Targeting LUNX Inhibits Non–Small Cell Lung Cancer Growth and Metastasis
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Abstract
There remains a great need for effective therapies for lung cancer, the majority of which are non–small cell lung cancers (NSCLC). Here, we report the identification of a novel candidate therapeutic target, LUNX, as a molecule overexpressed in primary NSCLC and lymph node metastases that is associated with reduced postoperative survival. Functional studies demonstrated that LUNX overexpression promoted lung cancer cell migration and proliferation by interactions with the chaperone protein 14-3-3. Conversely, LUNX silencing disrupted primary tumor growth, local invasion, and metastatic colonization. The finding that LUNX was expressed on cell membranes prompted us to generate and characterize LUNX antibodies as a candidate therapeutic. Anti-LUNX could down-regulate LUNX and reduce lung cancer cell proliferation and migration in vitro. Administered in vivo to mice bearing lung cancer xenografts, anti-LUNX could slow tumor growth and metastasis and improve mouse survival. Together, our work provides a preclinical proof of concept for LUNX as a novel candidate target for immunotherapy in lung cancer. Cancer Res; 75(6); 1080–90. ©2015 AACR.

Introduction
Lung cancer is the leading cause of cancer death worldwide. Non–small cell lung cancers (NSCLC), mainly adenocarcinomas and squamous cell carcinomas, represent approximately 80% of all lung cancers. NSCLCs are often diagnosed as advanced disease and confer a poor prognosis (1–3). Therefore, improvement in lung cancer diagnostics and treatment is urgently required.
Lung-specific X (LUNX; also known as BPIFA1, PLUNC, and SPLUNC1) is a member of the palate, lung, and nasal epithelium clone (PLUNC) protein family (4). Some reports have suggested that the human LUNX gene might be a potential marker for NSCLC, and LUNX mRNA has been identified in peripheral blood and mediastinal lymph nodes from patients with NSCLC (5–7). Bingle and colleagues have detected LUNX in carcinomas with glandular phenotypes by analyzing a limited panel of lung cancer tissues; however, no LUNX staining was detected in normal peripheral lung tissues by immunohistochemical analysis (8, 9). Northern blot analyses have demonstrated that LUNX mRNA is undetectable in human liver, brain, pancreas, skeletal muscle, adrenal gland, kidney, prostate, heart, stomach, spleen, mammary gland, adipose, and thyroid tissues (5). Our previous studies revealed that LUNX mRNA in peripheral blood and pleural fluid is one of the most specific genetic markers for NSCLC (10). However, there have been no statistical analyses of LUNX protein expression in large collections of primary tumor tissues, and the correlations between LUNX expression and clinical factors are poorly understood. It is unclear whether LUNX expression is associated with pathologic type and severity in patients with lung cancer. Therefore, the use of LUNX as a potential therapeutic target in NSCLC is uncertain.
In this study, we analyzed clinical NSCLC samples and determined that LUNX was overexpressed in primary tumors and enhanced in metastatic lymph nodes. In addition, increased LUNX immunoreactivity in patients with lung cancer was accompanied by decreased postoperative survival. Next, we found that LUNX promoted tumor growth, invasion, and metastasis by binding to 14-3-3 proteins and reducing their phosphorylation. The 14-3-3 proteins are a family of conserved acidic proteins that regulate numerous protein kinase signaling pathways. Studies have shown that 14-3-3 expression is upregulated in various types of cancer, and 14-3-3 expression is overexpressed and promotes tumor development in NSCLC (11–14).
LUNX expression is not detected in normal peripheral lung tissues; however, it is overexpressed in lung cancer and promotes lung cancer progression, thereby making LUNX a potential therapeutic target for lung cancer. Indeed, LUNX-targeted shRNA has been shown to reduce primary tumor growth, local invasion, micrometastasis formation, and metastatic colonization in multiple established lung cancer xenografts. We also detected LUNX protein in the cell membrane. Therefore, we generated a LUNX therapeutic antibody that demonstrated significant efficacy in in vitro and in vivo lung cancer models. The LUNX antibody directly downregulated LUNX protein expression and blocked its...
downstream pathways. Importantly, S-35-8 (the LUNX antibody) blocked metastasis and improved the survival of mice in preclinical models.

Materials and Methods

Antibody production

The S-35-8 (IgG2a-κ) LUNX hybridoma was raised against a His-tagged LUNX protein and screened by ELISA. The hybridoma was cultured in RPMI-1640. The S-35-8 antibody was purified by protein A affinity chromatography (GE Healthcare Bio-Sciences AB) and labeled as follows: S-35-8 Rho (S-35-8 LUNX antibody labeled with rhodamine) and S-35-8-FTTC (S-35-8 LUNX antibody labeled with FTTC).

Cell lines, plasmids, RNAi, and clinical samples

The A549, SK-MES-1, HFL1, SK-BR-3, HepG2, HO8910, NCI-H292, NCI-H1299, NCI-H358, and NCI-H460 cell lines were obtained from the Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, China) and passed in our laboratory for fewer than 6 months after receipt or revival. DNA fingerprinting and the isoenzymes test were used to authenticate the cell lines. The PG and Sk-lu-1 cell lines were obtained in 2003 from the ATCC, and the last short tandem repeat analysis was performed in May 2012. The plasmids were constructed in our laboratory. For a detailed description of each plasmid and sequences of the synthetic oligonucleotides, see the Supplementary Materials and Methods. Fresh specimens and paraffin-embedded tissues were collected from the Anhui Provincial Hospital, the First People’s Hospital of Hefei, and the Anhui Chest Hospital (Hefei, Anhui, China). The clinical characteristics of all patients with cancer in this study are shown in Supplementary Table S1. For details, see the Supplementary Materials and Methods.

ELISA

We established a double-antibody sandwich ELISA assay for LUNX protein using an S-20-15 (the first LUNX monoclonal antibody)-coated plate and S-35-8 (the second LUNX monoclonal antibody) for testing and TMB coloration (OD450 > 0.1, negative; OD450 < 0.1, positive). For details, see the Supplementary Materials and Methods.

Immunofluorescence, immunohistochemistry, Western blotting, flow cytometry, and apoptosis

Immunofluorescent, immunohistochemical, Western blotting, flow cytometric, and apoptotic assays were performed as previously described (15–17). The JNK inhibitor SP600125 was used in these studies (Beyotime). Images were captured with a Zeiss LSM710 or an Axioskop 2 plus microscope.

Proliferation, migration, and invasion assays

Cell proliferation was determined by cytometry. Cell migration and invasion were analyzed using wound-healing and Transwell permeable support systems (Catalog No. CBA-100-C, Cell Biolabs; refs. 18, 19). For details, see the Supplementary Materials and Methods.

GST-pulldown and immunoprecipitation assays

For the in vitro GST-pulldown assay, GST fusion protein–bound sepharose beads were incubated with the purified His-tagged LUNX fusion protein and GST or GST-14-3-3 θ (Biological Solution Specialists) overnight at 4°C. The beads were washed twice with PBS and 4 times with ice-cold PBS and then boiled in sample buffer. For the immunoprecipitation assay, whole cells were placed in lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl at pH 8.0, 1% NP-40, 0.5% deoxycholate) for 2 hours and then precleared by centrifugation. The supernatants were incubated with protein A/G-coupled sepharose beads (sc-2003, Santa Cruz Biotechnology) or anti-Flag sepharose beads (Sigma) coated with the indicated antibodies overnight at 4°C. The beads were washed twice with lysis buffer and 4 times with ice-cold PBS and then boiled in sample buffer (20, 21).

LUNX overexpression and knockdown assays in vivo

Male athymic Balb/c nude mice (5 weeks of age, Shanghai Experimental Animal Center, Shanghai, China) were used to establish subcutaneous and metastatic lung cancer xenograft models to analyze in vivo tumor growth, metastasis, and invasion. For the LUNX overexpression and knockdown experiments in the subcutaneous xenograft model, A549-mock, A549-LUNX, A549-sh-mock, A549-sh-LUNX1, or A549-sh-LUNX2 stable cell lines (2 × 10^6 cells) were injected subcutaneously into the axilla of the nude mice. For the shRNA experiments in the subcutaneous xenograft model, A549 cells (2 × 10^6 cells) were injected subcutaneously into the axilla of nude mice. When the tumors were palpable, the sh-LUNX1, sh-LUNX2, or sh-mock plasmids along with the DNA transfection reagent (Entranster-in vivo, Engreen) were injected into the tumor twice weekly. The tumor volume was calculated as (width^2 × length)/2 (22). For the LUNX overexpression and knockdown experiments in the lung cancer xenograft model and the orthotopic xenograft model, the A549-mock-luciferase, A549-LUNX-luciferase, A549-sh-mock-luciferase, A549-sh-LUNX1-luciferase, or A549-sh-LUNX2-luciferase stable cell lines (2 × 10^6 cells) were injected in the tail vein (intravenously) or the lung parenchyma of the nude mice. Tumor metastasis and invasion were analyzed 3 weeks later. The mice were injected with D-luciferin and imaged for 5 minutes using the IVIS 200 Imaging System (Caliper LS; ref. 18).

Determination of the therapeutic effects of the LUNX antibody

Male athymic Balb/c nude mice (5 weeks of age, from the Shanghai Experimental Animal Center) were used to establish subcutaneous and metastatic lung cancer xenograft models to assess the therapeutic effects of the LUNX antibody S-35-8. In the subcutaneous lung cancer xenograft model, A549 or NCI-H292 (5 × 10^6) cells were transplanted s.c. into the axilla of nude mice. The LUNX antibody (10, 20, or 30 mg/kg body weight), IgG, or PBS vehicle was injected intravenously twice weekly once the tumors were palpable. The tumor volume was calculated as (width^2 × length)/2. In the metastatic lung cancer xenograft model, A549-luciferase cells (2 × 10^6) were transplanted intravenously into nude mice. From the second day, the LUNX antibody (20 mg/kg body weight) or PBS vehicle was injected intravenously twice weekly. Tumor metastasis and invasion were analyzed after 3 and 5 weeks. The mice were injected with D-luciferin and imaged for 5 minutes using an IVIS 200 Imaging System (Caliper LS).

Statistical analyses

All data are presented as the mean ± SD and as statistical plots generated using GraphPad Prism 5. The differences between two
groups were determined using 2-tailed \( t \) tests. The differences among three or more groups were determined using one-way ANOVA followed by 2-tailed \( t \) tests. Kaplan–Meier analysis of the survival curves according to the LUNX level. \( P \leq 0.05 \) was considered significant.

**Results**

**LUNX is overexpressed in NSCLC cells, especially in tumor cell lymph node metastases**

We previously determined that LUNX mRNA in peripheral blood and pleural fluid is a specific genetic marker for NSCLC (10). To investigate LUNX expression in NSCLC, we assessed its protein levels in a series of cell lines and clinical NSCLC specimens. We found that the LUNX protein was highly expressed in NSCLC cell lines (NCI-H292, A549, NCI-H1299, and NCI-H358) and in tumor tissue from patients with NSCLC, whereas there was no expression in lung fibroblast (HFL1), breast adenocarcinoma (SK-BR-3), hepatocellular carcinoma (HepG2), or ovarian cancer (HO8910) cell lines, and only weak expression was detected in the paired paracancerous tissues (Fig. 1A and B). Several LUNX-positive cells in the paracancerous tissues may have been infiltrating tumor cells. Furthermore, KRAS and/or p53 mutations are quite frequent in NSCLC. Here, we detected LUNX expression both in KRAS-mutant NSCLC and in p53-mutant NSCLC cells (Supplementary Fig. S1A). Of the 150 total adenocarcinoma and squamous cell carcinoma samples, 80.6% (121 of 150) displayed elevated LUNX protein expression levels. LUNX expression was more frequently detected in primary tumor tissue from patients with adenocarcinoma (92%, 69 of 75) than from patients with squamous cell carcinoma (69.6%, 52 of 75). In patients with adenocarcinoma, a high percentage of specimens showed high or moderate LUNX expression. Moreover, in patients with squamous cell carcinoma, LUNX protein expression was associated with tumor–node–metastasis (TNM) stage and histologic grade, and increased LUNX protein expression was associated with increased clinical and pathologic severity (Fig. 1B and Supplementary Table S2).

**Figure 1.** LUNX is overexpressed in NSCLC cells, especially in the lymph node metastases of tumor cells. A, immunofluorescent staining for LUNX in NSCLC (NCI-H292, A549, NCI-H1299, and NCI-H358), lung fibroblast (HFL1), breast adenocarcinoma (SK-BR-3), hepatocellular carcinoma (HepG2), and ovarian cancer (HO8910) cell lines. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bar, 50 \( \mu \)m. B, IHC analysis of LUNX expression in 150 NSCLC tissue samples including adenocarcinoma and squamous cell lung carcinoma. The samples were classified according to TNM stage, histologic grade, or pathologic type. Kaplan–Meier curves of the 4-year postoperative survival were generated according to the LUNX staining grade in samples from 102 patients with NSCLC, 54 patients with squamous cell lung carcinoma, or 48 patients with lung adenocarcinoma. ELISA of LUNX expression in the pleural fluid of NSCLC and benign lung disease (pneumonia and tuberculosis) samples (\( \#\#\), \( P < 0.05 \)). IHC assessment of the correlation between the percentage of metastatic regional lymph nodes and LUNX staining status in patients with NSCLC (TNM stages II and III NSCLC samples; \( \#\#\), \( P < 0.01 \)). LUNX expression was classified into 1 of 4 categories according to the LUNX staining intensity: 1, faint or equivocal expression; 2, moderate expression; 3, intense expression; 4, no expression. The data are presented as the mean ± SD. Tumor, tumor tissue; Adjacent, matched paracancerous tissue. Scale bar, 200 \( \mu \)m. C, IHC analysis of LUNX expression in regional lymph node (mSLN) samples of patients with NSCLC (low expression of LUNX in primary tumor), statistical analysis testing the difference in expression level by the two types of tissues was performed using logistic regression model. Scale bar, 200 \( \mu \)m; \( P = 0.0038 \). D, immunofluorescent staining for LUNX in metastatic supravacular lymph node (mSLN) samples obtained by paracentesis. The patients were diagnosed with NSCLC or other cancers (lymphoma, breast cancer, hepatoma, and esophageal cancer). Scale bar, 100 \( \mu \)m.
To further investigate LUNX expression in NSCLC, we evaluated whether LUNX expression was associated with postoperative clinical outcomes. In the 4-year follow-up evaluation of 102 postoperative patients, increased LUNX immunoreactivity in patients with NSCLC was accompanied by significantly decreased postoperative survival (Fig. 1B and Supplementary Table S3). In some of these patients with NSCLC, increased LUNX expression was associated with significantly reduced 5-year overall post-surgery survival (Supplementary Fig. S1B). The Kaplan–Meier analysis of 48 patients with adenocarcinoma and a log-rank test showed no significant association of LUNX expression with overall survival (P = 0.2), possibly because of the small size and uneven sample distribution (Fig. 1B). These results indicated that patients with NSCLC presenting with high LUNX expression exhibited a worse prognosis than those with moderate or low LUNX expression.

In addition, we did not observe these results in samples from patients with benign lung diseases such as pneumonia or tuberculosis, and we did not detect LUNX expression in other organs or cancers (colon, liver, and breast; Supplementary Fig. S1C).

Intracellular proteins are released into the pleural fluid of patients with chest effusions due to necrosis. LUNX expression was detected in the pleural fluid of patients with NSCLC but not in the samples from patients with benign lung diseases (pneumonia and tuberculosis; Fig. 1B).

Next, to assess LUNX expression in metastatic tumor cells, we tested 30 NSCLC samples with low LUNX expression in the primary tumor and determined that LUNX expression was frequently higher in metastatic regional lymph nodes compared with the matched primary tumor tissue (Fig. 1C). Clinically, primary lung tumors typically metastasize to the supraclavicular lymph nodes. Therefore, by evaluating the LUNX expression in supraclavicular lymph node metastases, we detected that LUNX was overexpressed in supraclavicular lymph node metastases from patients with NSCLC but not with other types of cancer (lymphoma, breast, hepatoma, and esophageal; Fig. 1D). We tested 17 samples obtained via paracentesis and detected LUNX overexpression in 77.8% (7 of 9) of the samples from patients with NSCLC but not in any of the samples from the patients with other types of cancer (0 of 8; Fig. 1D).

In further study, we evaluated the association between LUNX expression and the incidence of regional lymph node metastasis. Samples from patients with TNM stages II and III lung cancer were investigated for LUNX expression, and we observed a positive association between a regional lymph node metastasis and LUNX expression (Fig. 1B).

These data demonstrated that LUNX was overexpressed in primary tumors and enhanced in the metastatic lymph nodes of patients with NSCLC but was rarely detected in benign lung diseases and other organs (colon, liver, and breast). Furthermore, high LUNX protein levels were associated with poor prognosis.

**LUNX promotes lung cancer cell proliferation, migration, and invasion**

LUNX protein expression was enhanced in metastatic lung cancer cells in several NSCLC samples, suggesting that LUNX may play a critical role in metastasis and invasion. To address this possibility, we evaluated LUNX expression in a panel of lung cancer cell lines with varying degrees of metastatic potential. The NCI-H292 and PG cells, which have a higher metastatic capacity than other tested lung cancer cell lines (A549, H460, SK-MES-1, and SK-Lu-1), expressed higher levels of LUNX (Fig. 2A and Supplementary Fig. S2A).

To further evaluate the role of LUNX in lung cancer cell migration and invasion, we stably overexpressed or depleted LUNX (using 2 different shRNAs against LUNX, sh-LUNX1 and sh-LUNX2) in 2 LUNX-positive NSCLC cell lines, NCI-H292 and A549, and found that LUNX overexpression promoted migration and invasion (Fig. 2C and D). Conversely, we demonstrated that LUNX silenced reduced tumor cell migration and invasion (Fig. 2C and D and Supplementary Fig. S2B).

Next, we assessed the role of LUNX in lung cancer cell proliferation. We found that LUNX overexpression increased proliferation of NCI-H292 and A549 cells. In addition, LUNX silencing in these cells reduced proliferation and Ki67 expression (Fig. 2B and Supplementary Fig. S2B and S2C).

These data suggested that LUNX expression is both necessary and sufficient to promote lung cancer cell proliferation, migration, and invasion.
cell migration and proliferation. LUNX-overexpressing NCI-H292 cells displayed increased cell migration and proliferation compared with mock-transfected (control) cells; however, when 14-3-3z and 14-3-3q were knocked down together, LUNX-overexpressing NCI-H292 cells were no more metastatic or proliferative than mock-transfected (control) NCI-H292 cells (Fig. 3C). Similar results were also observed in A549 cells (Supplementary Fig. S3E). These findings suggested that LUNX promotes lung cell migration and proliferation by targeting 14-3-3z and 14-3-3q.

In tumors, the 14-3-3 proteins bind to all Raf proteins and positively regulate Raf signaling, thereby functioning as direct effectors of Ras GTPase, which activates the Erk1/2 MAP kinase pathway (12, 27). In our experiments, silencing LUNX, 14-3-3z or 14-3-3q reduced phospho-Erk1/2 (Fig. 3D). The 14-3-3 proteins stabilize the activation-competent conformation of Raf to stimulate the next step in the MAP kinase cascade (27). We found that LUNX silencing had no effect on p38 phosphorylation but did decrease phospho-JNK levels (Fig. 3D and Supplementary Fig. S3F). Previous reports have shown that JNK is activated in a subset of NSCLC biopsies and promotes oncogenesis in the bronchial epithelium (28). Similarly, in our study, inhibiting JNK phosphorylation reduced NCI-H292 migration and proliferation (Supplementary Fig. S3H). LUNX silencing decreased phospho-JNK and its downstream targets phospho-ATF-2 and phospho-c-Jun (Supplementary Fig. S3G). LUNX silencing had no obvious effect on AKT phosphorylation and cell apoptosis (Supplementary Fig. S3F and S3I).

Together with the above results showing that LUNX promotes lung cancer cell proliferation and migration by binding to 14-3-3z and 14-3-3q and activating 14-3-3 pathways, these data indicated that LUNX may be a potential therapeutic target in lung cancer.

Targeting LUNX reduces lung cancer growth, metastasis, and invasion

To explore whether LUNX is an effective therapeutic target in lung cancer, we tested the relationship between LUNX levels and tumor progression by conducting multiple lung cancer xenograft experiments using stable LUNX-silenced or -overexpressing A549 cells.

Tumor growth, metastasis, and invasion are key steps in tumor progression. First, to assess the effect of targeting LUNX on reducing the lung cancer invasion metastasis cascade, we established a murine orthotopic xenograft model and monitored
tumor metastasis by D-luciferin-based bioluminescence. We generated stably LUNX-depleted, mock-depleted, LUNX-overexpressing, and mock-transfected cell lines, which were injected into nude mice via the lung parenchyma. The mice injected with stably LUNX-depleted cells had fewer distant metastases (sh-LUNX1 group, \( n = 0 \) of 5; sh-LUNX2 group, \( n = 1 \) of 5) than mice injected with stably mock-depleted cells (\( n = 3 \) of 5). In contrast, the mice injected with stably LUNX-overexpressing cell lines exhibited an increase in distant metastases (\( n = 5 \) of 5) compared with mice injected with stably mock-transfected cells (\( n = 3 \) of 5). The outgrowth of distant metastases in the LUNX-overexpressing group was markedly increased compared with the mock-transfected group (Fig. 4A). Local invasion is the initial step in tumor metastasis. In this orthotopic xenograft model, depleted LUNX reduced the ability of tumor cells to invade normal tissues and organs (Supplementary Fig. S4A).

In the metastatic cascade, micrometastasis formation and metastatic colonization are the final key steps. To investigate whether LUNX was involved in these steps, we established a tail vein injection model to imitate micrometastasis formation and metastatic colonization of disseminated tumor cells. LUNX stable overexpression increased the fluorescent area representing tumor foci in various organs, including the lungs, abdominal organs, and the thighbone. In contrast, stable silencing of LUNX reduced massive colonization and the number of micrometastasis foci throughout the body, and metastatic colonization was almost undetectable in all mice (Fig. 4B). Analysis of the number of metastatic lung nodes confirmed the enhanced colonization ability conferred by LUNX (Fig. 4B). The lung weights revealed that the elevated LUNX expression facilitated the metastatic colonization of tumor cells in the lung (Fig. 4B). Finally, using the same protocol, the mouse survival in the model was inversely proportional to LUNX expression (Fig. 4B). These data demonstrated that LUNX-targeted shRNA disrupted the local invasion, micrometastasis formation, and metastatic colonization of lung cancer cells in the initial and final steps of the invasion–metastasis cascade.

Next, to assess whether targeting LUNX reduced lung cancer growth, we analyzed subcutaneous tumor growth in a mouse xenograft model. We generated stably LUNX-depleted, mock-
depleted (control), LUNX-overexpressing, and mock-transfected (control) cell lines, which were injected subcutaneously into nude mice. The growth of LUNX-overexpressing tumors was more rapid than the mock-transfected tumors (control); conversely, LUNX-targeted shRNA reduced tumor growth (Fig. 4C and Supplementary Fig. S4C). In addition, the proliferation of the LUNX-targeted shRNA-treated tumors was reduced, as evidenced by the Ki67 staining (Supplementary Fig. S4B).

To further assess the potential of LUNX as a therapeutic target in lung cancer, we treated mice bearing subcutaneous tumors (A549 cells) with vectors expressing LUNX-shRNA or mock-shRNA along with a DNA transfection reagent. We found that LUNX-targeted shRNA suppressed lung cancer growth (Fig. 4D).

These data suggested that LUNX-targeted shRNA disrupts lung cancer growth, metastasis, and invasion and indicated that LUNX may be an effective therapeutic target in lung cancer.

LUNX antibody S-35-8 inhibits lung cancer proliferation and migration by downregulating LUNX

On the basis of the marked hydrophobicity of LUNX (29), we assessed LUNX localization on the membranes of cultured tumor cells. LUNX was detected in the purified membranes of NCI-H292 and A549 cells (Fig. 5A). Immunofluorescence staining and flow cytometry for LUNX showed membranous expression in NCI-H292 and A549 cells (no perforation; Fig. 5A).

LUNX has potential as a therapeutic target in lung cancer based on its weak expression in peripheral lung tissues, its overexpression in lung cancer cells, its localization on the cell membrane, and its ability to promote lung cancer progression. Therefore, we screened antibodies for the ability to recognize LUNX on the cell surface and to inhibit tumor cell proliferation and migration. We selected one antibody, S-35-8,
a mouse IgG2b clone that specifically bound to the LUNX protein (Supplementary Fig. S5A). This antibody did not induce apoptosis in the NCI-H292 cells but did inhibit A549, NCI-H292, NCI-H1299, and NCI-H358 cell migration and proliferation, which correlated with the antibody dose (Fig. 5B and Supplementary Fig. S5B–S5D).

To study the molecular mechanism of action of S-35-8, we performed Western blot analyses and showed that the S-35-8 treatment reduced LUNX protein levels and that increasing the dose of S-35-8 to 160 μg/mL led to complete blockage of LUNX expression. Previously, we demonstrated that LUNX modulates 14-3-3 activity by inhibiting 14-3-3 phosphorylation, contributing to lung cancer cell proliferation and migration. Here, we found that the S-35-8–induced LUNX downregulation led to 14-3-3 phosphorylation and subsequently reduced the activation of pathways downstream of 14-3-3, such as the Erk1/2 and JNK pathways (Fig. 5C).

To better understand the mechanism of S-35-8–mediated LUNX downregulation, we treated NCI-H292 and A549 cells with S-35-8-Rho (S-35-8 labeled with rhodamine; Fig. 5D and Supplementary Fig. S5E) and found that antibody endocytosis and antibody degradation were observed after 24 and 48 hours of treatment, respectively.

These data validated the use of a therapeutic LUNX antibody in lung cancer. The molecular mechanism of action of the antibody was based on its ability to reduce LUNX protein levels via antibody endocytosis.

The LUNX antibody S-35-8 inhibits lung cancer growth and metastasis in vivo

To analyze the therapeutic effect of S-35-8 in vivo, we established multiple mouse xenograft models and analyzed the effect of S-35-8 on tumor invasion, metastasis, and growth. First, to assess the effect of S-35-8 treatment on lung cancer metastasis, we implanted A549 tumors into mice via tail vein injection to establish a metastatic xenograft model and then treated the mice with PBS or S-35-8 (20 mg/kg). Next, we monitored the time to tumor micrometastasis formation and metastatic colonization. After 3 weeks of treatment, all PBS-treated mice (n = 6 of 6) had multiple bilateral lung tumors and 66.7% of them (n = 4 of 6) formed micrometastases that colonized the entire body (such as the humerus and enterocelia). In contrast, 50% of the S-35-8–treated mice (n = 3 of 6) showed signs of lung tumor growth, and none (n = 0 of 6) formed micrometastases or colonized the entire body. Similar results were observed after 5 weeks of treatment. These data suggest that treatment with S-35-8 significantly reduced tumor metastasis, including micrometastasis formation and metastatic colonization throughout the entire body (Fig. 6A).

An analysis of the number of metastatic lung nodes confirmed the therapeutic efficacy of S-35-8 (Fig. 6A). Finally, using the same protocol, improved mouse survival was observed after treatment with S-35-8 (Fig. 6A).

Next, to analyze the therapeutic effect of S-35-8 on tumor growth, we subcutaneously implanted A549 tumors into mice, treated the mice with PBS, IgG, or S-35-8 (20 mg/kg), and
monitored the time to tumor growth. S-35-8 treatment significantly reduced tumor growth (Fig. 6B). Consistent with these results, immunohistochemical analysis showed that Ki67 staining was reduced in tumors treated with S-35-8 (Supplementary Fig. S6A). Similarly, mice bearing palpable subcutaneous NCI-H292 tumors exhibited reduced tumor growth when treated with S-35-8 (Fig. 6C). The rapid growth of NCI-H292 tumors caused a significant reduction in mouse body weight; in contrast, S-35-8–treated mice maintained a normal body weight (Fig. 6C). In dose escalation studies of S-35-8, a dose of 30 mg/kg had significant inhibitory effects on NCI-H292 tumor growth (Fig. 6C).

We verified the delivery of S-35-8 into established orthotopic tumors in vivo via tail vein injection of S-35-8-FITC (S-35-8 labeled with FITC). Twelve hours after the injection, S-35-8-FITC had strongly accumulated in tumor tissues but showed relatively weak accumulation in normal lung tissue (Supplementary Fig. S6B).

Toxic side effects are an important factor that determines a drug’s clinical application. In our study, no cases of sudden death, pulmonary inflammation, or lung injury were observed upon treatment of wild-type mice with S-35-8. No adverse weight loss was noted even when the dose of S-35-8 was increased to 30 mg/kg body weight (Supplementary Fig. S6C).

These data suggest that the LUNX antibody S-35-8 suppresses lung cancer growth and metastasis.

Discussion

In this study, our results indicated that the LUNX protein was overexpressed in NSCLC cells, weakly expressed in normal lung tissue, and rarely detected in other normal organs. LUNX overexpression in NSCLC strongly correlated with poor prognosis. These data confirmed that LUNX is a potentially superior diagnostic marker or therapeutic target for lung cancer. LUNX (also named PLUNC) has been suggested to be a novel marker of gastric hepatoid adenocarcinoma, but LUNX staining has not been detected in primary hepatic carcinomas or in normal liver (30). The human LUNX family comprises 9 genes located in a single locus on chromosome 20q11. Until now, a small number of human LUNX proteins have been identified, and an even smaller number of these proteins have been studied in any detail (9, 31). As shown in Supplementary Fig. S1D, we assayed the mRNA levels of 8 genes with reported sequences in NSCLC. Only BPIFA4p mRNA was detected in the NCI-H292 cells other than BPIFA1 (LUNX), but the BPIFA4p locus type was found to be a pseudogene (Source: HGNC Symbol; Acc: HGNC: 20469).

The invasion–metastasis cascade can be simplified into two key steps: (i) cancer cell dissemination from the primary tumor to distant tissues and (ii) translocation of cells to form micrometastases and colonize distant organs (32). LUNX-targeted shRNA suppressed lung cancer cell invasion into the surrounding normal tissue and disrupted the formation of micrometastases and the metastatic colonization of tumor cells. Our results indicate that LUNX may be involved in the mechanism of lung cancer metastasis. In addition, the transfection of expression vectors encoding LUNX-targeted shRNA suppressed lung cancer growth by intratumor injection. These data suggested that LUNX is associated with lung cancer progression and indicated that targeting LUNX exerts an antitumor effect.
The major finding of our study was that the therapeutic targeting of LUNX inhibited tumor growth, metastasis, and invasion. An increase in LUNX detection in nasal secretions and sputum has been suggested to occur in inflammatory airway diseases (33, 34); however, we demonstrated that LUNX also exists in purified cell membranes, and membranous expression of LUNX was detected in lung cancer cells by immunofluorescent staining and flow cytometry. These data further confirmed that LUNX is a potential therapeutic target in lung cancer.

Next, we generated a therapeutic LUNX antibody, S-35-8, which slowed the growth and blocked the local invasion and metastasis of multiple established NSCLC xenografts. In addition, S-35-8 exerted its effects not only in KRAS-mutant NSCLC cells but also in NSCLC cells with wild-type KRAS or mutant p53. In our study, S-35-8 reduced LUNX expression on the cell surface by antibody endocytosis, and a potential mechanism of action involves the endocytosis and degradation of the LUNX-S-35-8 antigen–antibody complex. LUNX downregulation caused the 14-3-3ζ and 14-3-3θ inactivation and the inhibition of the Erk1/2 and JNK pathways, which might be a mechanism by which S-35-8 exerts its antitumor effect. The therapeutic benefit associated with antibody therapy is complex and most likely involves multiple mechanisms in vivo. The Fc function of an antibody is important for mediating tumor cell kill via antibody-dependent cell-mediated cytotoxicity (ADCC) in vivo, whereas effective tumor ADCC requires congeneric effector cells, target cells, and mononuclear antibodies; specific antibody affinity and subtypes may also be required (35). To improve the antitumor efficacy of S-35-8, additional studies are necessary to modify S-35-8 and develop its use in humans.

In our study, S-35-8 reduced tumor growth, micrometastasis formation, and metastatic colonization; therefore, it is highly likely that S-35-8 may be translatable to clinical applications. Toxic side effects are important factors that determine the clinical applicability of a drug. Therefore, toxicity is a question that needs to be addressed in this context, especially because LUNX has been reported to be involved in antibiotic of the upper airway, to be limited to a few nonciliated epithelial cells and to be elevated under inflammatory conditions, such as cystic fibrosis, chronic obstructive pulmonary disease, and allergic rhinitis (8, 9, 33, 36–38). LUNX regulates airway surface liquid volume by binding to ENaC in bronchial epithelial cultures (39). However, LUNX protein levels are relatively low in normal tissues, and LUNX staining is absent in small airways and peripheral lung (5, 8, 36). Therefore, to confirm the safety of this approach, further preclinical and clinical tests are necessary.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Zheng, M. Cheng, Q. Wang, X. Yu, R. Sun, Z. Tian
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Zheng, M. Cheng, Z. Tian, H. Wei
Writing, review, and/or revision of the manuscript: X. Zheng, B. Fu, Z. Tian, H. Wei
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Zheng, X. Fan, R. Sun, Z. Tian, H. Wei
Study supervision: Z. Tian, H. Wei

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References


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