Spi-B-mediated silencing of Claudin-2 promotes early dissemination of lung cancer cells from primary tumors

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Running title: Spi-B and lung cancer metastasis

Keywords: Spi-B, Lung cancer, Invasion, Claudin-2, Chromatin configuration

Financial Information

This work was supported by grants (91519331, 31371295 to Z. Liu, 81572271, 81372307 to Z.
Ma, 81572882 to Z. Yao, 81502538 to W. Du, 81402121 to Y. Jiang) from the National Natural Science Foundation of China, grant 2014CB910100 to Z. Liu from the Ministry of Science and Technology of China, grant 15JCZDJC34800 from Tianjin Municipal Science and Technology Commission to Z. Liu, grant 2016M591397 to X. Li from the China Postdoctoral Science Foundation, and grant 20140602 to W. Du from Tianjin Municipal University Science and Technology Foundation.

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Disclosure of Potential Conflicts of Interest
The authors declare no potential conflicts of interest.

Abstract
Dissociation from epithelial sheets and invasion through the surrounding stroma are critical early events during epithelial cancer metastasis. Here we find that a lymphocyte lineage-restricted transcription factor, Spi-B, is frequently expressed in human lung cancer tissues. The Spi-B-expressing cancer cells coexpressed Vimentin but repressed E-cadherin and exhibited invasive behavior. Increased Spi-B expression was associated with tumor grade, lymphatic metastasis and short overall survival. Mechanistically, Spi-B disrupted intercellular junctions and enhanced invasiveness by reconfiguring the chromatin structure of the tight junction gene
Claudin-2 (*CLDN2*) and repressing its transcription. These data suggest that Spi-B participates in mesenchymal invasion, linking epithelial cancer metastasis with a lymphatic transcriptional program.

**Introduction**

Every stage of cancer progression is accompanied with genetic and epigenetic dysregulation. Consequent aberrant activation and/or silencing of a series of functional genes confer premalignant epithelial cells with multiple distinct properties including unrestrained proliferation, resistance to cell death, evasion from immune destruction, and progression to frank malignancy (1-3). Epithelial cancer cells in their primary site are knit together by extensive intercellular junctions to form an epithelial cell sheet. To metastasize, individual or a small cluster cancer cells must first dissociate from their neighboring epithelial cells and invade the surrounding stroma. The specific epigenetic mechanisms controlling this process are poorly understood.

Spi-B (encoded by *SPIB*), an Ets family transcription factor, is expressed exclusively in mature B cells, T-cell progenitors, and plasmacytoid dendritic cells (4-6). B cells in *Spi-B*<sup>-/-</sup> mice are defective in B cell receptor (BCR) signaling and are unable to generate antibody responses to T-dependent antigens (7). Spi-B has been shown to regulate many genes that are important for BCR-mediated signaling including, Igυ heavy chain, Ig light chains (λ and κ), mb-1 (Igα), and the tyrosine kinases Btk (8). More recently, Spi-B has been detected in intestinal M cells, whose expression activates GP2 gene and endows M cells with antigen presentation capacity, thus playing essential role in controlling M cells differentiation (9).

Many reports have linked Spi-B with hematopoietic tumorigenesis. *SPIB* is recurrently amplified and occasionally translocated in the activated B cell-like subtype of diffuse large B cell
lymphoma (ABC DLBCL). Its expression is required for the survival of ABC DLBCL lines and contributes to apoptosis resistance via the PI3K-AKT pathway (10-12). In addition, gene expression profiling analysis has detected Spi-B in some malignant solid tumors including gastric cancer (13) and colorectal cancer (14). Immunohistochemical staining also detected Spi-B in hepatocellular carcinoma (15,16), suggesting that Spi-B may be aberrantly expressed in some solid tumors. However, the functional consequence of Spi-B expression in carcinomas is completely unknown.

Here, we report that Spi-B is expressed in invasive cancer cells in human primary lung cancer tissues. Expression of Spi-B in lung cancer cells downregulates Claudin-2 and thereby disrupting intercellular junctions and enhancing invasive behavior. These data identify an epigenetic process linking hematopoietic lineage gene control with local invasion in metastatic carcinoma cells, and suggest that Spi-B may be an effective biomarker for both prognosis and treatment of lung cancer.

Materials and Methods

Cells

HBEC cells are normal human bronchial epithelium immortalized by hTERT and CDK4, and were obtained from Dr. Jerry Shay (UT Southwestern Medical Center, Dallas, TX) in 2008 at the passage 30. A549, H460, HCC827, H1155, H69, H526, H82 and Lewis lung carcinoma (LLC1) cells were obtained from ATCC within the past 10 years and maintained in ATCC recommended media supplemented with 10% FBS, 100 U of penicillin/ml and 100 μg of streptomycin/ml. All experiments were performed within 1 month after thawing early-passage cells. A549, H460, HCC827, H1155, H69, H82 and H526 cells were authenticated in April 2017. DNA purified from above cell lines were tested by the short tandem repeat analysis method using Promega
PowerPlex 1.2 analysis system (Genewiz Inc.). Data were analyzed using GeneMapper4.0 software and then compared with the ATCC databases for reference matching.

**In vivo metastasis assay**

LLC1 expressing empty vector or *SPIB* were selected by cell sorting for GFP expression (FACS Vantage, BD). $10^5$ cells of each group in 100 μl saline were subcutaneously injected into 8-week-old C57BL/6 mice. Tumors in situ were excised 2 weeks after the inoculation of cells. 2 weeks after resection, the mice were sacrificed and metastatic nodules formation in the lungs was analyzed.

$10^6$ cells of LLC1 cells in 100 μl saline were injected into the tail vein of 8-week-old C57BL/6 mice. At one month following injection, the mice were sacrificed and metastatic nodules formation in the lungs was analyzed.

LLC1-luc cell line was established using a lentivirus encoding the luciferase gene, and stable clones were isolated by puromycin selection. GFP sorted LLC1-luc cells expressing empty vector, *SPIB*, or *CLDN2* and *SPIB* were subcutaneously injected as above. The whole lungs were immediately grinded in liquid nitrogen and total protein was used to detect tumor metastasis by assaying luciferase activity. All animal procedures were approved by Animal Care and Use Committee at Tianjin Medical University and conform to the legal mandates and national guidelines for the care and maintenance of laboratory animals.

**Immunohistochemical Analysis**

Lung cancer tissues were obtained from Tianjin Medical University Cancer Institute & Hospital in China. Samples were fixed in 4% paraformaldehyde at 4°C overnight and embedded in paraffin. Paraffin blocks were cut into 5 μm sections, and were immunostained with antibodies against Spi-B (Abcam) and Claudin-2 (Bioworld Technology), E-cadherin (BD Transduction...
laboratories), Vimentin (Sigma) and processed following the standard protocol for DAB staining. The use of all human lung cancer tissues and clinical data was approved by the Institutional Review Board of Tianjin Medical University. Informed consent was provided in accordance with the Declaration of Helsinki. Samples were deidentified prior to analysis.

**Invasion Assay**

Assays were performed in trans-well inserts with 8 μm pores (BD Biosciences) coated with 20% growth-factor-reduced Matrigel. Tumor cells in serum-free medium (2×10^5 cells per well) were seeded into the upper chamber and complete media was placed in the lower chambers as a chemo-attractant. The chambers were incubated for 20 h at 37°C with 5% CO₂. Experiments were performed in triplicate. Migrated cells on the undersides of filter membrane were fixed in 4% formalin and stained with crystal violet. The migrated cells were counted using light microscopy.

**Soft Agar**

Cells (1×10⁴) were resuspended in DMEM containing 10% FBS with 0.35% agarose and layered on top of 0.6% agarose in DMEM on 6-well plates. Cells were cultured for 21 days at 37°C with 5% CO₂. Experiments were performed in triplicate. Colonies were stained, analyzed morphologically and counted using light microscopy.

**3D Matrigel Culture**

These assays were optimized from previous publication (17). Tumor cells were detached with 0.25% trypsin-EDTA, centrifuged (1,000 rpm for 3 min), resuspended, and counted. Single cells (2×10³ per well performed in triplicate) were mixed into 0.4 ml of RPMI1640 medium supplemented with 2% FBS and 5% chilled growth factor reduced Matrigel (BD Biosciences), and cultured in suspension in 24-well ultra-low attachment plate (Corning) at 37°C for 14 days. Experiments were performed in triplicate. The organoids were categorized based on their
morphology (18).

**Chromosome conformation capture**

Chromosome conformation capture (3C) was performed as described previously (19). 10⁶ cells were cross-linked, lysed, and nuclei were digested with Dpn II. After ligation and subsequent DNA purification, the cross-linking frequencies between the anchor and test fragments were estimated by PCR reactions relative to standards. Three PCR products together containing from the CLDN2 gene promoter to all upstream regulatory elements were amplified, mixed at equal molar ratios, digested with Dpn II, and ligated at high concentrations to generate all possible ligation products. The cross-linking and ligation efficiencies between different samples were normalized by setting the highest cross-linking frequency to 1.0. Primers used in this study are provided in the Table S1.

**Chromatin Immunoprecipitation**

ChIP was performed as described previously (19). Antibody against H3K4me3 was from Millipore; antibody against flag M2-agarose was from Sigma. Results were quantified by real-time PCR with SYBR Green dye using the ABI Prism 7900 system (Applied Biosystems). All PCR signals from immunoprecipitation samples were referenced to their respective inputs to normalize for differences in primer efficiencies. Primers used in this study are listed in Table S1.

**Luciferase Assay**

DNA fragments of CLDN2 promoter and cis-regulatory elements upstream were amplified from HUVEC DNA using primers listed in Table S1. CLDN2 promoter was inserted into the XhoI and the Hind III site of the polylinker region pGL3-basic. Cis-regulatory elements were inserted into the Kpn I and Xho I site. Cell lines were transiently cotransfected either in triplicate or in duplicate with pRL-CMV Renilla luciferase reporter, which was used for normalization
Cell lines were harvested and assayed for luciferase activity using Dual-Luciferase™ reporter assay systems (Promega) following the manufacturers’ instructions at the time of 24-48 h after transfection.

**EMSA**

Nuclear proteins from A549 expressing Spi-B cells were extracted using a NucBuster protein extraction kit (Novagen) according to the manufacturer’s instructions. Double-stranded oligonucleotides corresponding to the potential Spi-B binding sites 5’-GGT CCC CAA ACA TTC CTC CCT CAG TGA CAC CTT TC-3’ and 5’-CAA TTG GTA GTT CCT CCC ACT TTT CA-3’ were end-labeled with biotin. Binding assays were performed in 10 μl of reaction mixture containing 2 μg of nuclear proteins, 10 mM Tris, 55 mM KCl, 1 mM dithiothreitol, 5% Glycerol, 0.05% NP40, 2.5 mM MgCl₂, 0.25 mM EDTA, 1 μg of poly (dI·dC) and 1 nM labeled probes at room temperature for 30 min. For supershift assays, 2 μg of nuclear protein extracts were incubated with Spi-B antibody for 30 min on ice before incubating with oligonucleotide. Reactions were analyzed by electrophoresis on a 6.0% non-denaturing polyacrylamide gel at 100 V for 1 h. After transfer, the membrane was immediately cross-linked for 1 min on a UV-light crosslinker instrument equipped with 254 nm bulbs. A chemiluminescent detection method utilizing a luminol/enhancer solution and a stable peroxide solution (Pierce, USA) was used as described by the manufacture and the membrane was exposed to X-ray films to visualize the bands.

**Transmission electron microscopy**

Cells in three-dimensional basement membrane gels were fixed in a fixative solution containing 2.5% glutaraldehyde. After being washed several times in PBS, the cells were then postfixed with 2% osmium tetroxide for 1h at room temperature in darkness, dehydrated in ascending
ethanol solutions and absolute acetone, immersed in 50% Epon812 in acetone and finally embedded in Epon812. Ultrathin sections were mounted on copper slot grids and stained with uranyl acetate and lead citrate, and then observed under a HITACHI HT7700 electron microscope.

**Expression Profiling**

The expression and functional profiles of genes were compared between A549 cells expressing empty vector and \textit{SPIB} using Agilent SurePrint G3 Human Gene Expression 8x60K v2 Microarray (Agilent Technologies). Analysis of functional profiling of Spi-B-regulated genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) based on the biological pathways from KEGG (Kyoto Encyclopedia of genes and genomes) database.

**Accession Numbers**

The Gene Expression Omnibus accession number for A549 cell expression profile data is GSE90645.

**Statistical Analysis**

Results were reported as mean ± SD unless otherwise noted. SPSS 18.0 was used for statistical analysis. Correlation of the expression levels between Spi-B and survival rates were determined with Kaplan-Meier analysis using Mantel-Cox log-rank testing (GraphPad Prism). Comparison of expression levels between Spi-B and Claudin-2 was determined by using the Pearson’s correlation test and the Spearman’s rank correlation test. A p-value of less than 0.05 was considered statistically significant for all tests.

**Results:**

\textit{Spi-B expression is correlated with poor prognosis in human lung cancer}
We studied Spi-B expression in 130 non-small cell lung cancers (NSCLCs), including lung adenocarcinoma and squamous lung carcinoma, in 14 small cell lung cancers (SCLCs), and in 10 tumor-adjacent normal lung tissues using immunohistochemical (IHC) staining. Epithelial cells in tumor-adjacent lung tissues did not express Spi-B. Lymphocytes in tumor-adjacent tissue expressed Spi-B (Figure 1Aa), consistent with previous reports (6). Both NSCLC and SCLC expressed Spi-B (Figure 1Ab-d). Interestingly, we noticed strong nuclear staining of Spi-B in cancer cells in the invasive strand at the tumor-stromal border in NSCLC tissue sections (Figure 1Ae). These Spi-B-expressing invasive NSCLC cells lacked the epithelial marker E-cadherin (Figure 1Af), but co-expressed the mesenchymal marker Vimentin (Figure 1Ag). These data indicate that Spi-B is expressed in cancer cells with mesenchymal attributes.

Quantification of staining based on the intensity of Spi-B nuclear staining and percentage of Spi-B positive tumor cells revealed higher expression of Spi-B in SCLCs than in NSCLCs (Figure 1B). In addition, higher Spi-B staining intensity was associated with higher histologic grade of NSCLC (Figure 1C, Analysis of variance, p = 0.0018787). To assess the prognostic significance of Spi-B, we examined expression levels in resected NSCLC from subjects with known clinical outcomes. Subjects with stage II disease (n = 73), whose tumors had low Spi-B staining intensity (staining scores ≤ 4, n = 47) had longer survival times than those whose tumors had high staining intensity (staining scores > 4, n = 26), with median survivals of 48 months (low Spi-B) versus 39.5 months (high Spi-B, p = 0.001, Figure 1D). Consistently, a highly significant negative correlation was found between Spi-B expression level and overall survival time in 9 SCLC subjects with available survival data (Figure 1E). Analysis of Spi-B staining intensity in another cohort of 62 primary NSCLC tissues (stage II), including 43 primary NSCLC tissues without lymphatic metastasis and 19 primary NSCLC tissues with different degrees of lymphatic
metastasis, revealed that increased Spi-B expression was associated with lymphatic metastasis (Figure 1F). These results indicate that the lymphocytes-restricted protein, Spi-B, is ectopically expressed in lung cancers, and its high expression level correlates with poor prognosis in human lung cancer.

**Spi-B expression promotes lung metastasis in vivo**

To test the functional consequences of Spi-B expression in lung cancers in vivo, lentiviral constructs were introduced into mouse Lewis lung carcinoma (LLC1) cells, which lack detectable endogenous Spi-B expression, and the metastatic capability was assessed. Subcutaneous engraftment of Spi-B-expressing LLC1 cells formed comparable size of primary tumors but produced significantly more lung metastatic nodules, compared with vector-transduced cells (Figure 2A and B), indicating a prometastatic role of Spi-B. However, direct intravascular inoculation of cells into the tail vein of syngeneic mice, which bypasses the local invasion and vascular invasion steps, showed a modest increase in numbers of metastatic nodule in lung in mice bearing Spi-B-expressing LLC1 cells, but this did not reach statistical significance (p = 0.0849) (Figure 2C and D). Thus, Spi-B expression may function at early events of metastasis, without markedly affecting the later metastatic cascades including circulation, extravasation and colonization.

We next examined Spi-B expression in 27 primary human lung cancer tissues and their matched lymphatic metastases. Consistently, enriched Spi-B expressing lung cancer cells were observed in lymphatic metastases compared to their corresponding primary tumor (Figure 2E). Quantitative analysis revealed significantly higher Spi-B staining intensity in lymphatic metastases than in primary lung cancer tissues (Figure 2F). Altogether, these data indicate that Spi-B expression promotes lung cancer metastasis via increasing early dissemination of cancer.
cells from their primary sites.

**Spi-B disrupts intercellular junctions and increases invasive capacity in vitro**

We next investigated the effects of Spi-B expression in established lung cancer cell lines in vitro. We examined Spi-B expression in various lung epithelial cell lines, including 1 immortalized human bronchial epithelial cell line (HBECs), 4 NSCLC cell lines and 3 SCLC cell lines. Spi-B mRNA was detected in 1 NSCLC cell line (H1155) and 3 SCLC cell lines (H526, H69, H82) (Figure 3A), consistent with the immunostaining results showing that more cases of SCLC expressed Spi-B than NSCLC. Given that Spi-B is primarily expressed in cancer cells with mesenchymal attributes in human primary NSCLC tissues, we tested whether Spi-B can induce the epithelial-mesenchymal-transition (EMT) program. Transient expression of Spi-B in HBECs caused elongated morphology in two-dimensional culture and inhibited expression of the epithelial marker E-cadherin in HBECs (Figure S1A and B), however, Spi-B did not alter expression of the mesenchymal marker Vimentin or the expression of EMT-related transcription factors (Snail1, Snail2, ZEB1 and Twist1) (Figure S1C), indicating that Spi-B alone is not sufficient to induce the EMT program; other oncogenic mutations may be required. Indeed, knockdown of Spi-B in H1155, a NSCLC line that expresses endogenous Spi-B and have mesenchymal phenotype, resulted in upregulation of E-cadherin and downregulation of Vimentin, Snail1, ZEB1 and Twist1 (Figure S1B and C), fostering epithelial characteristics of H1155. These data suggest that Spi-B might be essential in maintaining the mesenchymal phenotype of lung cancer cells although it is not able to induce the EMT program in epithelial cells.

Then, we transduced lentiviral constructs into A549 and H460 cells, two NSCLC lines that do not express endogenous Spi-B, and analyzed the resultant biological effect. Spi-B expression did
not alter the number of colonies formed in soft agar (Figure 3B and C). Interestingly, Spi-B expression caused significant morphological change of the clones in soft agar. Whereas control cells formed tightly organized spheres, 43% of Spi-B-expressing A549 cells and 38% of Spi-B-expressing H460 cells appeared dispersed and exhibited fewer cell-cell interactions (Figure 3B and C). In addition, while control cells developed into round or oval cell aggregates with strong cell-cell adhesion in three-dimensional basement membrane gels, a major portion of Spi-B-expressing cancer cells developed into a grape-like morphology with poorly adhesive colonies (Figure 3D and E). Transmission electron microscopy (TEM) showed impaired formation of tight and adherence junctions in Spi-B-expressing cells compared to control cells in three-dimensional basement membrane gels (Figure 3F and G). Consistently, knock-down of Spi-B in H526 cells resulted in tighter cell aggregates with strong cell adhesion in two-dimensional culture (Figure S2A). These data indicate that Spi-B expression disrupts intercellular junctions.

In addition, Spi-B overexpression caused enhanced invasiveness of A549 and H460 cells through Matrigel (Figure 3H and I). Conversely, knock down of Spi-B in H526 cells caused reduced invasion (Figure 3J). Spi-B overexpression also increased motility of A549 cells on collagen-coated plates in scratching assays (Figure 3K). Transient expression of Spi-B had no effect on cell proliferation or adherence to fibronectin, a major extracellular matrix component, as evaluated by BrdU incorporation analysis and replating assay (Figure S2B and C). Altogether, these data indicate that the major function of Spi-B in epithelial cancer cells is to disrupt the intercellular junctions and promote invasion.

**Spi-B downregulates tight junction genes**

To better understand the molecular mechanisms of Spi-B-mediated promotion of metastasis, we expressed Spi-B in A549 cells and used a microarray platform to analyze the resultant
expression profile. Genes for which expression was substantively altered in the stable Spi-B-expressing cell lines were functionally classified by gene ontology. Through analyses of enrichment within each category, two groups of genes that significantly changed expression levels (2-fold in either direction) after Spi-B overexpression were identified. Tree 1 included 278 downregulated genes and tree 2 contained 315 upregulated genes on Spi-B overexpression (Figure 4A). Functional profiling of these downregulated genes revealed that the major proportion of the genes was associated with regulation of cell interactions including regulation of actin cytoskeleton and tight junctions, which is consistent with the morphological changes observed in Matrigel and soft agar. The upregulated genes are involved in the BCR signaling pathway, which is also consistent with the physiological role of Spi-B. Individual Spi-B-regulated genes and subgroups of different molecular pathways are shown in Tables S2 and S3.

We used quantitative RT-PCR to confirm Spi-B-dependent differential transcription of 9 genes identified in the microarray analysis, including the downregulation of tight junction genes CLDN2, CLDN14, CGN, and CRB3, and the upregulation of PGF, FOS, RAC2 and TNFSF10, which promotes cancer metastasis, and upregulation of MMP9, which degrades extra cellular matrix and enhances tumor invasion (Figure 4B). The upregulation of secreted active MMP9 by Spi-B was further confirmed by gelatin zymography (Figure 4C). Consistently, knockdown of Spi-B in H1155 cells caused reciprocal changes in expression levels of these genes compared with expression of Spi-B in A549 cells (Figure 4B). These data indicate that Spi-B regulates tight junctions, proteases, and metastasis-related genes.

**Expression of Spi-B is negatively correlated with Claudin-2 in human primary lung cancer tissues**

Epithelial cells interact with neighboring cells through various intercellular junctions such as
tight, gap and adherens junctions. Tight junctions are associated with cell polarity and permeability (20). Given that overexpression of Spi-B in epithelial cancer cells resulted in the disruption of cell-cell adhesion and the loss of polarity, we focused on the tight junction genes that were downregulated by Spi-B expression. Claudin-2, a key component within tight junctions and whose downregulation was associated with breast cancer metastasis (21-23), was primarily expressed in bronchial epithelium in lung tumor-adjacent tissue (Figure S3A) and detected in some epithelial cells of alveoli in lung tumor-adjacent tissue (Figure S3B). Downregulation of Claudin-2 by Spi-B was confirmed by immunoblot at the protein level (Figure 5A).

To define the correlation between Spi-B and Claudin-2 in individual primary lung cancer tissues, we used IHC to study the expression levels of Spi-B and Claudin-2 in serial sections of NSCLC samples. The representative pictures are shown in Figure 5B. Claudin-2 was expressed in cancer cells that located in the central part of the lung cancer mass. These cancer cells did not express Spi-B. The invasive cancer cells at the tumor-stromal border expressed significant levels of Spi-B but lacked Claudin-2. When intratumoral staining was quantified, a highly significant negative correlation was found between Spi-B and Claudin-2 expression (Figure 5C). Therefore, endogenous Spi-B represses Claudin-2 in human lung cancer tissues.

**Spi-B promotes metastasis by repressing Claudin-2**

To assess whether Spi-B promotes cancer metastasis through Claudin-2 repression, we first tested the cellular effect of Claudin-2 depletion. The efficiency of Claudin-2 knock-down is shown in Figure 6A. Claudin-2 depletion in A549 cells exhibited similar cellular effects as Spi-B overexpression, including decreased cell-cell adhesion in soft agar, the loss of polarity in 3D Matrigel, and enhanced invasion through Matrigel (Figure 6B, C and D).

We next re-expressed Claudin-2 in Spi-B-overexpressing A549 cells (Figure 6E). As expected,
re-expression of Claudin-2 completely restored the formation of round cell aggregates with strong cell-cell adhesion in soft agar (Figure 6F), and decreased the proportion of grape-like organoids in three-dimensional basement membrane gels (Figure 6G). In addition, re-expression of Claudin-2 in Spi-B-transfected A549 cells partially abolished Spi-B-induced invasion through Matrigel (Figure 6H). However, re-expression of other Spi-B downregulated tight junction proteins, Claudin-14 and Crumbs 3, was not able to restore these changes in cellular behavior caused by Spi-B overexpression (Figure S4A, S4B and S4C). Therefore, the changes in cellular behavior caused by enforced Spi-B expression are primarily due to repression of Claudin-2.

To determine whether Spi-B promotes metastasis in vivo through Claudin-2 repression, we re-expressed Claudin-2 in Spi-B-expressing luciferase-labeled LLC1 cells and subcutaneously injected the cells into C57BL/6 mice as described above. Two weeks after removal of the primary tumors, the whole lung tissues were collected for protein extraction and to perform luciferase assays to quantify metastases. Re-expression of Claudin-2 reduced the numbers of both visible lung metastatic nodules and the metastatic luciferase-expressing LLC1 cells to baseline levels (Figure 6I and J). Therefore, we identified Claudin-2, a tight junction protein, as the main target of Spi-B in promoting metastasis of lung cancer cells. These results offer an explanation for the association of Spi-B with lymphatic metastasis and overall short survival in human lung cancer patients.

Thus, we propose a model whereby ectopic expression of Spi-B by some cancer cells at the tumor-stromal border disrupts intercellular junctions by silencing CLDN2 transcription, facilitating the dissociation of cancer cells from the primary epithelial carcinoma cell sheet on one hand, and promoting the dissociated cancer cells to invade through the surrounding stroma by increasing MMP9 expression on the other hand. Therefore, Spi-B-expressing cancer cells
have higher metastatic capability (Figure 6K).

**Spi-B establishes a long-range silencer-promoter interaction and represses CLDN2 transcription**

We next explored the mechanism by which Spi-B silences Claudin-2 expression. Claudin-2 is transcribed in A549 cells but not in H1155, H526, H69 and H82 cells (Figure 7A). This expression pattern for Claudin-2 is opposite from that which we found for Spi-B. CLDN2 contains three alternative promoters generating three differential transcript variants with distinct 5’ untranslated regions, yet encoding identical proteins. The ChIP analysis to scan 10 regions along CLDN2 for distribution of H3K4Me3, which marks active promoters, and H3K9acetyl, which associates with the promoter and early coding region of active genes, revealed enrichment of these two modified histones in P1 (Promoter1) in A549 cells (Figure 7B), indicating that P1 is the active promoter in A549 cells. To further study whether the CLDN2 gene is regulated directly by Spi-B, we examined the in vivo binding status of Spi-B in genomic loci of CLDN2 using chromatin immunoprecipitation (ChIP). Cis-regulatory elements are identified by DNase I hypersensitive sites, as reported in the ENCODE database, and potential Spi-B binding consensus sites were analyzed for Spi-B association (Figure 7C). The ChIP analysis revealed the occupancy of endogenous Spi-B in H526 cells, as well as the transfected Spi-B in A549 cells at three regions (sites 2, 4 and 8) upstream of the P1 (Figure 7C). We termed these three Spi-B occupied cis-regulatory elements as S1 (-26,001 to -24,893), S2 (-21,765 to -21,450) and S3 (-7,479 to -7,201), which are 25kb, 21kb and 7.5kb upstream of the active promoter, respectively.

Long range communication requires physical interaction (24,25). Assuming that S1, S2 or S3 controls CLDN2 gene transcription, physical interactions between these three cis-regulatory elements with the promoter are expected in active or silenced CLDN2. We therefore performed a
chromosome conformation capture (3C) assay to explore the chromatin configuration of the CLDN2 gene in A549 and H526 cells, focusing on DNA fragments carrying the S1, S2, S3 and the P1. Briefly, cross-linked chromatin was digested with Dpn II, diluted, re-ligated, and long range association frequencies were assessed with PCR. Indeed, physical interactions between the S3 and the P1 were detected in the active CLDN2 gene in A549 cells when using P1 as an anchor fragment (Figure 7D). In addition to the S3-P1 interaction, the S1-P1 interaction was also detected in the silenced CLDN2 gene in H526 cells (Figure 7D). The S2, however, did not interact with the promoter either in A549 or H526 cells (Figure 7D). Consistently, the H526-specific S1-P1 interaction was also detected when using the S1 as the anchor (Figure 7E). These results suggest that the physical interaction between the Spi-B-occupied cis-regulatory element S1 and the promoter may be associated with CLDN2 gene silencing. Then, we overexpressed Spi-B in A549 cells and evaluated the effect of Spi-B on chromatin configurations of CLDN2. Expectedly, transient expression of Spi-B caused juxtaposition of the S1 to the P1 but had no effect on the interaction between the S3 and the P1 (Figure 7F). Thus, aberrant activation of Spi-B in epithelial cancer cells establishes a physical interaction between a cis-regulatory element, S1, with the active promoter of CLDN2.

To investigate the functional consequence of juxtaposition of S1 to the P1 on CLDN2 transcription, we performed luciferase reporter gene assays, a commonly used method for promoter and cis-regulatory element (enhancer or silencer) characterization (26,27). We cloned the S1 or the S3 upstream of the P1 flanking the luciferase gene and transfected the constructs into HEK293 cells that normally express Spi-B (Figure 7G). While the S3 has no effect on the promoter activity, ectopic placement of the S1 upstream of the P1 completely reduced the promoter activity to the baseline level (Figure 7H), indicating that the S1 functions as a silencer.
of the P1. Two Spi-B-binding consensuses were identified within the S1. EMSA with super shift assay showed direct binding of Spi-B to these two sequences in vitro (Figure 7I). Cotransfection of SPIB shRNA or mutation of two Spi-B binding sites within the S1 partially restored promoter activity (Figure 7J), suggesting that in addition to establishing a silencer-promoter interaction Spi-B binding to the silencer represses promoter activity.

These data support a model whereby CLDN2 transcription requires decolocalization of an upstream silencer, S1, with the promoter. Spi-B associates with multiple upstream sites including those within the S1, establishing silencer-promoter interactions, and blocking CLDN2 transcription (Figure 7K).

**Discussion:**

For many years, the hypothesis that pathologic processes can be achieved by co-opting a series of physiological processes has been discussed. In the present study, we identified Spi-B, a lymphocyte restricted transcription factor, as a master modulator of invasion of lung cancer cells. Physiologically, expression of Spi-B starts in pre-B lymphocytes and reach a peak in immature and mature B lymphocytes (6,28), which parallels the process of expansion of developmentally mature B lymphocytes from bone marrow to peripheral circulation. Loss of Spi-B can cause an increased pre-B population within the bone marrow environment with diminished recirculating B lymphocytes (29). Therefore, Spi-B is critical for the departure of developmentally mature B lymphocytes from their developmental niche. Similarly, ectopic expression of Spi-B in lung cancer cells promotes their dissemination from the primary sites. From this point of view, Spi-B may activate functionally similar programs in lung carcinoma.

Spi-B is first identified as a transcriptional activator, directly activating c-Rel (29), P2Y10 (30), and p50 (31). Our results show that Spi-B can also act as a transcriptional repressor.
Multiple transcription factors including Miz-1 and GATA3 have been found to act as both a transcriptional activator and repressor. It was reported that whether they function as a transcriptional activator or a repressor depends on their interactions with other transcriptional regulators (32,33). Spi-B expression endows lung cancer cells with two properties that are normally required for expansion of developmentally mature B lymphocytes. Firstly, Spi-B downregulates proteins that constitute intercellular junction complexes, resulting in disruption of tight and adherens junctions. It has been documented that disruption of tight and adherens junctions impairs epithelial polarity and differentiation, inducing EMT and metastatic behavior (34,35). Consistent with that, Spi-B is mainly expressed in the invasive cancer cells at the tumor-stromal border in human primary lung cancer tissues. These Spi-B-expressing cancer cells co-express the mesenchymal marker Vimentin but lack the epithelial marker E-cadherin, and thus, exhibit mesenchymal attributes. Enforced expression of Spi-B in lung cancer cell lines downregulates E-cadherin expression and increases cell motility and invasive capability in vitro. Depletion of Spi-B in lung cancer cell lines that express endogenous Spi-B and have mesenchymal attributes promotes MET. These findings highlight the role of Spi-B in EMT program induction. Because Spi-B is not able to induce expression of Vimentin or EMT-TFs in HBECs, and it has been documented that transformation of HBECs requires series oncogenic mutations and/or genetic manipulation accompanied with microenvironmental induction (36-38), it is possible that Spi-B may promote EMT through disruption of intercellular junctions, but this effect requires other oncogenic mutations or specific microenvironment.

Secondly, Spi-B markedly enhance expression of MMP9, a key protease that is highly expressed in lymphocytes for matrix proteolysis to facilitate their departure from bone marrow (39-41), conferring carcinoma cells the ability to remodel the surrounding matrix. Multiple steps
of the invasion process require protease-mediated matrix remodeling. For example, protease is required for carcinoma cells to breach the basement membrane that confine the tumor to a local position, to liberate growth factor molecules that are tethered to the basement membrane or stroma, and to invade the stroma. Epithelial cells are unable to produce proteases. Carcinoma cells that retain epithelial markers follow behind stromal fibroblasts that remodel the extracellular matrix to invade (42). Mesenchymal cells are capable of matrix remodeling (42), which is consistent with our result that Spi-B is expressed in cancer cells with mesenchymal attributes and upregulates MMP9. Knockdown of MMP9 or overexpression of its inhibitor tissue inhibitor of metalloproteinases 1 (TIMP1) abrogated Spi-B-induced invasion through matrigel in vitro (Figure S5A and B). In addition to degrade matrix, MMP9 is involved in TGF-β activation (43), angiogenesis by increasing VEGF bioavailability (44) and is critical for both the invasion of the primary tumor and the formation of the metastatic niche (42,45-47).

Invasion is a fundamental step in tumor progression and a driving force for metastasis. Solid tumors invade the adjacent tissue by single cancer cells or a cluster of connected tumor cells (47,48). Two models have been proposed for individual cancer cell invasion: mesenchymal invasion or ameboid invasion. Mesenchymal invasion is characterized by an elongated morphology that requires extracellular proteolysis localized at cellular protrusions, whereas ameboid invasion is characterized by a rounded morphology and proteolysis independent (49). These two invasion modes are both important for carcinoma metastasis and interconvert into each other to adapt to the altered environment (50). Our results lead us to suggest that Spi-B participates in the mesenchymal invasion of lung cancers. The significant association of Spi-B expression in human lung cancers with invasive behavior substantiates its clinical significance in both prognosis and therapy.
Acknowledgements

The authors thank Baocun Sun at Tianjin Medical University for advice.

References


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**Figure Legends**
Figure 1. Spi-B is expressed in lung cancer cells and predicts poor prognosis. A. IHC staining with anti-Spi-B was performed on 10 tumor-adjacent normal lung tissues, 130 NSCLC, and 14 SCLC specimens. Representative fields show lack of Spi-B staining in normal lung epithelial cells but positive Spi-B staining in lymphocytes (arrows) in tumor adjacent tissue (a), positive Spi-B staining in human lung adenocarcinoma (b), squamous lung carcinoma (c) and SCLC (d). (e-g) Series sections of human primary lung cancer tissues stained with Spi-B (e), E-cadherin (f) and Vimentin (g). Scale bars are 50 μm. Arrows indicate the invasive cancer cells. B. The frequency of cases with no (0), low (0.1-3.9), or high (4.0-8.0) Spi-B staining stratified by IHC-defined lung cancer subtype. C. The frequency of cases with no (0), low (0.1-3.9), or high (4.0-8.0) Spi-B staining stratified by tumor grade. D. Kaplan-Meier survival rates for 73 subjects with stage II NSCLC disease with low (staining scores ≤ 4, n = 47, red line), versus high (staining scores > 4, n = 26, blue line) Spi-B expression were compared. Median survivals were 48 months (low Spi-B) versus 39.5 months (high Spi-B; p = 0.001). E. Semiquantitative scoring was performed and Spi-B scores were correlated with overall survival time of SCLC patients (r²=0.874, p=0.0002). F. Spi-B expression in human primary lung cancer tissues stratified by lymphatic metastasis.

Figure 2. Spi-B promotes lung metastasis. A. LLC1 cells expressing empty vector or SPIB were subcutaneously injected into 8-week-old female C57BL/6 mice. Two weeks later the primary tumors were removed. Another two weeks later the mice were sacrificed and the lung metastases were analyzed. Left panel shows the images of primary tumors. Middle panel shows the representative images of lungs. Black arrow indicates metastatic nodules. Right panel shows quantitation of lung metastatic nodules. B. Representative hematoxylin and eosin stain of lungs from mice subcutaneously injected with vector or SPIB-expressing LLC1 cells. Scale bars are 50
μm. C. LLC1 cells expressing empty vector or SPIB were injected into the tail vein of 8-week-old female C57BL/6 mice. One month later the mice were sacrificed and the lung metastases were analyzed. Left panel shows the representative gross lung pictures. Black arrow indicates metastatic nodules. Right panel shows quantitation of lung metastatic nodules. D. Representative hematoxylin and eosin stain of lungs from mice injected via tail vain with vector or SPIB-expressing LLC1 cells. Scale bars are 50 μm. E and F. IHC staining with anti-Spi-B was performed on 27 primary human lung cancer tissues and their matched lympho node metastases. Representative pictures of Spi-B staining in human primary lung cancer tissues and lymphatic metastases are shown in (E). Scale bars are 50 μm. Quantified Spi-B expression in primary human lung cancer tissues and lympho node metastases is shown in (F).

Figure 3. Spi-B disrupts intercellular junctions and promotes invasiveness and migration. A. RT-PCR shows Spi-B transcription in HBECs and various lung cancer cell lines. B and C. Spi-B-expressing cancer cells and control cells were allowed to grow in soft agar for 3 weeks, and colonies were counted (upper panels). Representative images of the clones are shown in the lower panel. The bar chart shows the percentage of mass (blue), and loose colonies (red). Error bars indicate means ± SD for a representative experiment performed in triplicate. Scale bars represent 100 μm. D and E. Phase-contrast micrographs of control and SPIB-expressing cancer cells cultured on Matrigel for 8 days. The bar chart shows the percentage of mass (blue) and grape-like (red). Error bars indicate means ± SD for a representative experiment performed in triplicate. Scale bars represent 25 μm. F and G. TEM sections shows intercellular junctions of control and SPIB-expressing cancer cells cultured on Matrigel for 8 days. The bar chart shows the number of tight junctions per field. Error bars indicate means ± SD for eight fields in a representative experiment. Scale bars represent 0.2 μm. H and I. Control and SPIB-expressing
cancer cells were subjected to an invasion assay. Error bars indicate means ± SD for a representative experiment performed in triplicate. Scale bars represent 100 μm. J. H526 cells expressing control or shRNA against Spi-B were subjected for invasion assay. The efficiency of Spi-B knockdown is shown in upper panel. Error bars indicate means ± SD for a representative experiment performed in triplicate. K. Images of wound-healing assay showed the motility of control and SPIB-expressing A549 cells.

Figure 4. Changes of gene expression profiling upon Spi-B expression. A. Functional profiling of genes differentially expressed between control A549 cells and SPIB-expressing A549 cells. Double-headed arrows indicate 315 genes upregulated and 278 genes downregulated by Spi-B. Representative Spi-B-induced (red) and Spi-B-repressed genes (green) are listed vertically (left) and under each molecular pathway (right). B. Quantitative RT-PCR was performed to confirm the transcriptional change of indicated genes identified in the microarray. RNA was purified from control or SPIB-expressing A549 cells and control or Spi-B shRNA-expressing H1155 cells. Relative expression is shown as fold differences relative to GAPDH. C. Representative zymograms showed gelatinolytic activity of MMP9 in control or Spi-B-expressing A549 cells.

Figure 5. Spi-B negatively correlates with Claudin-2 in human lung cancer tissues. A. Immunoblots show expression of Claudin-2, Spi-B and Actin. B. IHC for Spi-B and Claudin-2 was performed on 39 human primary lung cancer specimens (stage I-II). Representative fields show strong Spi-B staining in peripheral lung cancer cells. Cancer cells in the center field of the tumor mass are Spi-B negative. Antibodies to Claudin-2 stain the cancer cells in the center field of the tumor mass; peripheral cancer cells are negative for Claudin-2 staining. C. Semi-quantitative scoring was performed and Spi-B scores were correlated with Claudin-2 scores ($r^2=0.236$, $p=0.00054$).
Figure 6. Spi-B disrupts intercellular junctions and promotes invasion by silencing Claudin-2 expression. A. Immunoblots show the efficiency of Claudin-2 knockdown. B. A549 cells were transduced with control or shRNAs against Claudin-2 as indicated. The cells were grown in soft agar for 3 weeks. The representative images of the clones are shown in the left panel. The bar chart shows the percentage of mass (blue), and loose colonies (red). Error bars indicate means ± SD for a representative experiment performed in triplicate. Scale bars are 100 µm. C. A549 cells were transduced with control or shRNAs against Claudin-2 as indicated and plated in Matrigel. Phase contrast of acini were shown in left panel and quantified in right panel. The bar chart shows the percentage of mass (blue) and grape-like (red). Error bars indicate means ± SD for a representative experiment performed in triplicate. Scale bars represent 25 µm. D. A549 cells were transduced with control or shRNAs against Claudin-2 as indicated and subjected to an invasion assay. Error bars indicate means ± SD for a representative experiment performed in triplicate. E. A549 cells were transduced with Spi-B or Spi-B and Claudin-2 as indicated. Immunoblots show expression of Spi-B, Claudin-2, and actin. F. A549 cells were transduced with Spi-B or Spi-B and Claudin-2 as indicated. The cells were grown in soft agar for 3 weeks. The representative images of the clones are shown in the left panel and the percentages of clones with differential morphology are quantified as the right panel. The bar chart shows the percentage of mass (blue), and loose colonies (red). Error bars indicate means ± SD for a representative experiment performed in triplicate. Scale bars are 100 µm. G. A549 cells were transduced with Spi-B or Spi-B and Claudin-2 as indicated and plated in Matrigel. Phase contrast of acini are shown in left panel and quantified in right panel. The bar chart shows the percentage of mass (blue) and grape-like (red). Error bars indicate means ± SD for a representative experiment performed in triplicate. Scale bars represent 25 µm. H. A549 cells
were transduced with Spi-B or Spi-B and Claudin-2 as indicated and subjected to an invasion assay. Error bars indicate means ± SD for a representative experiment performed in triplicate. Scale bars represent 100 μm. I-J. LLC1-Luc cells were transduced with Spi-B or Spi-B and Claudin-2 as indicated. The cells were subcutaneously injected into C57BL/6 mice. Two weeks later, the primary tumors were removed and the lung metastases were analyzed. The images of the primary tumors (left panel), the lung (middle panel) and lung metastatic nodules (right panel) are shown in (I). The proteins were extracted from the whole lung tissue and subjected to luciferase assay. The luciferase activity of each lung is shown in (J). * indicates p<0.05. K. Schematic showing the role of Spi-B in epithelial cancer metastasis. Spi-B is activated in the cancer cells at the tumor-stromal border. Spi-B expression downregulates tight junction protein Claudin-2, facilitating dissociation of cancer cells from the epithelial cell sheet on one hand, and promoting local invasion through upregulation of MMP9 on the other hand.

Figure 7. Spi-B establishes S1-CLDN2 promoter interactions. A. RT-PCR shows transcription of CLDN2 and GAPDH. B. Quantitative ChIP shows distribution of H3K4me1 and H3K9ac histone modifications in A549 cells. Location of regions assessed by ChIP is shown in schematic. Error bars represent SD of three independent chromatin preparations. C. Quantitative ChIP was performed showing association of Spi-B in H526 cells and Spi-B-Flag in transfected A549 cells with regions 2-4. Schematic showing DNase I hypersensitive sites (vertical lines) and Spi-B binding consensus (red stars). Bar graphs show fold enrichment of Spi-B binding. D and E. 3C was used to calculate cross linking frequency between chromatin segments to assess proximity in A549 (Claudin-2-expressing, solid circles) and H526 (Claudin-2-nonexpressing, open circles) cells. Vertical lines represent Dpn II restriction sites; arrows indicate PCR primer sites and direction. Anchor symbols mark anchoring primer for each data set. Cross linking frequency
between different segments and P1 (D), S1 (E) is shown. Top panels show representative PCR products. Mean ± SD of 3 independent chromatin preparations is shown. F. Cross-linking frequency is shown using the P1 as anchor in A549 cells transiently expressing Spi-B (open circles) or vector control (solid circles). Mean ± SD of three independent experiments is shown. G. RT-PCR shows transcription of SPIB and GAPDH. H. Luciferase reporter activity is shown with ectopic placement of S1 or S3 adjacent to the P1. Error bars indicate means ± SD for a representative experiment performed in triplicate. I. EMSA shows mobility shift of probe with S1 sequence containing the two Spi-B consensus sites, with supershift following anti-Spi-B treatment. J. Cotransfection of Spi-B shRNA or double mutation of two Spi-B binding consensus within S1 partially abrogated S1 activity in luciferase assay. Error bars indicate means ± SD for a representative experiment performed in triplicate. K. Schematic showing the mechanism by which Spi-B represses Claudin-2 transcription. Spi-B associates with silencer S1 and establishes physical interaction between S1 and P1, thereby repressing P1 activity.
Figure 1
Figure 2

A

Vector

SPIB

Primary tumor

Vector

SPIB

Lung

B

C

D

E

F

p=0.001

Vector

SPIB

p=0.0849

Vector

SPIB

p<0.0001

primary lung cancer

lymph node metastases

SPIB expression
Figure 3
Figure 4
Figure 5
Figure 6
Spi-B-mediated silencing of Claudin-2 promotes early dissemination of lung cancer cells from primary tumors

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Cancer Res Published OnlineFirst July 28, 2017.

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