved in lung cancer progression are poorly understood.

Here we identify LOX as a target negatively regulated by LKB1 in both de novo murine lung tumors and human NSCLC

cell lines. We further provide evidence that LOX potentiates lung cancer progression elicited by LKB1 deficiency via ECM remodeling, and may serve as a potential therapeutic target for lung cancer therapy.

Results

LOX Expression Level Correlates with LKB1 Status in Lung Cancer. We have previously shown that loss of function of *Lkb1* promoted lung cancer progression and metastasis in mouse model driven by *Kras*^{G12D} mutant (6). Integrative bioinformatic analysis on microarray datasets derived from lung tumors in *Kras* and *Kras/Lkb1*^{L/L} mice and from human NSCLC cell lines with or without *LKB1* (6) has identified a series of differentially expressed genes, among which *LOX*, previously shown to be involved in breast cancer metastasis (13), drew much of our attention. Significantly increased *Lox* expression (~15-fold assessed by quantitative RT-PCR) was observed in murine lung tumors with *Lkb1* deficiency (Fig. 1A), but not in those with *p53* deficiency (SI Appendix, Fig. S1). This is further confirmed by immunofluorescence staining on lung tumor sections (Fig. 1B). Consistently, increased LOX activities were observed in sera from mice with *Lkb1*-deficient lung tumors (Fig. 1C). Study of a panel of human NSCLC cell lines revealed that high LOX expression levels were evident in all three lines with mutant *LKB1* but not in majority lines with wild-type *LKB1* (SI Appendix, Fig. S2). To examine the clinical relevance of LOX levels to lung cancer progression, LOX activities in sera from a cohort of 80 lung adenocarcinoma patients were measured. LOX activity correlated significantly with clinical stages and metastasis status, but not with sex or smoking status (Table 1). Reevaluation of published microarray dataset of a cohort of 111 lung cancer patients (19) revealed significant association of high LOX expression with shorter overall survival in lung adenocarcinoma patients (19 vs. 49 mo median survival, $P = 0.0009$; Fig. 1D).

LKB1 Negatively Regulates LOX Transcription Through mTOR-HIF-1 α Signaling Axis. We next sought to investigate whether up-regulation of *LOX* is the direct effect of *LKB1* deficiency. Ectopic expression of LKB1 in human NSCLC cell lines A549 dramatically decreased *LOX* mRNA levels, protein levels, and enzymatic activities

Author contributions: Y.G., Q.X., H.M., G.G., and H.J. designed research; Y.G., Q.X., H.M., L.L., J.L., Y.F., J.W., X.H., J.Z., Y.S., and G.W. performed research; Y.G., Q.X., H.M., Z.F., R.P., H.C., K.-K.W., G.G., and H.J. analyzed data; and Y.G., G.G., and H.J. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. B.E.J. is a guest editor invited by the Editorial Board.

¹Y.G., Q.X., and H.M. contributed equally to this work.

²G.G. and H.J. contributed equally to this work.

³To whom correspondence may be addressed. E-mail: hbji@sibs.ac.cn or gxge@sibs.ac.cn.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1004952107/-DCSupplemental.

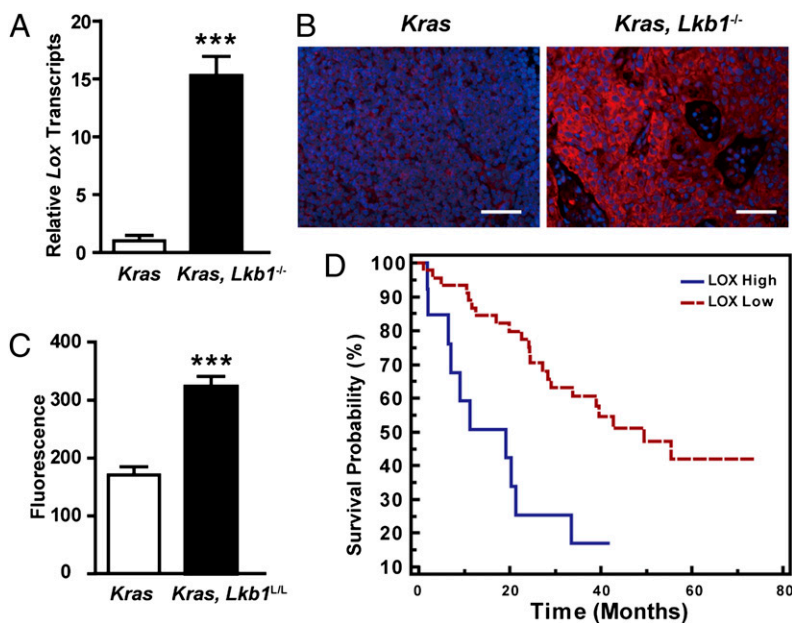


Fig. 1. LKB1 down-regulates LOX in lung cancer. (A) Real-time PCR quantification of *Lox* mRNA levels in *Kras* and *Kras/Lkb1^{-/-}* lung tumors (three in each group). (B) LOX immunofluorescent staining on *Kras* and *Kras/Lkb1^{-/-}* lung tumor sections. (Scale bars: 100 μ m.) (C) LOX enzymatic activity assay on serum samples from mice with *Kras* ($n = 6$) or mice with *Kras/Lkb1^{-/-}* lung tumors ($n = 7$). (D) Kaplan–Meyer plots show that lung cancer patients with high LOX expression levels had statistically significant shorter survival ($P = 0.0009$) than patients with low LOX expression levels. Data were presented as means \pm SEM. Statistical analyses were performed using Student's *t* test. *** $P < 0.001$.

(Fig. 2A). This regulation was also seen in LKB1 mutant cell line CRL-5800 and wild-type LKB1 cell line CRL-5807 (SI Appendix, Fig. S3B and D). *LKB1* knockdown in HTB-182 cells, a human NSCLC cell line with wild-type *LKB1*, resulted in a significant increase of *LOX* mRNA, protein levels, and enzymatic activities (SI Appendix, Fig. S4A and B).

The altered *LOX* mRNA levels strongly indicate transcriptional level regulation. Consistently, ectopic expression of LKB1 dramatically decreased *LOX* promoter activity in multiple NSCLC cell lines, including A549, CRL-5800, and CRL-5807 (SI Appendix, Figs. S3A and C and S5A). Hypoxia-inducible factor 1 α (HIF-1 α) was reported to regulate *LOX* expression in breast cancer via the conserved hypoxia-responsive elements (HREs) in *LOX* promoter (13). Ectopic LKB1 expression in A549 cells significantly decreased HIF-1 α protein level (Fig. 2B), as well as its transcriptional activity (SI Appendix, Fig. S5B). Conversely, knockdown of LKB1 increased HIF-1 α protein level in HTB-182 cells (SI Appendix, Fig. S4A). Overexpression of HIF-1 α or HIF-1 α -PA mutant, a stable form of HIF-1 α , increased *LOX* promoter activity, transcription (SI Appendix, Fig. S5C and D), and LOX

protein levels in A549 cells (Fig. 2C). More importantly, reintroduction of HIF-1 α could rescue the inhibitory effect of LKB1 on *LOX* promoter activity (Fig. 2D), mRNA level (SI Appendix, Fig. S5E), protein level (Fig. 2E), and enzymatic activity (SI Appendix, Fig. S5F). Similar results were seen in other NSCLC cell lines, including CRL-5800 and CRL-5807 (SI Appendix, Fig. S3). HIF-1 α knockdown significantly decreased *LOX* expression (Fig. 2F and SI Appendix, Fig. S5G and H). As a multifunctional protein kinase, LKB1 is involved in multiple signaling pathways, among which mTOR pathway has been shown to regulate HIF-1 α (20). Inhibition of mTOR activation using either rapamycin or PP242 significantly decreased *LOX* promoter activity and LOX mRNA and protein levels in A549 cells (Fig. 2G and H and SI Appendix, Fig. S6A–C). Consistent with a previous report (21), mTOR inhibition significantly decreased HIF-1 α transcriptional activity through down-regulation of HIF-1 α protein level (Fig. 2H and SI Appendix, Fig. S6C and D). Ectopic expression of HIF-1 α rescued the inhibition of LOX enzymatic activity by rapamycin treatment in A549 cells (SI Appendix, Fig. S6E), indicating that HIF-1 α mediates the regulation of LOX transcription downstream of mTOR. Therefore, LKB1 regulates *LOX* transcription directly through mTOR-HIF-1 α signaling axis.

Table 1. LOX serum activity correlates with human lung adenocarcinoma progression and metastasis

Characteristic	No.	LOX activity (mean \pm SD)	<i>P</i> value
Clinical stage			
I/II	36	1365.18 \pm 1022.10	
III/IV	44	2322.54 \pm 2441.68	0.022*
Metastasis			
Nonmetastatic	33	1332.24 \pm 1075.75	
Metastatic	47	2284.55 \pm 2361.16	0.018*
Gender			
Male	40	1633.64 \pm 1165.18	
Female	40	2149.80 \pm 2545.95	0.249
Smoking status			
Nonsmoker	52	2038.49 \pm 2282.30	
Smoker	28	1619.17 \pm 1250.39	0.371

Nonmetastatic, no metastasis; metastatic, lymph node metastasis or distant metastasis.

*Denotes significant difference.

LOX Mediates Lung Cancer Cell Anchorage-Independent Growth and Migration. LKB1 is a multitask tumor suppressor involved in regulation of cell proliferation and cell migration. We then asked whether LOX contributes to altered cell migration and/or cell proliferation in *LKB1*-deficient lung cancer cells. Consistent with previous reports (6), ectopic LKB1 expression significantly reduced lung cancer cell proliferation, anchorage-independent growth, and cell migration (Fig. 3A and B and SI Appendix, Fig. S7A–E). LOX overexpression had no effect on cell proliferation, nor could it rescue the inhibition on cell growth by LKB1 in A549 and CRL-5807 cells (SI Appendix, Fig. S7C and E). Consistently, neither LOX knockdown in A549 or CRL-5844 cells nor inhibition of LOX enzymatic activity via β -aminopropionitrile (BAPN) treatment in A549 cells had significant effect on cell proliferation (SI Appendix, Fig. S7F–K), suggesting molecules other than LOX may mediate the antiproliferation effect of LKB1. However, reintroduction of LOX into A549 and CRL-5807 cells ectopically expressing LKB1 could partially rescue the inhibitory effects of LKB1 on anchorage-independent cell growth and cell migration

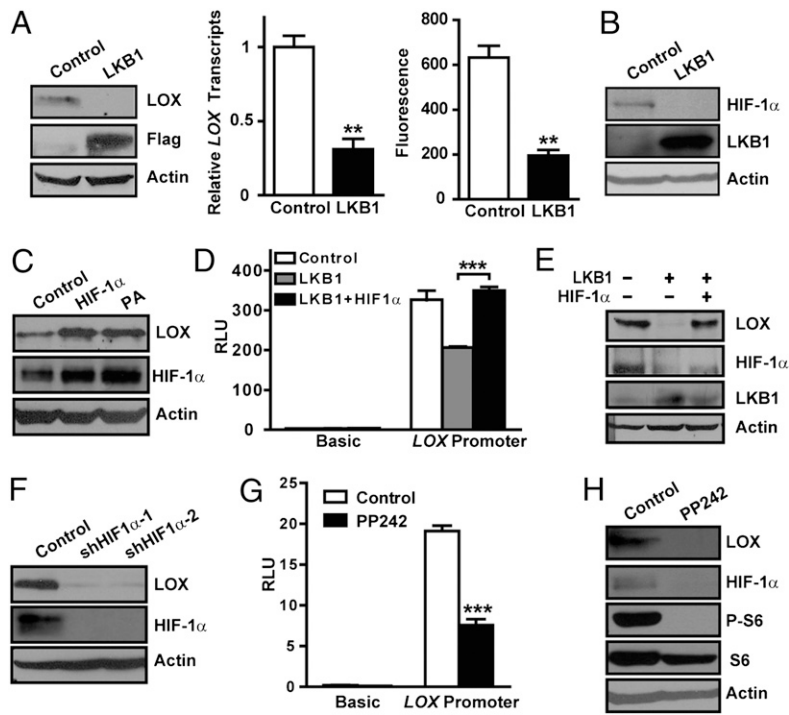


Fig. 2. HIF-1 α mediates LOX transcription downstream of LKB1. (A) Down-regulated LOX mRNA, protein levels, and activities in A549 cells reconstituted with LKB1. Anti-Flag is used for detection of Flag-LKB1. (B) Ectopic LKB1 expression down-regulated HIF-1 α protein level in A549 cells. (C) Expression of either HIF-1 α or PA mutant, a stable form of HIF-1 α , up-regulated LOX levels in A549 cells. (D and E) Reintroduction of HIF-1 α rescued the inhibition of LOX promoter activity (D) and down-regulation of LOX protein levels (E) by LKB1 in A549 cells. (F) Knockdown of HIF-1 α decreased LOX protein levels in A549 cells with 200 μ M CoCl₂. (G and H) mTOR inhibitor PP242 treatment significantly decreased LOX promoter activity (G) and protein levels (H) in A549 cells. Data are presented as mean \pm SEM. Statistical analyses were performed using Student's *t* test. ***P* < 0.01, ****P* < 0.001.

(Fig. 3 A and B and *SI Appendix*, Fig. S8 A and B). The oxidase activity of LOX is necessary as LOX mutated in Lys320 or Tyr355, residues critical for LOX enzymatic activity, failed to rescue the inhibition of anchorage-independent cell growth and cell migration by LKB1 (Fig. 3 A and B). Conversely, LOX knockdown in A549 and CRL-5844 cells resulted in a significant decrease of anchorage-independent cell growth and cell migration abilities (Fig. 3 C and D and *SI Appendix*, Fig. S8 C and D).

LOX Inhibition Alleviates Lung Cancer Malignancy Triggered by LKB1 Deficiency. To test if LOX is essential for lung carcinogenesis provoked by *LKB1* deficiency in vivo, BAPN, a widely used LOX pharmacological inhibitor, was administrated to previously established lung cancer mouse model with or without *Lkb1* deficiency (6). BAPN treatment resulted in efficient inhibition of LOX activity (*SI Appendix*, Fig. S9A). BAPN treatment for 4 wk significantly decreased both tumor number and tumor volume in *Lkb1*-deficient

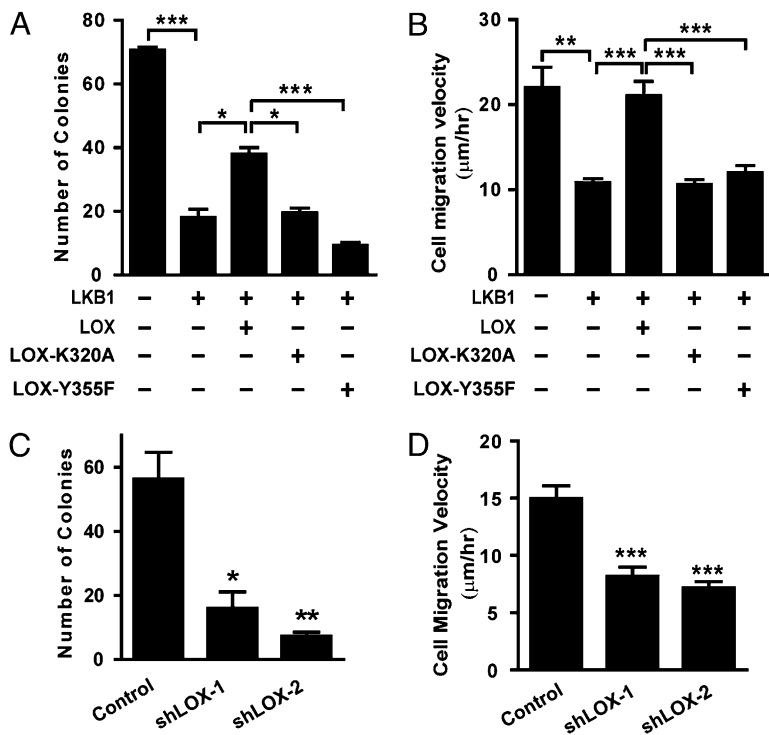


Fig. 3. LOX mediates lung cancer cell anchorage-independent growth and migration. (A and B) Ectopic expression of wild-type, but not enzymatically inactive LOX in A549 cell rescued the inhibitory effect of LKB1 on anchorage-independent cell growth (A) and cell migration (B). (C and D) Knockdown of LOX in A549 cells significantly impaired anchorage-independent cell growth (C) and cell migration (D). Data are presented as mean \pm SEM. Statistical analyses were performed using Student's *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

mice model revealed by histological inspection, whereas it had no obvious effect on the progression of tumors with wild-type *Lkb1* (Fig. 4A and B and *SI Appendix*, Fig. S10A and B). Interestingly, BAPN treatment dramatically reduced the number of large LKB1-deficient tumors ($>0.5 \text{ mm}^2$) but had no much effect on the number of those small tumors (Fig. 4C), indicating that LOX inhibition mainly impairs tumor progression. LOX activity inhibition resulted in much less cell proliferation, indicated by Ki67 positive staining, and enhanced tumor cell apoptosis, indicated by cleaved Caspase-3 staining (Fig. 4D and *SI Appendix*, Fig. S9). The roles of LOX in lung cancer metastasis were further confirmed by i.v. injection of A549 cells into nude mice. Knockdown of LOX in A549 cells resulted in significantly less tumors and smaller tumors in the mouse lung (*SI Appendix*, Fig. S9C and D).

Excess Collagen Deposition Increases Lung Cancer Cell Proliferation and Invasiveness Through Activation of $\beta 1$ Integrin Signaling. LOX functions as a key enzyme in collagen cross-linking, and therefore facilitates collagen deposition (12). We hypothesized that increased LOX activity in LKB1-deficient lung tumors may result in altered composition and architecture of collagen matrix and thus contribute to cancer progression. As shown by Sirius red staining, active ECM remodeling featured with collagen-rich fibrotic loci is evident in *Lkb1*-deficient tumors but not in those with wild-type *Lkb1* (*SI Appendix*, Fig. S11A). BAPN treatment greatly diminished fibrotic loci in *Lkb1*-deficient lung tumors (*SI Appendix*, Fig. S11B), indicating LOX activity is responsible for the dense collagen deposition in *Lkb1*-deficient lung tumors. We next asked if the altered extracellular matrix, featured with excessive collagen deposition in LKB1-deficient lung tumors, facilitates cancer cell malignant transformation. Previous studies suggested that increased stiffness of the extracellular matrix promoted breast epithelial cell transformation (22). Similar to that observed in breast cancer cells, increased collagen concentration resulted in changed cell morphology and enhanced invasion ability of A549 cells in 3D matrigel culture in a collagen concentration-dependent manner (*SI Appendix*, Fig. S11C), underscoring fundamental roles of ECM remodeling with tensional change in cancer cell transformation and invasion ability. Immunocytochemistry analyses revealed disrupted cell polarity and increased cell proliferation within collagen-rich environment (Fig. 5A). $\beta 1$ integrin is the major cell-surface collagen receptor. $\beta 1$ integrin blocking antibody significantly attenuated A549 cell invasiveness in collagen-

rich matrigel and reversed the disruption of cell polarity (Fig. 5A). Percentage of proliferating cells in collagen-rich matrigel was also decreased after $\beta 1$ integrin antibody treatment (Fig. 5A). Binding of $\beta 1$ integrin to collagen ligand activates intracellular signaling, including phosphorylation and activation of focal adhesion kinase (FAK) and Src. Knockdown of FAK impaired the aggressive cell behavior in collagen-rich matrigel, in a manner similar to that in $\beta 1$ integrin blocking antibody treatment (Fig. 5B). Taken together, these data support a fundamental role of excess collagen deposition in lung cancer progression in response to elevated LOX activity through activation of $\beta 1$ integrin signaling.

Discussion

The high mortality of lung cancer is mainly attributable to the poor understanding of lung cancer progression and metastasis. LKB1 loss-of-function mutations, which contribute to $\sim 30\%$ of NSCLCs, warrant studies to explore downstream targets for development of effective cancer treatment strategies.

Here we identify LOX as an essential mediator of LKB1-deficiency-elicited lung cancer progression through ECM alteration, especially collagen matrix remodeling. LOX activity inhibition significantly alleviates LKB1-deficient lung cancer malignancy and invasion, underscoring the essential roles of LOX in cancer progression in lung tumors with LKB1 loss-of-function mutation. LKB1 loss triggers up-regulation of LOX expression through mTOR-HIF-1 α signaling axis. Hypoxia and/or up-regulation of hypoxia-inducible factors are frequently observed in many types of cancers. Gene transcription mediated by HIF-1 α promotes characteristic tumor behavior, including angiogenesis, invasion, metastasis, dedifferentiation, and enhanced glycolytic metabolism (23). VEGF, the well-known downstream target of HIF-1 α , was also proposed to be involved in LKB1-deficient tumor progression. Sunitinib inhibition of VEGFR kinase activity resulted in a prolonged survival in *Kras/Lkb1^{L/L}* lung cancer mice via suppression of primary tumor growth without much impact on the malignancy progression (24). Thus, LOX and VEGF, two downstream targets of HIF-1 α , may function synergistically in LKB1-deficient tumor progression.

Several recent studies have highlighted LOX as an important promoter of tumor malignancy. Erler et al. (13, 25) have shown that LOX is essential for hypoxia-induced tumor metastasis in human breast cancer, and further provide evidence that LOX facilitates the metastatic process via type-IV collagen cross-linking in the basement membrane and formation of “premetastatic

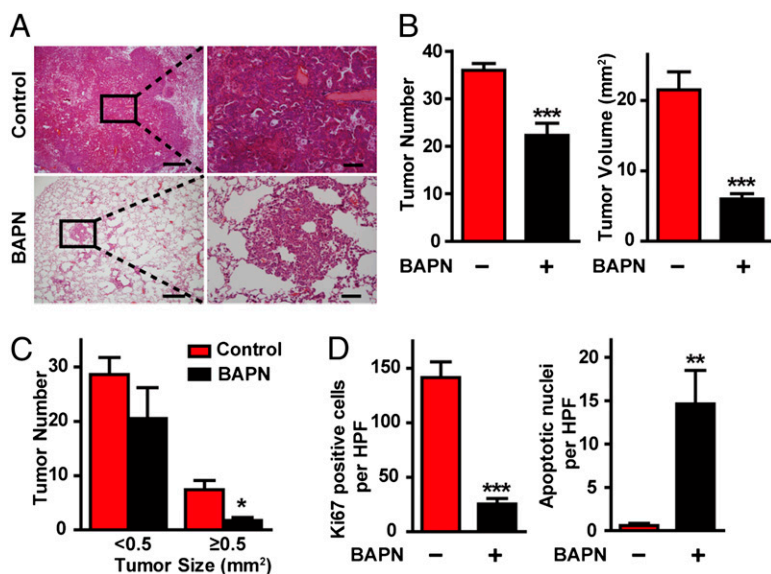


Fig. 4. LOX inhibitor BAPN significantly alleviates lung tumor progression in de novo murine lung cancer with *Lkb1* deficiency. (A) BAPN treatment significantly decreased the size of murine lung tumors. (Scale bars: *Left*, 500 μm ; *Right*, 100 μm .) (B and C) Quantification of tumor volume and numbers in H&E-stained lung sections from *Kras/Lkb1^{L/L}* mice treated with BAPN or saline (eight mice in each group). (D) Quantitative proliferative and apoptotic indices in lung tumors from control and BAPN treatment group determined from more than 200 high-power fields (HPF). Data are presented as mean \pm SEM. Statistical analyses were performed using Student's *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

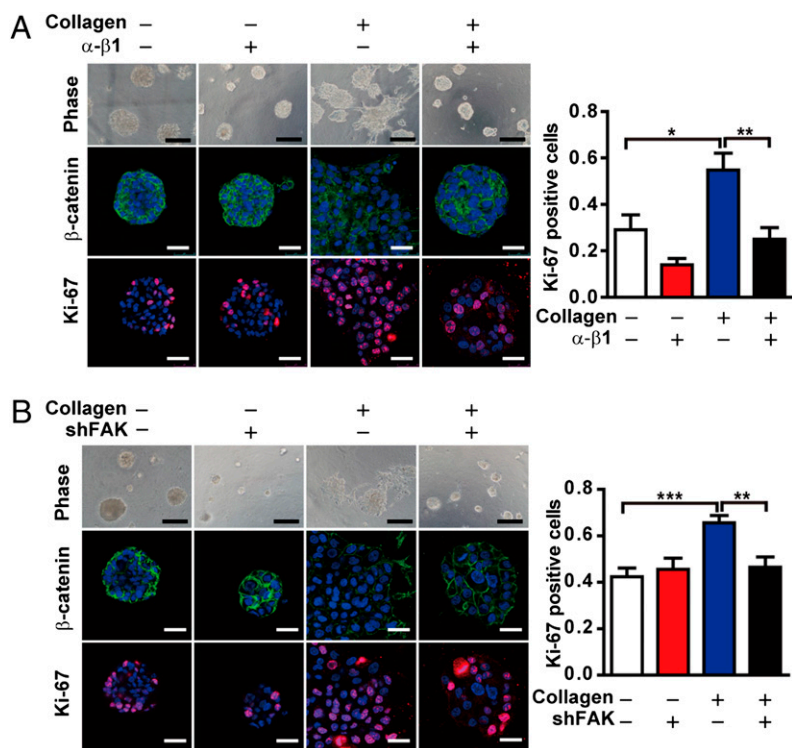


Fig. 5. Excess collagen deposition increases lung cancer cell proliferation and invasiveness through activation of β 1 integrin. (A) β 1 integrin antibody (20 μ g/mL) blocked collagen-dependent cell morphology change and cell proliferation in 3D culture. (B) Knockdown of FAK retains A549 cell morphology and proliferation in collagen-rich environment. (Scale bars: Top, 200 μ m; Middle and Bottom, 25 μ m.) (Right) Quantification of percentage of proliferating cells. Experiments were performed in duplicates and repeated three times.

niche.” Our data in lung cancer mouse model clearly show that the main function of LOX is to promote advanced-stage lung cancer progression and metastasis without much impact on initial neoplastic transformation. It is noteworthy that LOX mediates cell migration provoked by LKB1 loss with little impact on cell proliferation in 2D cell culture. However, lung cancer cells are more proliferative in collagen-rich matrigel, compared with those cultured in the absence of exogenously added collagen. LOX activity inhibition also affected lung tumor growth and progression in de novo animal model. The discrepancy could be due to the difference between 2D and 3D cell culture systems, and LOX may alter cancer cell growth potential via ECM remodeling.

Despite that breakdown of surrounding matrix is believed to be prerequisite for tumor metastasis, histopathological analyses had clearly shown correlation of poor outcome in patients with fibrotic lesions in a variety of cancers, including lung cancer, underscoring the essential roles of ECM remodeling during tumor progression and metastasis. The matrix stiffness, as well as the ECM composition and architecture, play fundamental roles in cell fate determination. Normal breast epithelial cells in stiff 3D microenvironment share characteristics with transformed breast cancer cells in disrupted cell adherent junction, enhanced cell proliferation, failure in lumen formation, and aberrant activation of intracellular signaling pathways and cytoskeleton rearrangement (22). Our data show that lung cancer triggered by LKB1 loss is frequently accompanied with fibrotic foci and significant ECM remodeling. The dense collagen matrix microenvironment provokes the increase of cancer cell invasion ability through activation of Src and FAK downstream of β 1 integrin signaling, whereas β 1 integrin blocking antibody and depletion of FAK significantly decreased cancer cell proliferation and invasiveness in a collagen-rich environment. The fundamental role of ECM remodeling is pivotal for lung cancer associated with LOX cross-linking of collagen and is of great importance for novel therapeutic strategies. The recent work by Weaver and coworkers (26) has also highlighted the importance of ECM remodeling in a broad range of cancer progression.

LOX was reported to be deregulated in multiple cancers, including breast cancer, head and neck cancer, and lung cancer (16). Our data show that high LOX expression or serum activity significantly correlates with lung cancer poor prognosis, and cancer stages and metastasis, respectively. A previous study found no correlation between *LKB1* loss-of-function mutations and clinical outcome in stage I and II NSCLC patients (9). Although a larger-scale analysis of *LKB1* mutation and/or expression is necessary to reach a conclusive point, it is conceivable that signaling components, e.g., mTOR and/or HIF-1 α , which regulate LOX gene expression downstream of *LKB1*, may be commonly hyperactivated at late stages of lung cancer in a *LKB1*-dependent or -independent manner. From this aspect, our finding of increased LOX serum activity in advanced stages of lung cancer patients may provide an even broader application for lung cancer prognosis.

In conclusion, we provide strong evidence that LOX is an important downstream mediator of lung cancer progression and metastasis provoked by *LKB1* deficiency. The identification of LOX links molecular pathways that control the progression and metastasis in lung cancers with *LKB1* mutations to stromal ECM remodeling through collagen cross-linking and β 1 integrin signaling activation. LOX is a potentially important therapeutic target for lung cancer treatment and a promising biomarker for lung cancer prognosis.

Materials and Methods

Additional information is provided in [SI Appendix](#).

Reporter Gene Assay. Luciferase activities were measured 48 h after transfection using the Dual-Luciferase Assay Kit (Promega) on a GloMax 20/20 Luminometer. pRL-SV40 or pEGFP-C1 was cotransfected as internal control. Experiments were performed in triplicate and repeated at least three times.

Mouse Treatment. *Kras*^{G12D}, *Lkb1*^{L/L}, and *P53*^{L/L} mice were originally generously provided by T. Jacks (Cambridge, MA) and R. Depinho (Boston), respectively. Lung cancer mice models with *Kras*, *Kras/Lkb1*^{L/L}, and *Kras/P53*^{L/L} mice were generated as described previously (6). All mice were housed in a pathogen-free environment at Shanghai Institute of Biochemistry and Cell

Biology and treated in strict accordance with protocols approved by the Institutional Animal Care and Use Committee of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Detailed information is provided in *SI Appendix*.

Human Serum Sample Analysis. The study was approved by the local ethic committees in Fudan University Shanghai Cancer Center, and sera were collected with the written consent of patients from July 2007 to March 2009. Additional details are provided in *SI Appendix*.

Histopathological Analysis and Immunological Studies. Histopathological analysis and immunological studies were performed as described (6, 27–29). Additional details are provided in *SI Appendix*.

LOX Enzymatic Activity Assay. Serum from mice, human lung cancer patients, and phenol-red free conditioned media (CM) from confluent cells were collected for LOX enzymatic activity assessment as described (30). See *SI Appendix* for detailed information.

Statistical Analysis. Data were analyzed by Student's *t* test; $P < 0.05$ was considered significant. Survival analysis on previously published microarray data (19) was performed using the Kaplan–Meier method. A *P* value < 0.05 was considered statistically significant.

ACKNOWLEDGMENTS. The authors acknowledge Drs. William Kaelin (Boston), Celeste Simon (Philadelphia), Caroline Damsky (San Francisco), Charles Shanley (Royal Oak, MI), Jianfeng Chen (Shanghai, China), Jun-Lin Guan (Ann Arbor, MI), Jing Fang (Shanghai, China), Larry Fisher (Bethesda), and Liang Chen (Boston) for sharing reagents; Dr. Tyler Jacks (Cambridge, MA) for providing the *Kras*^{G12D} mice; Drs. Dangsheng Li, Lei Zhang, Jun-Lin Guan, Xiaofan Wang, and Steve Weiss for invaluable comments on the manuscript; and Ye Wang, Feng Liu, Wei Bian, Xiaoyan Wang, and Fei Li for technical support. This work was supported by National Basic Research Program of China Grants 2010CB912102 and 2010CB529703, National Natural Science Foundation of China Grants 30740084, 30871284, and 30971495, Chinese Academy of Sciences Grants 2008KIP101 and 2008KIP102, and Science and Technology Commission of Shanghai Municipality Grant 08PJ14105. H.J. and G.G. are scholars of the Hundred Talents Program of the Chinese Academy of Sciences.

- Jemal A, et al. (2006) Cancer statistics, 2006. *CA Cancer J Clin* 56:106–130.
- Tomlinson IP, Houlston RS (1997) Peutz–Jeghers syndrome. *J Med Genet* 34:1007–1011.
- Westerman AM, et al. (1999) Peutz–Jeghers syndrome: 78-year follow-up of the original family. *Lancet* 353:1211–1215.
- Sanchez-Cespedes M (2007) A role for LKB1 gene in human cancer beyond the Peutz–Jeghers syndrome. *Oncogene* 26:7825–7832.
- Wingo SN, et al. (2009) Somatic LKB1 mutations promote cervical cancer progression. *PLoS ONE* 4:e5137.
- Ji H, et al. (2007) LKB1 modulates lung cancer differentiation and metastasis. *Nature* 448:807–810.
- Sanchez-Cespedes M, et al. (2002) Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. *Cancer Res* 62:3659–3662.
- Matsumoto S, et al. (2007) Prevalence and specificity of LKB1 genetic alterations in lung cancers. *Oncogene* 26:5911–5918.
- Koivunen JP, et al. (2008) Mutations in the LKB1 tumour suppressor are frequently detected in tumours from Caucasian but not Asian lung cancer patients. *Br J Cancer* 99:245–252.
- Ding L, et al. (2008) Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* 455:1069–1075.
- Erler JT, Weaver VM (2009) Three-dimensional context regulation of metastasis. *Clin Exp Metastasis* 26:35–49.
- Kagan HM, Li W (2003) Lysyl oxidase: Properties, specificity, and biological roles inside and outside of the cell. *J Cell Biochem* 88:660–672.
- Erler JT, et al. (2006) Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* 440:1222–1226.
- Kirschmann DA, et al. (2002) A molecular role for lysyl oxidase in breast cancer invasion. *Cancer Res* 62:4478–4483.
- Kirschmann DA, Seftor EA, Nieva DR, Mariano EA, Hendrix MJ (1999) Differentially expressed genes associated with the metastatic phenotype in breast cancer. *Breast Cancer Res Treat* 55:127–136.
- Payne SL, Hendrix MJ, Kirschmann DA (2007) Paradoxical roles for lysyl oxidases in cancer—a prospect. *J Cell Biochem* 101:1338–1354.
- Peyrol S, Galateau-Salle F, Raccourt M, Gleyzal C, Sommer P (2000) Selective expression of lysyl oxidase (LOX) in the stromal reactions of broncho-pulmonary carcinomas. *Histol Histopathol* 15:1127–1135.
- Borczuk AC, Kim HK, Yegen HA, Friedman RA, Powell CA (2005) Lung adenocarcinoma global profiling identifies type II transforming growth factor-beta receptor as a repressor of invasiveness. *Am J Respir Crit Care Med* 172:729–737.
- Bild AH, et al. (2006) Oncogenic pathway signatures in human cancers as a guide to targeted therapies. *Nature* 439:353–357.
- Shaw RJ, et al. (2004) The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell* 6:91–99.
- Brugarolas JB, Vazquez F, Reddy A, Sellers WR, Kaelin WG, Jr (2003) TSC2 regulates VEGF through mTOR-dependent and -independent pathways. *Cancer Cell* 4:147–158.
- Paszek MJ, et al. (2005) Tensional homeostasis and the malignant phenotype. *Cancer Cell* 8:241–254.
- Keith B, Simon MC (2007) Hypoxia-inducible factors, stem cells, and cancer. *Cell* 129:465–472.
- Gandhi L, et al. (2009) Sunitinib prolongs survival in genetically engineered mouse models of multistep lung carcinogenesis. *Cancer Prev Res (Phila)* 2:330–337.
- Erler JT, et al. (2009) Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell* 15:35–44.
- Levental KR, et al. (2009) Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* 139:891–906.
- Ji H, et al. (2006) The impact of human EGFR kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies. *Cancer Cell* 9:485–495.
- Ge G, Greenspan DS (2006) BMP1 controls TGFbeta1 activation via cleavage of latent TGFbeta-binding protein. *J Cell Biol* 175:111–120.
- Junqueira LC, Bignolas G, Brentani RR (1979) Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J* 11:447–455.
- Palamakumbura AH, Trackman PC (2002) A fluorometric assay for detection of lysyl oxidase enzyme activity in biological samples. *Anal Biochem* 300:245–251.