Neutrophils recruited by NKX2-1 suppression via activation of CXCLs/CXCR2 axis promote lung adenocarcinoma progression

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Abstract

**Background:** Lung adenocarcinoma (LUAD) progression is dependent on the immune tumor microenvironment through paracrine signaling. NKX2-1 is the lineage-specific transcription factor that serves as a well-characterized pathology marker to define LUAD with progressive impact in patients. However, the involvement of NKX2-1 in modeling the tumor immune microenvironment is still unclear. Here, we demonstrated that NKX2-1-low tumors expedite tumor progression in LUAD by recruiting tumor-promoting neutrophils.

**Method:** Single-cell RNA sequencing and Visium in situ capturing profiling were used to characterize the infiltration of neutrophils in orthotopic syngeneic tumors. Clinical relevance of NKX2-1 expression and disease status were evaluated by immunohistochemical analysis of LUAD tissue arrays and the overall survival analysis was performed by using TCGA dataset. Chemokine secretion was analyzed by chemokine array and validated by qRT-PCR. ATAC-seq was used to confirm the modulatory role of NKX2-1 on the chromatin accessibility of CXC chemokine genes.

**Results:** NKX2-1 downregulation was observed in high-grade LUAD with increased neutrophil recruitment and infiltration. NKX2-1 knockdown promoted the expression and secretion of CXCL1, CXCL2, CXCL3, and CXCL5 in LUAD cells. ATAC-seq revealed the restrictive regulation of NKX2-1 on the promoters of CXCL1, CXCL2, and CXCL5. Single-cell RNA sequencing and Visium in situ capturing revealed that the infiltrated neutrophils exhibited strong cell-cell communication through the activation of CXCLs/CXCR2 signaling with increased tumor growth and vice versa when inhibited with CXCR2 antagonist SB225002.

**Conclusion:** This study revealed that NKX2-1 negatively regulates the infiltration of tumor-promoting neutrophils by suppressing CXCLs/CXCR2-dependent mechanisms. Hence, targeting CXCR2 in NKX2-1-low tumors is a potential antitumor therapy that may improve LUAD patient outcomes.

INTRODUCTION

Lung cancer is the most prevalent and aggressive cancer worldwide. Non-small-cell lung carcinoma (NSCLC) comprises approximately 85% of lung cancer cases, and in turn can be classified into lung adenocarcinoma (LUAD), squamous cell carcinoma (SCC), and large cell lung carcinoma (LCLC) [1]. LUAD is a common NSCLC subtype associated with the highest mortality, increased recurrence rate, and short relapse-free survival [2]. Despite the advancements in lung cancer treatment, the prognosis of LUAD patients remains poor due to the challenges posed by metastasis and drug resistance [3]. High-grade LUAD is characterized by elevated tumor plasticity manifested in frequent cellular and molecular transitions, such as epithelial-mesenchymal transition (EMT), a process critical for metastasis [4]. Previous studies have shown that the alteration of the tumor microenvironment (TME) affects the infiltration of immune cells with concurrent LUAD tumor progression [5]. However, the detailed mechanisms governing the LUAD-associated immune microenvironment which involves a complex
The interplay of immune cells, cytokines, and paracrine-driven molecules interacting with the tumor, remains unclear.

The immune microenvironment comprises an intricate network of immune cells, cytokines, and various molecules interacting with cancer cells within the TME [6]. Among them are neutrophils, a type of innate immune cells that play a vital role in responding to infection and inflammation [7]. Neutrophils are abundant in the bloodstream, comprising approximately 70% of all white blood cells in humans and 10–20% in mice [8]. The activation of CXCR2 is a critical step in triggering the migration of neutrophils from the bone marrow [9], whereas CXCR4 regulates the retention of neutrophils in the bone marrow [10]. In solid tumors, neutrophils can exhibit anti-tumorigenic activity involving cytotoxicity against tumor cells, as well as promote tumor growth, angiogenesis, metastasis, and immune evasion [8, 11]. The infiltration of tumors with neutrophils is mediated through the chemotactic effects of CXC chemokines by binding to their receptor, CXCR2 [12]. For instance, CXCL8 is a common ligand of CXCR2 that is mostly secreted by different types of tumors and controls neutrophil recruitment [13]. Additionally, the activation of CXCL8/CXCR2 signaling promotes tumor angiogenesis, metastasis, chemoresistance, and tumor progression [14, 15].

Cell plasticity facilitates the transition between cell lineages and the emergence of drug resistance in certain cancer subtypes, contributing to increased tumor aggressiveness [16]. These transitions are characterized by massive changes in gene expression programs regulated by master regulator transcription factors (TFs). Among them is NK2 homeobox 1 (NKX2-1), which is predominantly expressed during lung and thyroid development [17] and serves as a lineage-specific TF that determines alveolar cell identity [18]. In lung cancer, NKX2-1 regulates the identity of LUAD through the enforcement of differentiation programs, meanwhile, NKX2-1 downregulation confers worse disease outcomes in poorly differentiated tumors [19]. On the contrary, the process of transdifferentiation from adenomatous to more aggressive squamous histological type is associated with elevated transcriptional activity of SOX2, a well-known TF in squamous cell carcinoma [20]. SOX2 is known to change the phenotype of squamous cell carcinoma to club and alveolar type 2 (AT2) cells [20]. Furthermore, it enhances the generation of neural progenitor cells in lung epithelial cells [21]. The balance between SOX2 and NKX2-1 plays a critical role in determining the shifts in cellular lineage that modulate lung cancer progression due to the property of lineage-specific TFs to govern differentiation status that is typically associated with the degree of tumor malignancy [18].

Recently, it has been demonstrated that neutrophil plasticity and heterogeneity underlie adverse events that may result in the discontinuation of immunotherapy [8]. SOX2 has been characterized as an important TF that recruits neutrophils into the TME through the regulation of CXCL3 and CXCL5 expression, thus facilitating cancer progression [16]. However, the implication of NKX2-1 in attracting immune cells to the TME and mediating the progression of LUAD remains unclear. Here, we demonstrated that low expression of NKX2-1 correlates with high neutrophil infiltration which predicts poor clinical outcomes in LUAD patients. Our single-cell and Visium in situ capturing NGS analyses revealed a strong cell-cell communication between NKX2-1-low tumors and neutrophils through CXC chemokines regulation
and the activation of CXCLs/CXCR2 signaling. The inhibition of CXCR2 chemokine receptor with specific inhibitor SB225002 decreased the infiltration of tumor-promoting neutrophils with reduced tumor growth in NKX2-1-low tumor.

MATERIALS AND METHODS

Cell culture

HCC827 (delE746_A750), H1975 (L858R/T790M), HCC827/GR, and H1975/AZDR lung cancer cells were obtained from Dr. Yu-Ting Chou’s laboratory (National Tsing Hua University, Taiwan). HCC827/GR and H1975/AZDR cells were originally established by treating HCC827 and H1975 cell lines with the increased concentrations of gefitinib and osimertinib for 6 months, and the surviving cells were pooled together and cultured [24]. HL-60 (CCL-240) promyeoloblast cell line was obtained from the American Type Culture Collection (ATCC). These cells were cultured with RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, and 100 U/mL penicillin. Lewis lung carcinoma (LL2) cell line was obtained from Dr. Nien-Jung Chen’s laboratory (National Yang-Ming Chiao Tung University). These cells were maintained in DMEM medium supplemented with 10% FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin. All cells were maintained at 37°C with 5% CO2 and were all tested negative for mycoplasma contamination. Gefitinib and osimertinib were purchased from Selleck Chemicals (Houston, TX, USA), and dissolved in dimethyl sulfoxide (DMSO; MP Biomedicals, Santa Ana, CA, USA) at a concentration of 10 mmol/L.

Animal experiments

All animals used in this study were bred and maintained according to the Guidelines for Laboratory Animal Welfare in the Taipei Veterans General Hospital under the supervision of the Department of Medical Research of Taipei Veterans General Hospital (IACUC No. 2021-047 and 2022-097).

Female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Eight to ten-week-old mice were used in all the experiments. The mice were housed and maintained under specific-pathogen-free (SPF) conditions in an animal facility. In in vivo experiments, C57BL/6 mice and Lewis lung carcinoma (LL2) cell lines were used. CXCR2 inhibitor (SB225002) was dissolved in 1% DMSO, 20% polyethylene glycol 400, 5% Tween 80, and 74% ddH2O. SB225002 was administered at 10 mg/kg by intraperitoneal injection every other day. Control groups received solvent (1% DMSO, 20% PEG 400, and 5% Tween 80). On day 0, LL2 cells expressing luciferase reporter and eGFP (shCtrl and shNKX2-1) cells were collected and resuspended in PBS. For the subcutaneous tumor model, 100 μL cell suspension containing 5 × 10^5 cells was injected subcutaneously into the flank region, while treatment started when the tumors were palpable. Mice were sacrificed on days 22–23. For the orthotopic lung cancer model, 20 μL cell suspension containing 5 × 10^5 cells was injected through intrathoracic injection. The treatment started on day 1 and ended on day 5. Mice were sacrificed on day 7.

Ethic statements and human samples
The experimental procedures and protocols involving human samples were conducted according to the tenets of the Declaration of Helsinki and were approved by the Institutional Review Board of Taipei Veterans General Hospital (protocol no. 2020-04-009B and 2020-10-003B). Human tissue samples were obtained after taking informed consent from the patients.

**Plasmids, shRNAs, and cell transfection**

shCtrl (pLKO.1), human shNKX2-1 (TRCN0000020449_NM_003317 and TRCN0000020450_NM_003317), and mouse shNKX2-1 (TRCN0000086265 and TRCN0000086267) were purchased from Academia Sinica RNAi core (Taipei, Taiwan). NKX2-1 (pcDNA3.1(+) wt TTF-1; 49989) overexpression plasmid, and pHAGE PGK-GFP-RES-LUC-W (46793) plasmid were obtained from Addgene (Watertown, MA, USA). To obtain lentivirus particles, the lentivirus vector was co-transfected with packaging and envelope plasmids (psPAX2 and pMD2G) into HEK 293T cells. Viral supernatants were collected 72 h after transfection, followed by ultracentrifugation at 82,700g for 2 h. Cells were infected with lentivirus and 8 µg/ml polybrene (Sigma-Aldrich, St Louis, MO, USA) according to the instructions of Addgene (http://www.addgene.org/). Subsequently, the cells were selected with puromycin (2 µg/ml) to establish stable cell lines.

For transient transfection, TransIT-LT1 Transfection Reagent (Mirus Bio, Madison, WI, USA) was used. All procedures were conducted according to the manufacturer's guidelines. Plasmids used in this study are listed in Supplementary Table 3. qRT-PCR and western blot were used to validate the knockdown efficiency by shRNAs.

**RNA extraction and qRT-PCR analysis**

The total RNA was exacted using RNeasy Mini Kit (QIAGEN, Hilden, Germany) and quantified by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). 1 µg of total RNA was subjected to first-strand complementary DNA synthesis using the SuperScript III Reverse Transcriptase Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. qRT-PCR reactions were performed using the SYBR Green kit in an ABI 7900 sequence detection system (Thermo Fisher Scientific) following the manufacturer's guidelines. The primers were designed using Primer Express Software v3.0.1 (Thermo Fisher Scientific) and are listed in Supplementary Table 1. The specificity of all primers was computer-tested using BLAST (National Center for Biotechnology Information, Bethesda, MD, USA) by homology search with the human or mouse genome and further confirmed by dissociation curve analysis. The relative expression of mRNA was determined by the 2−ΔΔCT method and normalized to the endogenous expression of GAPDH mRNA.

**Western blotting**

The cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing proteinase and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The total protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal protein concentrations were resolved by SDS-PAGE, transferred onto PVDF membranes (MilliporeSigma,
Burlington, MA, USA), and blocked with 5% skimmed milk in Tris-buffered saline with Tween 20. The membranes were incubated overnight at 4°C with primary antibodies listed in Supplementary Table 2. On the next day, the membranes were incubated with HRP-conjugated secondary antibodies. Immunoblots were visualized using the Immobilon Western Chemiluminescent HRP Substrate (MilliporeSigma). The blots were tested with GAPDH or α-tubulin antibodies to confirm equal protein loading.

**Human chemokine array**

For secreted protein expression analysis, the Proteome Profiler Human Chemokine Array Kit (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer’s protocol.

**RNA-seq analysis**

The purified RNA was used to prepare the sequencing library by TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) following the manufacturer’s instructions. Briefly, mRNA was purified from 1 µg of total RNA by oligo (dT)-coupled magnetic beads and fragmented into small pieces under elevated temperature. The first-strand cDNA was synthesized using reverse transcriptase and random primers. The adaptors were ligated after the generation of double-stranded cDNA and adenylation of 3’ ends of DNA fragments. The products were enriched by PCR and purified with AMPure XP system (Beckman Coulter, Brea, CA, USA). The libraries were qualified by Qsep400 System (BiOptic Inc., New Taipei City, Taiwan) and quantified by Qubit 2.0 Fluorometer (Thermo Fisher Scientific). The qualified libraries were then sequenced on an Illumina NovaSeq 6000 platform with 150 bp paired-end reads generated by Genomics, BioSci & Tech Co., New Taipei City, Taiwan.

The low-quality reads from the raw data were removed using the fastp (version 0.20.0 software). The filtered reads were aligned to the reference genomes using HISAT2 (version 2.1.0). The software featureCounts (v2.0.1) in the Subread package was applied to quantify the gene abundance. Differentially expressed genes were identified by DESeq2 (version 1.28.0) [4] or EdgeR (version 3.36.0). The functional enrichment analysis of Gene Ontology (GO) terms was implemented in an R package clusterProfiler (version 4.0.0).

**Chromatin immunoprecipitation quantitative real-time PCR (ChIP-qPCR)**

ChIP-qPCR was performed using a High Cell Number Chromatin Immunoprecipitation kit (Diagenode, Denville, NJ, USA). 1 × 10^7 cells/ml resuspended in PBS were processed according to the manufacturer’s instructions. Chromatin was sonicated using a Bioruptor sonicator (Diagenode) according to the manufacturer’s protocol and examined with an electrophoresis assay for shearing optimization. Enriched DNA was quantified by performing real-time PCR using SYBR Green qPCR Master Mix (Thermo Fisher Scientific) in an ABI 7900 sequence detection system (Thermo Fisher Scientific) following the manufacturer’s guidelines. The antibodies and primers used in ChIP-qPCR assays are listed in Supplementary Tables 1 and 2. The enrichment signal was normalized to input DNA.
Assay for transposase-accessible chromatin with sequencing (ATAC-seq)

ATAC-seq libraries were prepared by using an ATAC-seq kit (Diagenode; #C01080001) following the manufacturer's instructions. In brief, \(5 \times 10^6\) H1975 (shCtrl and shNKX2-1) cells were harvested, washed and the pellet was lysed. Nuclei were extracted, and tagmentation was performed according to the manufacturer's instructions. DNA was isolated by using a spin column and the transposase-processed DNA fragments were amplified by using the 2X High-Fidelity Master mix with 1 µl barcoded primers (#C01011035) for 13 cycles. AMPure XP beads (Beckman Coulter) were used to purify the DNA following the manufacturer's protocol. Qubit Flex Fluorometer was used to assess the DNA quality and integrity.

Sequencing was performed and the fastq files were analyzed by using ENCODE ATAC-seq pipeline for single-end reads with default parameters. The genome fasta file was processed by the build_genome_data.sh script (supplied with the ATAC-seq pipeline). The output mapping and peak files of each sample were further analyzed. The resulting files were visualized by using IGV viewer [55]. MEME suites 5.5.4 was used to find the motif enrichment.

Tissue microarrays

LUAD tissue microarray slides (TMAs) were obtained from US Biomax (Rockville, MD, USA). LC641 microarray panel contained 64 cases of LUAD, while the LC10013c microarray panel contained 48 cases of LUAD with matched adjacent normal lung tissues. TMA immunostaining was performed according to the manufacturer's protocol. The antibodies used are listed in Supplementary Table 2. The scores of immunoreactivity patterns of all tissues from TMA were examined at the Department of Pathology, Taipei Veterans General Hospital, under the supervision of the Department of Medical Research and Education of Taipei Veterans General Hospital.

Chemotaxis assay of neutrophils and co-culture experiment

8 µm pore size FluoroBlok cell culture inserts (Corning Inc., Corning, NY, USA) were used to perform a chemotaxis transwell migration assay. HL-60 cells were seeded in the transwell’s upper chamber, and the medium from LUAD cells (HCC827 and H1975) was added to the lower chamber. After 6 h incubation, the migrated cells were fixed with methanol and stained with propidium iodide. The stained cells were viewed under a microscope and further quantified using ImageJ.

For the co-culture experiment, the medium from shCtrl/H1975 or shNKX2-1/H1975 cell culture was used to culture HL-60 cells for 5 days. The medium was changed every other day.

Flow cytometry analysis

Lungs containing tumor nodules were collected after mice were sacrificed. The mouse lung tissues were dissected and cut into small pieces. Dissected tissues were incubated with 1 mg/mL collagenase type I
and DNase 1 in RPMI 1640 basic medium for 1 h at 37°C. After incubation, the digested tissue was passed through a 70 µm cell strainer to improve cell dissociation.

Red Blood Cell Lysis Buffer (154 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4) was added to the single-cell suspension to lyse red blood cells. The digested cells were washed three times and resuspended in PBS. 1 × 10⁶ cells/mL were stained with 1 µl fluorescence-conjugated antibodies (BD Biosciences, Franklin Lakes, NJ, USA; dilution 1:100) for 30 min in 100 µl PBS at 4°C. The cells were washed twice before flow cytometry analysis. Antibodies used are Pacific Blue-labeled mouse anti-human CD11b Antibody, PerCP/Cyanine5.5-labeled rat anti-mouse CD170 (Siglec-F), APC/Cyanine7- labeled rat anti-mouse Ly-6G, PerCP-labeled rat anti-mouse Ly-6C, APC/Cyanine7-labeled mouse anti-human CD11b, Human Integrin alpha M/CD11b PE-conjugated mouse anti-mouse (R&D; FAB16991P), PE/Cyanine7- labeled mouse anti-mouse Ly-6G, BV421-labeled rat anti-Mouse Siglec-F. Data acquisition was performed on BD FACSCanto II System Flow Cytometer (BD Biosciences) and was analyzed by FlowJo Software.

**Single-cell RNA-Sequencing (scRNA-seq)**

scRNA-seq libraries of LL2 expressing luciferase reporter- and eGFP cells (shCtrl, shNMX2-1, and shNMX2-1 treated with CXCR2 antagonist) were generated by using a Chromium Controller instrument (10x Genomics, Pleasanton, CA, USA) and Chromium Single Cell 3’ Reagent Kits v2 according to the manufacturer's instructions. scRNA-Seq reads were aligned to the mouse reference genome dataset and quantified using Cell Ranger version 6.1.0 (10x Genomics default pipeline. Raw count matrix files were imported into the R package Seurat version 4.1.1 for downstream processing. First, we used the package SoupX to remove ambient RNA contamination from raw scRNA-seq data. Cells with a gene number more than 500 and a mitochondrial gene proportion < 0.2 were selected for downstream analysis. The matrices used regularized negative binomial regression to normalize UMI count data by “SCTransform” (v2) and regress out the percentage of mitochondrial genes in each cell. We removed the doublet by “Doubletfinder” (estimate doublet ratio by 8‰ for every additional 1000 cells). After quality control and “SCTransform” (v2) normalization, the subsequent steps are based on the standard process of the “Seurat” package. 3000 feature genes are selected to integrate data. For the integrated data, “RunPCA” is used for data dimension reduction. The uniform manifold approximation and projection algorithm is used for visualizing the dimension reduction data by the “RunUMAP” function. Then we clustered cells by “FindNeighbors” and “FindClusters” functions based on the Leiden algorithm. FindConservedMarkers was used for cell type annotation based on gene markers. The findmakers function was used to find the differential gene analysis between each group. We used R package iTALK (0.1.0) to perform the crosstalk between tumor and immune cells.

**Spatial transcriptomics**

Formalin-fixed paraffin-embedded (FFPE) samples passed the RNA quality control (DV200 > 50%) on shCtrl/LL2 tumors (shCtrl), and shNMX2-1/LL2 tumors. They were further processed for Visium in situ capturing construction and sequencing. The tissue was prepared according to the Visium CytAssist Spatial Gene Expression for FFPE-Tissue Preparation Guide (#CG000618; 10x Genomics, Pleasanton, CA,
USA). The sequencing was performed by Genomics, BioSci & Tech Co. (New Taipei City, Taiwan). The Space Ranger pipeline v2.0.1 (10× Genomics) and the mm10-2020-A reference were used to process FASTQ files. The sequencing results were guaranteed to be accurate as follows: For shCtrl sample: Number of spots under tissue: 2642; mean reads per spot: 80303; median genes per spot 8365; number of reads: 212,160,122; valid barcodes: 99.2%; valid UMIs: 100%; sequencing saturation: 26.7%. For shNKX2-1 sample: Number of spots under tissue: 1973; mean reads per spot: 89390; median genes per spot 8719; number of reads: 176,365,674; valid barcodes: 99.2%; valid UMIs: 100%; sequencing saturation: 26.7%. UMAP and violin plots were run and plotted using Loupe Browser (10× genomics, Pleasanton, CA, USA). Trajectory analysis and pathway enrichment analysis were performed and plotted using Partek flow software (Partek Incorporated, Chesterfield, MO, USA).

**Statistical analysis**

Statistical analyses of data were presented as mean ± standard deviation by using Microsoft Excel and GraphPad Prism. Two-tailed unpaired Student’s t-test was used for the two-group comparison. One-way ANOVA was used for multiple-group comparisons. Survival curves were plotted by the Kaplan-Meier method and assessed by a log-rank test. The criterion for significance was considered $p < 0.05$.

**RESULTS**

**Low expression of NKX2-1 is associated with aggressive LUAD**

The downregulation of NKX2-1 is known to be associated with poorly differentiated lung adenocarcinoma (LUAD) tumors exhibiting high metastatic potential, and conversely, NKX2-1 is upregulated in less malignant well-differentiated LUAD tumors [22]. To validate the effects of NKX2-1, we proceeded to investigate the protein expression levels of NKX2-1 across different grades of LUAD by conducting immunohistochemistry (IHC) staining on LUAD tissue microarray. As can be seen on the representative IHC images (Fig. 1A) or from quantification of the IHC staining (Fig. 1B), the protein expression of NKX2-1 in grade 3 LUAD tissues is markedly lower than in grade 1 and 2 tissues. Moreover, quantification of IHC staining based on LUAD staging showed that stage IIIB and stage IV LUAD tissues were characterized by low expression of NKX2-1 compared to stage IA, IB, IIA, IIB, and IIIA LUAD tissues (Fig. 1C). Furthermore, survival analysis using The Cancer Genome Atlas (TCGA) dataset showed that high NKX2-1 expression levels correlated with better relapse-free survival and overall survival outcome (Fig. 1D and E). To further understand the functional role of NKX2-1 in cancer progression, we validated the differential role of NKX2-1 in EGFR tyrosine kinase inhibitor (EGFR-TKI) resistance in LUAD. Previously, it was shown that low expression of NKX2-1 is associated with EGFR-TKI resistance in LUAD patients by the mechanism of lineage switch from adenocarcinoma to squamous cell carcinoma [23]. Therefore, we investigated the protein and mRNA expression levels of NKX2-1 alongside EMT markers in previously established EGFR-TKI-resistant LUAD cell lines, gefitinib-resistant HCC827/GR and osimertinib-resistant H1975/AZDR [24]. Both EGFR-TKI-resistant LUAD cell lines demonstrated significantly higher resistance...
to EGFR-TKIs (Supplementary Fig. 1A). Meanwhile, the expression of NKX2-1 on both protein and mRNA levels was virtually non-existent in EGFR-TKI-resistant LUAD as compared with their respective parental controls, allowing to classify these cells as NKX2-1-low cells (Supplementary Fig. 1B and C). Earlier findings have also shown that EGFR-TKI resistance fosters the malignant transformation of cells through the induction of epithelial-mesenchymal transition (EMT) [25]. Therefore, we investigated the morphology of EGFR-TKI-resistant cells and the expression of EMT markers. Our results showed that gefitinib-resistant HCC827/GR and osimertinib-resistant H1975/AZDR cell lines displayed more mesenchymal-like morphology compared with their respective parental controls (Supplementary Fig. 1D). Immunoblotting and qRT-PCR analysis also showed significant downregulation of epithelial markers (E-cadherin and EpCAM) and upregulation of mesenchymal markers (vimentin, fibronectin, ZEB1, and ZEB2) in EGFR-TKI-resistant cell lines as compared to the parental counterparts (Supplementary Fig. 1B and C). Following the induction of EMT, we also found that both EGFR-TKI-resistant cell lines demonstrated increased migration and invasion capacity in a transwell assay (Supplementary Fig. 1E and F). These results support the notion that EMT is clinically associated with acquired resistance to EGFR-TKIs [26, 27].

In this study, we sought to establish both in vitro and in vivo models to dissect the effect of NKX2-1 downregulation in LUAD. We efficiently knocked down NKX2-1 expression in NKX2-1-high LUAD cell lines (HCC827 and H1975) using shRNA constructs (Supplementary Figs. 2A and B). qRT-PCR indicated that NKX2-1 knockdown resulted in significant upregulation of mesenchymal markers, vimentin, ZEB1, and ZEB2, in both HCC827 and H1975 cells (Figs. 1F and G) and the downregulation of epithelial marker, E-cadherin, in H1975 cells (Fig. 1G).

The induction of EMT by NKX2-1 knockdown led to increased migration in both cell lines, as evaluated by transwell migration assay (Fig. 1H). Therefore, we proceeded to explore the regulatory role of NKX2-1 in in vivo tumorigenicity by using both the orthotopic LUAD mouse model and the experimental model of LUAD distal metastasis. NKX2-1 was knocked down in murine Lewis lung carcinoma cells (LL2) expressing luciferase reporter and eGFP (Supplementary Fig. 2C) and these cells were subjected either to orthotopic implantation to the lung or intra-tail injection (Fig. 1I). The orthotopically implanted cells with NKX2-1 knockdown (shNKX2-1/LL2) generated tumors characterized by increased growth in the recipient mice, compared to the implanted cells transfected with a scrambled control shRNA (shCtrl/LL2) (Fig. 1J). Furthermore, qRT-PCR results showed the upregulation of vimentin and fibronectin, and the downregulation of E-cadherin in the tumors derived from shNKX2-1/LL2 cells, indicative of the induction of EMT after NKX2-1 knockdown (Supplementary Fig. 1I). IHC staining of EMT markers in the excised tumor tissues also indicated that shNKX2-1/LL2-derived tumor cells underwent EMT (Fig. 1K), which was consistent with the in vitro findings (Fig. 1F and G). In addition to demonstrating the metastatic potential in the orthotopic model, we intravenously injected shCtrl/LL2 or shNKX2-1/LL2 cells into the mice via the tail vein (Fig. 1I and L). Mice injected with shNKX2-1/LL2 cells exhibited a significant increase of luciferase signal at the upper torso compared to the shCtrl/LL2-injected mice (Fig. 1M). This result was in line with the significant tumor nodules observed in the lungs of shNKX2-1/LL2, but not shCtrl/LL2-injected mice (Fig. 1L). These results indicated that low expression of NKX2-1 in LUAD contributed to increased tumor growth (Fig. 1J) and higher metastasis potential (Fig. 1M). In summary, the clinical data,
cellular models, and animal experiments demonstrated that low expression of NKX2-1 was associated with features related to advanced cancer status and worse clinical outcomes (Fig. 1N).

**NKX2-1 expression negatively correlates with neutrophil infiltration**

The tumor microenvironment (TME) can affect clinical outcomes due to the ability of the innate or adaptive immune cells to exert either tumor-promoting or tumor-suppressing effects [28]. The most abundant immune cells that infiltrate tumors are neutrophils and macrophages, which may exert pro-tumorigenic effects by driving angiogenesis, extracellular matrix remodeling, metastasis, and immunosuppression [29]. Therefore, we aimed to elucidate the effect of NKX2-1 on the complex interactions between malignant and immune cells within the TME. Tumor Immune Estimation Resource (TIMER) dataset [30] was used to systematically analyze possible correlation between NKX2-1 differential expression and immune infiltrations (Fig. 2A). The analysis showed that the infiltration of CD8+ T cells, macrophages, neutrophils, and dendritic cells negatively correlated with NKX2-1 expression levels in LUAD (Fig. 2B). Given that the correlation with neutrophil infiltration was the most statistically significant and given the growing evidence of its oncogenic role in LUAD [8, 18, 23], we analyzed the expression of a set of neutrophil markers such as ITGAM, CEACAM8, ELANE, and CXCR2 by IHC staining of NKX2-1-negative and positive LUAD tissues.

**NKX2-1-low tumors promote neutrophil infiltration via CXC chemokines**

Chemokines secreted by both tumor cells and TME play a central role in regulating the recruitment of immune cells such as neutrophils [31]. Notably, the infiltration of tumor-promoting neutrophils leads to increased tumor progression [31] and is normally associated with high-grade aggressive tumors [32]. Given that this observation is not always the same in all tumors due to their striking heterogeneity, we performed single-cell RNA sequencing (scRNA-seq) and Visium in situ capturing profiling on shCtrl/LL2 and shNKX2-1/LL2-derived tumor tissues to comprehensively characterize the infiltrated immune cells upon NKX2-1 knockdown at high resolution. For scRNA-seq, shCtrl/LL2 and shNKX2-1/LL2 cells were orthotopically implanted into mouse lungs and the harvested tumors were dissociated into viable single cells (Fig. 3A), which were further shown to pass standard scRNA-seq quality control (Supplementary Fig. 3A – D). To further characterize the distinct cell types from shCtrl/LL2 tumors and shNKX2-1/LL2 tumors, preliminary clustering was applied to all the cells by using uniform manifold approximation and projection (UMAP) analysis, and FindAllMarker package was used to find the different markers in each cluster (Fig. 3B). Meanwhile, the crosstalk between tumor cells and different cell types of the TME was further analyzed by using iTALK R package (Figs. 3C and D). The results indicated a significantly strong interaction of tumor cells with neutrophils (Fig. 3C). By further analysis of the cell-cell interactions, we found that the communication between tumor cells and neutrophils could occur through CXCLs/CXCR2 signaling activation (Fig. 3D). Enrichment analysis of gene ontology biological processes (GO-BP) showed enrichment in response to stress, regulation of programmed cells death, myeloid leukocyte
migration, inflammatory response terms (Fig. 3E). To further visualize the enriched GO related to myeloid cells, UMAP analysis also revealed the increase in myeloid leukocyte migration from our scRNA-seq (Fig. 3F), suggesting that high myeloid cells such as neutrophils, macrophages etc. migrated to shNKX2-1/LL2 tumors when compared to shCtrl tumors. To further validate the infiltration of immune cells in NXX2-1-low tumors, Visium in situ capturing profiling of shCtrl/LL2 tumors (shCtrl) and shNKX2-1/LL2 tumors was performed. Tissues were collected with a capture area of 8 x 8 mm, and ~ 5,000 gene expression spots were chosen (Fig. 3G). The results also showed an increase in myeloid leukocyte activation and intracellular signal transduction upon NXX2-1 downregulation (Fig. 3G). To validate the infiltration of neutrophils, we applied flow cytometry analysis to identify CD11b<sup>+</sup> Ly-6G<sup>+</sup> cells, commonly recognized as tumor-associated neutrophils (TANs) [33, 34]. We observed a significant increase in CD11b<sup>+</sup> Ly-6G<sup>+</sup> neutrophils in tumor-bearing lungs from the recipient mice intravenously injected with either clone of shNKX2-1/LL2 cells, compared to control mice transplanted with shCtrl/LL2 cells or the healthy lungs (Fig. 3H and I). Altogether, our findings demonstrated an increase in neutrophil infiltration within NXX2-1-low tumors, potentially mediated by the activation of cell surface receptor signaling pathways.

**NXX2-1 downregulation affects the expression of CXC chemokines**

Having illustrated the role of CXC chemokines in mediating cell-cell communication between tumor cells and neutrophils by scRNA-seq analysis (Fig. 3E), we further proceeded to assess the regulatory role of NXX2-1 on CXC chemokine expression and secretion in human LUAD cell culture model. RNA-seq analysis was performed on HCC827 cells subjected to shRNA-mediated NXX2-1 knockdown (Supplementary Fig. 2B). Functional enrichment analysis of the genes positively regulated by NXX2-1 knockdown, revealed that the most enriched GO-BP terms were related to neutrophil activation, neutrophil-mediated immunity, and myeloid cell activation involved in immune response (Fig. 4A), and the most enriched GO molecular function (GO-MF) terms were related to CXCR chemokine receptor binding, and CCR6 chemokine receptor binding (Fig. 4B). These cell culture model observations which indicate the role of CXC chemokines in neutrophil recruitment were consistent with our in vivo mouse model upon the functional elimination of NXX2-1 (Fig. 3G). More specifically, our RNA-seq analysis identified the upregulation of several neutrophil chemotactic genes such as CXCL1, CXCL2, CXCL3, CXCL5, and CXCL8 upon NXX2-1 knockdown (Fig. 4C). Among them, CXCL8 has been extensively characterized as a mediator of neutrophil mobilization and attraction [35], therefore, we decided to explore the role of other CXC chemokines in NXX2-1-low tumors. The validation of RNA-seq data by qRT-PCR confirmed that the knockdown of NXX2-1 by two shRNAs contributed to the upregulation of CXCL1, CXCL2, CXCL3, and CXCL5 mRNA levels in HCC827 and H1975 LUAD cells (Fig. 4D and E).

**NXX2-1 suppresses the chromatin accessibility of CXC chemokine genes at the promoter regions**
Following the observations that NKX2-1 suppresses the expression of various CXC chemokines in LUAD cells (Fig. 4), we sought to elucidate the mechanism involved in NKX2-1-mediated suppression of CXC chemokine expression. We hypothesized that NKX2-1 could exert its action by affecting the chromatin structure. Therefore, the open chromatin regions in shCtrl/H1975 and shNKX2-1/H1975 cells were sequenced by an assay for transposase-accessible chromatin using sequencing (ATAC-seq), a sequencing method based on the insertion of sequencing adapters by hyperactive Tn5 transposase [36].

To evaluate the effect of NKX2-1 on the overall pattern of chromatin accessibility, we estimated the abundance of reads with increased and decreased accessibility in shCtrl/H1975 and shNKX2-1/H1975 samples. Indeed, the open chromatin ATAC-seq reads tended to be enriched with large number of genes at the transcription start sites (TSS) in shNKX2-1/H1975 samples than in shCtrl/H1975, indicative of the potential role of NKX2-1 as a negative gene regulator of gene expression (Fig. 5A). Generally, the distribution pattern of open chromatin peaks across different functional genomic elements exhibited an increase within less than 1kb of the promoter in shNKX2-1 sample (26.06%) when compared to the shCtrl/H1975 (16.32%) (Fig. 5B). To determine the changes in chromatin accessibility following NKX2-1 knockdown, we evaluated the differential accessibility between shNKX2-1/H1975 and shCtrl/H1975 cells across the genome (Fig. 5C). The enriched open chromatin regions resulting from NKX2-1 knockdown were predominantly located on chr18, chr13, chr6, and chr4 (Fig. 5C). Interestingly, chr4 where the cluster of CXC chemokine genes is located, was one of the most chromatin-accessible chromosomes resultant from NKX2-1 knockdown. As was shown by GO-BP enrichment analysis, the enriched open chromatin regions resultant from NKX2-1 knockdown were mostly located at the promoters of genes associated with the positive regulation of secretion by cells and positive regulation of protein secretion (Fig. 5D), which was consistent with our hypothesis. On the other hand, more closed chromatin structure in shNKX2-1/H1975 cells as compared to shCtrl/H1975 cells was present at the promoters of genes associated with mitochondrial gene expression and mitochondrial translation (Fig. 5D). Notably, our ATAC-seq results showed an increase in open chromatin-associated reads at the promoter regions of CXCL1, CXCL2, and CXCL5 genes following the knockdown of NKX2-1 in H1975 cells (Fig. 5E). This suggested that NKX2-1 could potentially modulate the chromatin structure at the promoter regions of these chemokine genes. We used ChIP-qPCR to validate this observation and showed that the knockdown of NKX2-1 in H1975 (originally NKX2-1-high cell line) led to a decrease in the occupancy of NKX2-1 at the promoter regions of CXCL1, CXCL2, and CXCL5 (Fig. 5F). In contrast, the overexpression of NKX2-1 in H1975/AZDR (originally NKX2-1-low cell line) increased the occupancy of NKX2-1 on the promoter regions of the same genes (Fig. 5F). Next, we sought to identify the potential NKX2-1-binding sequence motifs that might be related to the regulation of cytokines expression. ATAC-seq reads were analyzed by using Find Individual Motif Occurrences (FIMO) software to predict NKX2-1-binding motif sequences. Our results showed three most prevalent NKX2-1-binding motif sequences present among the ATAC-seq reads close to the canonical motif sequence (Fig. 5G). Collectively, our data suggest that the direct binding of NKX2-1 negatively regulates chromatin accessibility at the promoters of CXC chemokine genes (Fig. 5H), which is consistent with the suppression of CXC chemokine expressions by NKX2-1 (Fig. 4).
Neutrophils attracted by NKX2-1-low tumor cells exhibit cancer-promoting properties

In light of the known role of CXCL chemokines as potent neutrophil chemoattractants [37], we conducted a chemotaxis assay to validate the functionality of NKX2-1-suppressed CXCL chemokines (Fig. 6A). As was shown by transwell chemotaxis assay, HL-60 cell migration was significantly stimulated by the medium conditioned by shNKX2-1-transfected H1975 and HCC827 cells in contrast to medium conditioned by shRNA-transfected control (shCtrl) (Fig. 6B). To investigate the details of global expression programs occurring in the recruited neutrophils, we performed RNA-seq analysis on HL-60 cells cultured in the medium derived from shCtrl and shNKX2-1-transfected H1975 cells. Revigo tool [40] was utilized for GO analysis of the genes positively regulated in HL-60 cells in the medium conditioned by shNKX2-1/H1975 cells, and the results showed the enrichment of the following GO-BP terms: secretion by cells, neutrophil activation, inflammatory response, immune response, neutrophil chemotaxis, etc. (Fig. 6C).

The phenotypic manifestation of neutrophils is related to their ability to adapt to different inflammatory contexts in the TME. For instance, previous reports have demonstrated that tumor-promoting or tumor-suppressing functions of neutrophils are determined by the functional characteristics exhibited through specific markers [38] related to neutrophil activation and cytokine status [39]. To identify the neutrophil phenotypes, we applied hierarchical clustering to identify the distinct gene expression patterns in HL-60 cells cultured with the medium conditioned by NKX2-1 knockdown cells. The analysis showed an increase in the expression of certain pro-inflammatory genes such as CCL3, CCL5, IL1B, CXCL8, CCL4, among others (Fig. 6D). Earlier studies have indicated that the antitumor phenotype (N1) of TANs is marked by the elevated levels of TNFα, CCL3, ICAM-1, and the decreased level of arginase. Conversely, the pro-tumor phenotype (N2) of TANs is characterized by high levels of expression of CCL2, CCL3, CCL4, CCL8, CCL12, CCL17, CXCL1, CXCL2, IL8, and CXCL16 chemokines [34]. To confirm the phenotypic effects on HL-60 cells cultured in the conditioned medium derived from shNKX2-1-transfected H1975 and HCC827 cells, we conducted qRT-PCR analysis, and our results revealed a significant increase in the mRNA expression levels of pro-tumor genes CCL2, MMP9, and IL8 (CXCL8) (Fig. 6E). Meanwhile, HL-60 treated by shNKX2-1/H1975-conditioned medium exhibited a significant decrease in the mRNA expression levels of antitumor genes encoding IFNβ1 and IFN-γ, while shNKX2/HCC827 medium showed no significant impact on the expression levels of these genes (Fig. 6E).

Furthermore, Visium in situ capturing profiling was performed to identify and characterize the transcriptomes of the infiltrated neutrophils in in vivo mouse model. The GO-BP enrichment analysis of the genes overexpressed in the tumors across all regions derived from shNKX2-1/LL2 cells as compared to shCtrl/LL2 cells showed a prevalence of such processes as positive regulation of intracellular signal, regulation of cell communication, regulation of cell population proliferation, and regulation of tumor necrosis factor superfamily cytokine production (Fig. 6F). To analyze the regions within tumor cross sections with enriched neutrophil infiltrations, we identified upregulation of some neutrophil related
genes: $S100a9$, $Serpine1$, $Mt2$, $Nos2$, $Mt1$, $IL33$, $Adm$, and $Ero1l$.[40–42]. The regions with increased above-threshold expression of these neutrophil-related genes (with defined log fold-change thresholds of more than 1) were defined as infiltration-positive while the regions with below-threshold expression were defined as infiltration-negative. Our results revealed that neutrophil-positive regions exhibited enrichment of GO-BP terms of cell communication and cell population proliferation compared to the neutrophil-negative regions in both shCtrl and shNKX2-1 tumors (Fig. 6G). While neutrophil-negative regions had slight difference based on the GO terms related to the regulation of cell communication and cell population proliferation (Fig. 6G). Our results also revealed that positive regulation of intracellular signal transduction and the regulation of tumor necrosis factor production were not affected in neutrophil-positive regions (Fig. 6G). Ccl3 and Ccl4 were the most upregulated genes related to the regulation of cell communication in shNKX2-1 tumor-infiltrated neutrophils when compared to the shCtrl tumor-infiltrated neutrophils (Fig. 6H), which was consistent with our initial observations (Fig. 6D). At the same time, Cdkn1a and Mif were the most upregulated genes implicated in the regulation of “cell population proliferation” in shNKX2-1 tumor-infiltrated neutrophils when compared to the shCtrl tumor-infiltrated neutrophils (Fig. 6H). The abundance of neutrophil-positive regions was much higher in shNKX2-1 as compared to shCtrl tumors as can be seen on cross sections of Visium in situ capturing visualization and these regions markedly overexpressed genes of “regulation of cell communication” and “regulation of cell population proliferation GO-BP annotations” (Fig. 6I). In summary, our findings suggest that low expression of NKX2-1 induces specific phenotypic properties in neutrophils, which potentially contribute to increased cancer progression through cell communication and cell proliferation. In other words, low levels of NKX2-1 in cancer cells may create a pro-tumor immune microenvironment, fostering malignant tumor development.

**In vivo targeting of CXCLs/CXCR2 signaling with SB225002 reduces tumor growth and neutrophil infiltration**

The identified NKX2-1-regulated CXC chemokines are known to share a common receptor, CXCR2. CXCR2 is a crucial chemokine receptor that facilitates neutrophil chemotaxis [43]. The CXCLs/CXCR2 signaling is associated with increased cancer progression in LUAD [14] aside from its significant role in recruiting neutrophils to inflamed sites [14, 44, 45]. Previous reports have shown the potential of interfering with CXCLs/CXCR2 signaling to reduce tumor growth and enhance the efficiency of immunotherapy in different cancers [14, 44, 46]. Since our findings are indicative of the modulatory role of NKX2-1 in LUAD TME, we sought to investigate whether targeting the CXCL/CXCR2 signaling pathway could suppress NKX2-1-low LUAD tumor growth and neutrophil infiltration. shCtrl/LL2 and shNKX2-1/LL2 cells were orthotopically and subcutaneously injected with and without intravenous administration of SB225002, a specific inhibitor of CXCR2. The experimental course for subcutaneous and orthotopic LUAD models was 18 days and 5 days, respectively, with SB225002 delivered every second day (Fig. 7A and B). The gross necropsy findings showed a remarkable tumor suppression by CXCR2 antagonism compared with the vehicle recipient mice upon tail vein administration of SB225002 in the subcutaneous mice model (Fig. 7A). Monitoring the tumor growth in the orthotopic models by using the IVIS imaging system showed
consistently with the subcutaneous mouse model observation of suppressed tumor growth by CXCR2 antagonism (Fig. 7B).

Altogether, our results demonstrate that low expression of NKX2-1 fosters tumor growth, and targeting CXCLs/CXCR2 axis with SB225002 mitigates the tumor growth induced by NKX2-1 downregulation.

To further assess whether the CXCLs/CXCR2 signaling is essential for NKX2-1-dependent modulation of neutrophil infiltration, scRNA-seq analysis was performed with a major focus on the neutrophil population after inhibiting CXCR2 with SB225002. Specifically, shCtrl/LL2 and shNKX2-1/LL2 cells were orthotopically implanted into mouse lungs with or without subsequent administration of SB225002 (Fig. 7B). The harvested tumors were dissociated into viable single cells which were shown to pass standard scRNA-seq quality control (Supplementary Fig. 3A – D). Different neutrophil markers were used to identify the neutrophil population by using FindAllMarker package (Supplementary Fig. 3E). Unsupervised clustering was performed on the neutrophil population, resulting in six clusters (Fig. 7C and D). Each subpopulation was independently validated through unsupervised clustering. Indeed, NKX2-1 knockdown increased the neutrophil population, while SB225002 treatment decreased it (Fig. 7C and D). Additionally, pseudo-time analysis was used to delineate the single-cell lineage order based on the gene expression profile obtained from the scRNA-seq analysis (Fig. 7E). The distribution pattern of cell percentages across six neutrophil clusters showed similarity between shCtrl/LL2 tumor with vehicle administration and SB225002-treated shNKX2-1/LL2 tumor, but was different in vehicle-treated shNKX2-1/LL2 tumor (Fig. 7F). This observation suggests that blocking the CXCLs/CXCR2 signaling can reverse the effect of NKX2-1 knockdown in terms of both the quantity and the landscape of infiltrated neutrophils. Notably, the neutrophil population of cluster 3 consistently exhibited an enrichment pattern across three different samples (shNKX2-1/LL2 tumors with vehicle administration, shCtrl/LL2 tumors with vehicle administration, and shNKX2-1/LL2 tumors with SB225002 administration). The result demonstrated significant enrichment in shNKX2-1 + vehicle, compared to the shCtrl + vehicle and shNKX2-1 + SB225002 (Fig. 7F). To gain deeper insights into the potential role of neutrophil cluster 3 in tumor progression, we explored the underlying cancer-promoting genes within the cluster 3. Violin plot illustrated the gene expression score, revealing a significant upregulation of cancer-promoting genes in shNKX2-1/LL2 + vehicle compared to both shCtrl/LL2 + vehicle and shNKX2-1/LL2 + SB225002 (Fig. 7G). These genes included Cdkn1a, Plaur, Ptgs2, Cox17, Lilrb4q, G0s2, Egr1, and Cxcl2, with previous reports suggesting an increase in cancer progression (Supplementary Table 1). Concurrently, ridgeline plot visually depicted the expression distributions of these differentially expressed genes (Fig. 7H). To further unravel the molecular events occurring in NKX2-1-low tumor in comparison to NKX2-1-low tumor treated with SB225002, we analyzed the upregulated genes in shNKX2-1 + vehicle and shNKX2-1 + SB225002. The result revealed 14 overlapping genes between shNKX2-1 + vehicle and shNKX2-1 + SB225002, 101 non-overlapping genes in shNKX2-1 + vehicle and 263 genes in shNKX2-1 + SB225002 (Fig. 7I). GO-BP analysis demonstrated a decrease in the enrichment of cellular response to chemokine, cellular response to lipopolysaccharide, and others among the overlapped genes common between shNKX2-1 + vehicle and shNKX2-1 + SB225002 as compared to shNKX2-1 + vehicle and shNKX2-1 + SB225002 only (Fig. 7J). Meanwhile, the non-overlapping genes in shNKX2-1 + vehicle exhibited enrichment in the cellular response to chemokine,
response to lipopolysaccharides, positive regulation of monocyte differentiation, and other terms, compared to the overlapped enrichment (Fig. 7J). The non-overlapping genes in shNKX2-1 + SB225002 showed the enrichment in cellular response to chemical stress, response to lipopolysaccharide, etc., in comparison to the overlapped enrichment (Fig. 7J). Altogether, our scRNA-seq analysis indicated that the knockdown of NKX2-1 not only increased the neutrophil population but specifically attracted neutrophils with cancer-promoting properties. This phenomenon could be counteracted by inhibiting the CXCLs/CXCR2 signaling with a CXCR2 antagonist. Our data demonstrated the pivotal role of the CXCLs/CXCR2 signaling in NKX2-1-low tumor progression and cancer-promoting neutrophil infiltration in LUAD. This suggests a potential method to control the malignant progression of NKX2-1-low LUAD tumors. Collectively, these findings emphasize that the CXCLs/CXCR2-dependent mechanism is essential for tumor progression and neutrophil infiltration in NKX2-1-low LUAD.

Networks engaged by NKX2-1-low tumor-activated neutrophils in in vitro and in vivo models

In lung cancer, the activation of TME neutrophils introduces complexity to the inflamed environment by triggering additional mechanisms [31]. Our observations indicated that the downregulation of NKX2-1 promotes the recruitment and infiltration of neutrophils into LUAD tumors through the secretion of chemokines such as CXCL1, CXCL2, CXCL3, and CXCL5 into the TME (Fig. 2–4), further fostering tumor-promoting effects (Figs. 6 and 7). Notably, among the CXC chemokines, CXCL1, CXCL2, and CXCL5 are involved in the paracrine network that mediates tumor progression and metastasis [47]. To further evaluate the molecular events triggered by the tumor-promoting neutrophils attracted by NKX2-1-low LUAD tumors, we analyzed both scRNA-seq samples (NKX2-1-low tumors in comparison with the control) (Fig. 3B) and RNA-seq data from HL-60 cells co-cultured with shNKX2-1/H1975 conditioned medium compared to HL60 co-cultured with shCtrl/H1975 conditioned medium, i.e., in vivo and in vitro models, respectively (Fig. 6A and B). GO-BP enrichment analysis showed that neutrophils induced in NKX2-1-low LUAD tumor exhibited enrichment in chemokine production (20% upregulated genes), interleukin 17 (IL-17) production (34.21% upregulated genes), and tumor necrosis factor-mediated signaling pathway (31.31% upregulated genes) compared to the control tumor sample (Fig. 8A). Previous report has shown that IL-17 production and tumor necrosis factor are related to tumor growth [47], this implies that the enrichment of tumor necrosis factor-mediated signaling pathway and IL-17 production in neutrophils underlies high inflammation leading to increased tumor growth. Additionally, the co-cultured shNKX2-1/H1975-conditioned medium with HL-60 showed enrichment in neutrophil chemotaxis (51.49% upregulated genes), neutrophil migration (43.59% upregulated genes), and neutrophil activation involved in the immune system (71.43% upregulated genes) compared to the control group (Fig. 8A). This suggested that neutrophils engaged by NKX2-1-low cancer cells play a role in promoting tumor progression by modulating the tumor surrounding with tumor-promoting molecules and inflammatory cells. Graphical representation comparing the two models (scRNA-seq from NKX2-1-low tumor and shNKX2-1/H1975 conditioned medium co-cultured with HL-60) (Fig. 8B) is consistent with our previous analyses (Fig. 8A). To further validate the genes involved in these enriched pathways, a network analysis was conducted. The results indicated a significant gene communication between Tnfrsf1b, Tnfaip3, Sphk1, Snai2, Il6r, and Il18 in NKX2-1-low tumors (Fig. 8C) while in HL-60 cocultured with shNKX2-
conditioned medium inferred a significant gene communication between CXCL2, CCL2, CCL3, LGALS3, TNFAIP6, S100A8 and S100A9 (Fig. 8D). Altogether, our studies demonstrated the modulatory role of NKX2-1 in the immune microenvironment of LUAD through the enrichment of chemokine expression and secretion. This promotes recruitment and infiltration of neutrophils into the tumor, further contributing to tumor progression (Fig. 8E).

**DISCUSSION**

Neutrophils are innate immune cells that play a crucial role in addressing infection and inflammation [7]. In solid tumors, neutrophils are the most potent pro-inflammatory cells, exhibiting a high intra-tumor density that correlates with tumor metastasis and angiogenesis based on the stimulated cytokines and growth factors [39]. The chemotactic recruitment of neutrophils is facilitated by chemokines such as CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8, by binding to their receptor CXCR2 [39]. Beyond their chemotactic role, CXC chemokines contribute to tumor cell proliferation, metastasis, chemoresistance, angiogenesis, invasion, and migration [47]. Concurrently, the activation of CXCLs/CXCR2 signaling fosters tumor angiogenesis, progression, and chemoresistance [14]. In lung cancer, lineage-specific transcription factors (TFs) regulate cancer cell identity by influencing the expression of other genes that shape the TME [18]. However, how the infiltrated immune cells, particularly neutrophils, affect poorly differentiated LUAD tumors with low NKX2-1 expression remains unclear. In this study, we demonstrated that LUAD tumors with NKX2-1 depletion exhibited increased infiltration of tumor-promoting neutrophils via the activation of CXCLs/CXCR2 signaling.

CXCR2 is a pivotal chemokine receptor with tumor-promoting effects, that facilitates the recruitment of leukocytes to tissues during inflammation and tumor growth [43]. In LUAD, CXCR2 is associated with tumor invasion, angiogenesis, and metastasis [14, 48]. Additionally, the CXCLs/CXCR2 axis also plays a crucial role in promoting drug resistance in LUAD [14]. In this context, we demonstrated that the downregulation of NKX2-1 leads to the upregulation of CXCL1, CXCL2, CXCL3, and CXCL5 which are all ligands of CXCR2. Notably, while IL-17 is recognized for promoting cancer progression by enhancing pro-tumorigenic phenotypes in lung cancer [49] and CXC chemokine expression in breast cancer [50] its specific role in the context of NKX2-1-low LUAD warrants further exploration. In this study, we demonstrated that NKX2-1 can suppress the expression of CXC chemokines and trigger IL-17 production (Fig. 8), leading to an increase in neutrophil recruitment. Previous reports have established that NKX2-1 can directly bind to the proximal promoter regions or the various intra- and inter-genic regions of the regulated genes [51]. On account of this observation, our ATAC-seq analysis revealed that the downregulation of NKX2-1 resulted in open chromatin structure at the promoter-TSS of CXCL1, CXCL2, and CXCL5 genes (Fig. 5). Furthermore, we identified NKX2-1-binding motifs that could govern the regulation of these cytokine genes (Fig. 5). This implies that the binding activity of NKX2-1 at the regulatory elements of CXCL1, CXCL2, and CXCL5 promotes the repressive states of these genes. Notably, this is consistent with prior studies showing NKX2-1 binding at the promoter and the first exon of murine Cxcl5 [19]. Consequently, targeting the CXCLs/CXCR2 axis could potentially enhance the clinical outcomes for NKX2-1-low LUAD patients.
To characterize the neutrophil population influenced by NKX2-1 loss in LUAD malignancy, we applied scRNA-seq and Visium in situ capturing to investigate the immune microenvironment heterogeneity in NKX2-1-low tumors. These novel tools offer unprecedented insight into cellular biology [52, 53]. Our quantitative analyses revealed that NKX2-1 downregulation led to increased neutrophil infiltration, a phenomenon mediated by CXC chemokines. Notably, this effect was abrogated by CXCR2 antagonism (Fig. 7). While most studies often focus on neutrophil plasticity, cellular density, or maturation, the full potential of the genes expressed by neutrophils in the TME remains underexplored. It is crucial to highlight that our scRNA-seq and Visium in situ capturing unveiled the expression of cancer-promoting genes in the infiltrated neutrophils. This identification was achieved through unsupervised clustering which revealed the broad gene expression patterns within the heterogenous neutrophil population. Notably, one of such clusters exhibited a particularly high pattern of expression of tumor-promoting cancer-promoting genes, which included Cdkn1a, Plaur, Ptgs2, Cox17, Lilrb4q, G0s2, Egr1, and Cxcl2, many of them encoding secreted tumor-promoting factors (Fig. 7). Neutrophil infiltration correlates with tumor aggressiveness via tumor grades in human gliomas [32] and previous reports have also shown that the infiltration of neutrophils is related to an aggressive type of pancreatic tumor [54]. Importantly, our study demonstrates that the interplay between NKX2-1, CXC chemokine axis, and neutrophil infiltration is associated with tumor aggression marked by high expression of tumor-promoting genes by infiltrated neutrophils (Fig. 3–7).

CONCLUSION

Our study delineates the role of NKX2-1 as a bona fide modulator of the immune tumor microenvironment of NKX2-1-low LUAD, whose loss induces the expression/secretion of CXC chemokines leading to increased recruitment and infiltration of tumor-promoting neutrophils. These neutrophils exhibit cancer-promoting properties with strong cell-cell communication within tumor tissue. This creates a pro-oncogenic TME which ultimately drives increased tumor progression. Our findings provided compelling evidence for the involvement of the NKX2-1/CXC chemokine signaling axis in mediating neutrophil infiltration and LUAD progression. Targeting the NKX2-1/CXC chemokine signaling may represent a promising therapeutic strategy to suppress tumorigenesis and improve the survival outcome for clinical LUAD patients with NKX2-1-low malignant tumors.

Abbreviations

NKX2-1: NK2 homeobox 1 or Thyroid transcription factor-1

LUAD: Lung adenocarcinoma

TCGA: The Cancer Genome Atlas

TIMER dataset: Tumor Immune Estimation Resource

EMT: Epithelial-mesenchymal transition
CXCR2: CXC Motif Chemokine Receptor 2
SCLC: Small-cell lung cancer
NSCLC: Non-small cell lung cancer
EGFR: Epidermal growth factor receptor
EGFR-TKI: EGFR tyrosine kinase inhibitor
TAN: Tumor-associated neutrophils
TME: Tumor microenvironment
TF: Transcription factor
scRNA-seq: Single-cell RNA sequencing
IHC: Immunohistochemistry
qRT-PCR: Real-Time Quantitative Reverse Transcription PCR
ChIP-qPCR: ChIP–Quantitative Polymerase Chain Reaction
GO: Gene Ontology term
GO-BP: Biological process of GO term
GO-MF: Molecular function of GO term

Declarations

The authors declare no potential conflicts of interest.

Ethical Approval

The collection of human tissue samples was conducted with informed consent in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects. The experimental procedures and protocols involving human samples were conducted according to the tenets of the Declaration of Helsinki and were approved by the Institutional Review Board of Taipei Veterans General Hospital (Protocol No. 2020-04-009B and 2020-10-003B).

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

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Availability of data and materials

The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files

Consent for publication

All authors agreed to publish the manuscript.

References


**Figures**
**Figure 1**

**Low expression of NKX2-1 is associated with aggressive LUAD.** (A) A representative image of NKX2-1 IHC staining performed on tumor tissue microarrays of different grades of LUAD. (B and C) Histoscore (H-score) quantification of NKX2-1 IHC staining performed on tissue microarray of different LUAD grades (B) and stages (C). The data are presented as means ± SD error bars, **p<0.01, ***p<0.001, ****p<0.0001, ns - not significant (Student’s t-test). (D and E) Kaplan-Meier curves showing the overall survival (D) and
disease-free survival of NKX2-1 LUAD patients from the TCGA dataset. \( p \)-values were calculated by the log-rank test. (F and G) qRT-PCR analysis showing the expression levels of EMT markers in HCC827 (F) and H1975 (G) cells transfected with NKX2-1-targeting shRNAs (shNKX2-1 #1 and shNKX2-1 #2). Mean fold changes (N=3) relative to shRNA control (shCtrl) are shown with SD error bars, \( \times p<0.05, \times \times p<0.01, \times \times \times p<0.001, \times \times \times \times p<0.0001, \) ns – not significant (Student’s t-test). (H) Transwell migration assay showing the migratory capacity of HCC827 and H1975 cells upon NKX2-1 knockdown. Mean numbers of migrated cells (N=3) are shown with SD error bars, \( \times p<0.05, \times \times p<0.01, \times \times \times p<0.001, \) ns – not significant (Student’s t-test). (I) Schematic representation showing the design of experiments performed on both orthotopic and tail vein injected mouse models by using shNKX2-1/LL2 and shCtrl/LL2 cells. (J) Photographs (top panels) and GFP fluorescence images (bottom panels) of tumors derived from orthotopically injected shCtrl/LL2 (left panel) and shNKX2-1/LL2 cells (middle and right panels). (K) IHC staining of NKX2-1 and EMT markers performed on the tumors derived from orthotopically injected shCtrl/LL2 and shNKX2-1/LL2 cells. Left panel: representative images. Right panel: H-score quantification. (L and M) Photographs (L) and bioluminescent signal visualization (M) of tumors derived from shCtrl/LL2 (left panel) and shNKX2-1/LL2 cells (middle and right panels) injected via tail vein. (N) An illustration showing the role of NKX2-1 in LUAD progression through cancer metastasis.
Figure 2

NKX2-1 expression negatively correlates with neutrophil infiltration. (A) Pipeline analysis of the correlation between NKX2-1 expression and different immune cell infiltrates. (B) Scatter plots showing the correlation between NKX2-1 expression and the indicated immune cell infiltrate levels in LUAD from TIMER dataset analysis. The gene expression levels against tumor purity are displayed first because genes highly expressed in the tumor microenvironment are expected to exhibit negative associations with
tumor purity and vice versa. (C) H-score quantification of the expression levels of the selected neutrophil markers (ITGAM, CEACAM8, ELANE, and CXCR2) performed on tumor tissue microarrays of NKX2-1-positive and negative LUAD tissues. Data are presented as means ± SD error bars, ****p < 0.0001 (Student's t-test). (D) TCGA dataset analysis showing the correlation between NKX2-1 and the selected neutrophil markers (ITGAM, CEACAM8, ELANE, and CXCR2) in LUAD. (E) Relapse-free survival curve showing the survival of LUAD patients with high or low neutrophil infiltration from TCGA dataset analysis. p-values were calculated by the log-rank test. (F) IHC staining of the indicated neutrophil markers in the cross sections of shCtrl/LL2 and shNKX2-1/LL2-derived tumors from mouse orthotopic model. Left panel: representative images. Right panel: H-score quantification. (G) A schematic illustration showing that NKX2-1-low LUAD tumors are characterized by high infiltration of neutrophils. The representative images (Supplementary Fig. 2D) and the quantification of the IHC staining (Fig. 2C) showed an upregulation of these markers in NKX2-1-negative LUAD tissues as compared to NKX2-1-positive tissues. Furthermore, TCGA dataset analysis confirmed the negative correlation of NKX2-1 expression with the selected neutrophil markers (ITGAM, CEACAM8, ELANE, and CXCR2) in LUAD patients (Fig. 2D). In addition, the survival analysis showed that low infiltration of neutrophils in relapse-free LUAD patient tumors was associated with better survival outcomes (Fig. 2E). Moreover, IHC staining of the tumor tissues derived from shNKX2-1/LL2 cells orthotopically implanted into mouse lungs (Fig. 1I) also showed elevated expression of ITGAM, CEACAM8, ELANE, and CXCR2 as compared to the control (Fig. 2F). In conclusion, our data suggest that low expression of NKX2-1 correlates with high neutrophil infiltration and the abundance of neutrophil infiltration predicts poor clinical outcomes for LUAD patients (Fig. 2G).
Figure 3

NKX2-1-low tumors promote neutrophil infiltration via CXC chemokines. (A) Diagram demonstrating the experimental procedure for scRNA-seq and Visium in situ capturing. (B) UMAP plot from scRNA-seq showing the distinct cell populations across two samples (shCtrl/LL2 and shNKX2-1/LL2-derived tumors). (C and D) iTALK analysis of scRNA-seq validating the crosstalk between tumor cells and immune cells (C) and cell-to-cell communication (D) between cell types and the involved genes. (E) Gene
ontology biological process (GO-BP) enrichment analysis of DEGs in shNKX2-1/LL2 tumors compared to shCtrl/LL2 tumors identified by scRNA-seq. (F) UMAP plot from scRNA-seq showing myeloid leukocyte migration in shCtrl/LL2 and shNKX2-1/LL2 tumors. (G) Hematoxylin-eosin staining (H&E) section annotations in shCtrl/LL2 and shNKX2-1/LL2 tumors (top panel) and representative image from Visium in situ capturing showing the activation of intracellular signal transduction and myeloid leukocyte activation. (H and I) Flow cytometry identification of Ly-6G$^{+}$CD11b$^{+}$ cells from healthy lungs and metastasized tumor-bearing lungs (shCtrl/LL2 and shNKX2-1/LL2) (H). The quantification of Ly-6G$^{+}$CD11b$^{+}$ cells (I) is shown as means ± SD error bars, **$p<0.01$ (Student’s t-test). (J and K) Schematic illustration showing that NKX2-1-low tumors induce neutrophil infiltration via the activation of cell-surface receptor signaling, exacerbating the clinical outcomes of LUAD patients by recruiting neutrophils into the TME.
Figure 4

**NKX2-1 downregulation affects the expression of CXC chemokines.**  
**(A and B)** Functional enrichment analysis showing the enrichment of GO biological process (GO-BP) (A) and molecular function (GO-MF) (B) terms among the genes positively regulated by NKX2-1 knockdown.  
**(C)** Hierarchical clustering heatmap showing the differential gene expression (left panel) and the expression of CXC chemokines (right panel) upon NKX2-1 knockdown.  
**(D and E)** qRT-PCR analysis showing the expression level of CXC
chemokines in HCC827 (D) and H1975 (E) resulting from NKX2-1 knockdown. The mean fold changes (N=3) relative to scrambled shRNA control (shCtrl) are shown with SD error bars, *p<0.05, **p<0.01, ***p<0.001 (Student’s t-test). (F and G) Chemokine array analysis showing the secretion levels of the indicated chemokines following NKX2-1 knockdown in H1975 cells (F) and overexpression in H1975/AZDR cells (G). Left panel: representative dot plot. Right panel: densitometry quantification of dot blots. *p<0.05, **p<0.01, ***p<0.001, ns – not significant (Student’s t-test). (H) TCGA dataset analysis showing the correlation between NKX2-1 expression and CXCL1, CXCL2, CXCL3, and CXCL5 expression levels. Furthermore, as was shown by chemokine array, the knockdown of NKX2-1 in H1975 cells led to increased secretion of CXCL1, CXCL5, and CXCL7 (Fig. 4F). Conversely, the overexpression of NKX2-1 in H1975/AZDR (EGFR-TKI-resistant) cells significantly decreased the secretion of CXCL1, CXCL5, CXCL7, and CXCL8 (Fig. 4G). TCGA dataset analysis also indicated a negative correlation between the expression of NKX2-1 and CXCL1, CXCL3, and CXCL5. However, there was no observed correlation between the expression of CXCL2 and NKX2-1 (Fig. 4H). To summarize, our data demonstrate the role of NKX2-1 as a suppressor of the expression and secretion of CXC chemokines in LUAD.
**Figure 5**

**NKX2-1 suppresses the chromatin accessibility of CXC chemokine genes at the promoter regions.**

**(A)** Heatmap representation of the chromatin accessibility at the transcription start sites (TSS) of multiple genes and 10 kb upstream and downstream regions. **(B)** Annotation for the localization of ATAC-seq peaks showing the distribution of sequenced reads across the indicated functional genomic elements. The peaks located 1 Kb upstream and 100 bp downstream of the TSS were defined as promoter-TSS. **(C)**
The distribution of open chromatin areas across the chromosomes is shown as the ratio of signals from shNKX2-1/H1975 vs shCtrl/H1975. (D) GO-BP analysis of genes with open (top panel) and closed chromatin structure (bottom panel) at the promoter regions resultant from NKX2-1 knockdown. (E) Integrative Genomics Viewer (IGV) representation of ATAC-seq signals at the promoter regions of CXC chemokine genes in shCtrl/H1975 and shNKX2-1/H1975 samples. The red line denotes a given range across the two samples. (F) ChIP-qPCR analysis showing the binding of NKX2-1 at the promoter region of CXCL1, CXCL2, and CXCL5 genes in shNKX2-1/H1975 cells and H1975/AZDR overexpressing NKX2-1. Mean fold changes (N=3) relative to input are shown with SD error bars *p<0.05, **p<0.01, ***p<0.001 (Student's t-test). (G) Most enriched canonical NKX2-1 binding motifs across ATAC-seq reads. (H) Schematic illustration showing the potential mechanisms of NKX2-1 regulating the expression of CXC chemokines by altering chromatin structure.
Figure 6

Neutrophils attracted by NKX2-1-low tumors exhibit cancer-promoting properties. (A) Schematic illustration demonstrating the experimental procedure for chemotaxis assay by using HL-60 and conditioned medium from LUAD cell lines. (B) Chemotaxis assay showing the migratory capacity of HL-60 cells in response to the conditioned media (CM) derived from H1975 (left panel) and HCC827 (right panel) subjected to NKX2-1 knockdown or. Mean numbers of migrated cells (N=3) are shown with SD.
error bars, *p<0.05, **p<0.01, ***p<0.001 (Student's t-test). (C) Revigo scatter plot from RNA-seq showing enriched GO-BP terms among the genes upregulated in HL-60 cells cultured with conditioned media derived from shNKX2-1/H1975 as compared to shCtrl/H1975 as shown by RNA-seq analysis. (D) Hierarchical clustering heatmap from RNA-seq showing the differential gene expressions (left panel) and neutrophil-related genes (right panel) in HL-60 cultured with the conditioned media derived from shCtrl/H1975 and shNKX2-1/H1975. (E) qRT-PCR analysis showing the expression levels of pro-inflammatory genes and anti-inflammatory genes in HL-60 cultured with conditioned medium derived from H1975 and HCC827 cells subjected to NKX2-1 knockdown. The mean fold changes (N=3) relative to shCtrl are shown with SD error bars, bars **p<0.01, ***p<0.001, ****p<0.0001, ns – not significant (Student's t-test). (F) GO-BP enrichment analysis of the genes upregulated in the whole tumor regions derived from shNKX2-1/LL2 cells as compared to shCtrl/LL2 from Visium in situ capturing profiling. (G) GO-BP enrichment analysis of the upregulated genes in tumors derived from shNKX2-1/LL2 cells as compared to shCtrl/LL2 from Visium in situ capturing profiling. The regions with increased define log fold-change thresholds of more than 1 were defined as infiltration-positive while the regions with below-threshold expression were defined as infiltration-negative. (H) Violin plots showing the expression level of the most upregulated genes in shNKX2-1 and shCtrl infiltrated neutrophils of the “regulation of cell communication” and “regulation of cell population proliferation” GO-BP annotations. (I) Visium in situ capturing representative images showing the neutrophil-positive regions and overlapping regions of expression “regulation of cell communication” and “regulation of cell population proliferation” GO-BP annotations.
Figure 7

In vivo targeting of CXCLs/CXCR2 signaling with SB225002 reduces tumor growth and neutrophil infiltration. (A) Subcutaneous mouse model. Top panel - schematic representation of experimental design, bottom panel - tumor growth curve for 18 days following subcutaneous injection. Administration of SB225002 and vehicle control in the shNKX2-1/LL2 tumor. Data are shown as means ± SD error bars (n=4, ***p<0.001 (Student's t-test) (B) Orthotopic mouse model. Top panel - schematic representation of
experimental design, bottom panel - bioluminescent signal visualization, photographs GFP fluorescence images of tumors derived from shCtrl/LL2+vehicle, shNKX2-1/LL2+vehicle, and shNKX2-1/LL2+SB225002 tumors. (C and D) Unsupervised clustering of UMAP plot from scRNA-seq on infiltrated neutrophils subpopulations shown in six clusters (C) and in separate samples of shCtrl+vehicle, shNKX2-1+vehicle, and shNKX2-1+SB225002 cells (D). (E) Pseudo-time visualization showing the single-cell lineage order based on the gene expression profile from scRNA-seq analysis. (F) Graphical representation of cell percentages according to neutrophil clusters with more than one read for the corresponding gene. (G) Violin plot showing the gene expression score of upregulated cancer-promoting genes, expressed in shCtrl+vehicle, shNKX2-1+vehicle, and shNKX2-1+SB225002 cells. (H) Ridgeline plot showing the visualized expression distributions of the differentially expressed cancer-promoting genes in shCtrl+vehicle, shNKX2-1+vehicle, and shNKX2-1+SB225002 cells. The expression of these genes is plotted on the x-axis. (I) Venn diagram showing the overlapping and unique genes between shNKX2-1+vehicle and shNKX2-1+SB225002 tumor. (J) GO-BP analysis showing the enriched terms between shNKX2-1+vehicle and shNKX2-1+SB225002 (14 common genes), 101 genes unique for shNKX2-1+vehicle and 263 genes unique for shNKX2-1+SB225002.
Networks engaged by NKX2-1-low tumor-activated neutrophils in *in vitro* and *in vivo* models. (A) Pie chart representation showing the enriched GO-BP terms with the upregulated gene percentage from scRNA-seq from NKX2-1-low sample compared to the control group and RNA-seq from HL-60 co-cultured with conditioned medium from shNKX2-1 cells compared with shCtrl. (B) Graphical representation from the enriched GO-BP enrichment analysis from scRNA-seq from NKX2-1-low tumors compared to the control.
group and RNA-seq from HL-60 cocultured with conditioned medium from shNKX2-1/H1975 cells compared with shCtrl. (D and C) Network analysis showing different genes involved in the enriched GO-BP analysis from scRNA-seq from NKX2-1-low sample compared to the control group (C) and RNA-seq from HL-60 co-cultured with conditioned medium from shNKX2-1/H1975 cells compared with shCtrl (D). (E) Illustrative overview of unconventional molecular profiling techniques such as scRNA-seq and Visium in situ capturing employed to unravel the modulatory role of NKX2-1 in the immune microenvironment of LUAD. NKX2-1 downregulation induces the expression and secretion of CXCL1, CXCL2, CXCL3, and CXCL5, culminating in increased neutrophil recruitment and infiltration into LUAD tumors consequently fostering increased cancer progression.

**Supplementary Files**

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