

ARTICLE



Cancer-associated fibroblasts drive lung adenocarcinoma progression via THBS2-mediated epithelial-mesenchymal transition

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The role of cancer-associated fibroblasts (CAFs) in the initiation and invasion phases of human lung adenocarcinoma (LUAD) development is not fully understood. In this study, we utilized single-cell RNA sequencing, spatial transcriptomics, and a combination of in vivo and in vitro models to decode the dynamics of tumor-stroma interactions during human LUAD progression, focusing primarily on adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIA), and invasive adenocarcinoma (IAC). **We identified a matrix CAF (mCAF) subtype characterized by high THBS2 expression, which was closely associated with poor clinical outcomes, tumor recurrence, and the invasive dynamics of LUAD.** Spatial transcriptomics and multiplex immunohistochemistry analysis revealed that this CAF subpopulation was closely associated with tumor cells, with clear spatial colocalization. In vivo and in vitro experiments demonstrated that THBS2 secreted by these mCAFs directly binds to SDC4 on tumor cells, enhancing tumor epithelial-mesenchymal transition (EMT) programs. **This study highlights THBS2+ mCAFs as key regulators of tumor-stroma interactions and identifies the THBS2-SDC4-EMT axis as a potential therapeutic target in LUAD.**

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INTRODUCTION

The progression of lung adenocarcinoma (LUAD) involves several pathological stages, including adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIA), and invasive adenocarcinoma (IAC) [1, 2]. AIS is regarded as a pre-invasive stage, MIA represents an early invasive phase, and IAC is fully invasive. While the 5-year postoperative overall survival rates for AIS and MIA approach 95–100% [3], the prognosis for IAC is significantly worse, with survival rates dropping to 50–80% [4, 5]. Therefore, elucidating the transition from AIS to IAC is crucial for understanding the underlying factors contributing to these disparities in survival and for identifying key intervention points.

During the progression from AIS to IAC, cancer cells breach the basement membrane and adopt histological patterns beyond the lepidic growth pattern [1]. These cells acquire malignant traits, such as epithelial-mesenchymal transition (EMT), which induces

significant changes in their morphology and behavior. Importantly, this transformation is not solely dictated by the cancer cell genome; rather, it arises from intricate interactions between tumor cells and the signals emanating from the surrounding tumor microenvironment (TME), particularly through stromal interactions at the tumor-epithelium interface [6]. Thus, the multi-step development and progression of LUAD are orchestrated by these dynamic interactions, driving the transition from non-invasive to more invasive cancer stages.

The latest World Health Organization (WHO) classification of lung cancer (5th edition) emphasizes the definition of “invasion” in invasive adenocarcinoma by specifying the presence of fibroblastic foci and neofibroplasia, which are indicative of active stromal remodeling [1]. This emphasis highlights the pivotal role of cancer-associated fibroblasts (CAFs) in driving the invasion process. CAFs facilitate cancer cell invasion by secreting matrix

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metalloproteinases (MMPs) that degrade the extracellular matrix (ECM), creating pathways for tumor infiltration [7–9]. Additionally, CAFs release growth factors (such as TGF- β) and cytokines that promote the EMT process in cancer cells [10–13]. This transition leads to the loss of cell polarity, enhanced motility, and increased invasiveness, enabling cancer cells to breach the basement membrane and infiltrate surrounding tissues, ultimately resulting in invasive cancers. Moreover, CAFs support cancer cell survival and spread through direct physical interactions [14].

Research on early-stage LUAD has primarily focused on the IAC tissues, while studies exploring the transition from AIS to IAC remain underdeveloped, particularly at the high-resolution single-cell level. Single-cell RNA sequencing (scRNA-seq) has emerged as a powerful tool to dissect cancer heterogeneity at the single-cell level and construct comprehensive gene expression maps. However, current investigations into the cellular landscapes of pre-invasive LUAD remains limited [15, 16], and the mechanisms by which CAFs contribute to the progression from a pre-invasive state to an invasive phenotype warrant further elucidation.

Here, we identified THBS2+ matrix CAFs (mCAFs) as critical contributors to LUAD initiation and invasion during the progression from AIS to IAC. These mCAFs are characterized by elevated THBS2 expression, active ECM remodeling, and strong interactions with tumor cells. Importantly, we discovered SDC4 as a novel receptor for THBS2 and demonstrated that THBS2 binding to SDC4 on tumor cells activates the EMT pathway. This activation drives the acquisition of invasive tumor phenotypes and is closely associated with poor clinical outcomes in LUAD. Overall, our findings underscore the pivotal role of the THBS2-SDC4 axis in LUAD invasion and highlight its potential as a therapeutic target.

METHODS AND MATERIALS

Single-cell RNA-seq data collection and processing

This study collected and analyzed two scRNA-seq datasets: GSE189357 and GSE164789, which encompassed 297,019 cells of LUAD, covering stages from AIS and MIA to IAC. The Seurat R package was implemented to analyze single-cell data. Scrublet was used to identify potential doublets [17]. High quality cells (cells with 500–40,000 UMI/cell, 500–5000 genes/cell, and <15% mitochondrial genes) were retained. Cell-level normalization was performed using the NormalizeData function. The top 1500 highly variable genes were selected using the FindVariableFeatures function. Batch effects across different cohorts were corrected with the Harmony R package [18]. The shared nearest neighbor (SNN) graph was constructed using the FindNeighbors function, based on edge weights between cells. Cell clusters were then identified using the Louvain algorithm through the FindClusters function.

The initial clustering was conducted with the FindClusters function, and each cluster was annotated using known markers: T/natural killer cells (CD3D, CD3E, TRAC, KLRF1, KLRD1, FGFBP2, PRF1); B cells (CD19, CD79A, CD79B, MS4A1); Plasma cells (TNFRSF17, IGHG1, IGHA1, MZB1); Myeloid cells (LYZ, CST3, CD68); Dendritic Cells (ITGAX, CD1C, THBD, FCER1A); Epithelial cells (EPCAM, CAPS, SNTN, SCGB1A1); Fibroblast (COL1A1, COL3A1, DCN, ACTA2, FAP); Endothelial cells (VWF, CLDN5, CDH5, PECAM1, ENG, ACKR1). Malignant and non-malignant epithelial cells were differentiated by analyzing large-scale copy-number variations (CNVs) in individual cells using the InferCNV R package [19]. To map the lineage trajectory of fibroblasts, the Monocle2 R package [20] was applied. CellChat was employed to analyze cell-cell communication networks in scRNA-seq data [21].

Spatial transcriptomics data collection and analysis

The 10X Visium spatial transcriptomics data were obtained from GSE189487 dataset. We scored gene features using the AddModuleScore function in Seurat R package, with highly scored spots viewed as possible spatial locations where the cell subpopulation is located to present cell-cell interactions. The SpaGene [22] was used to infer ligand-receptor interactions by ligand-receptor co-localization.

Bulk transcriptome data collection and analysis

Ten LUAD transcriptome datasets with clinical information were collected from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases, including TCGA-LUAD, GSE14814, GSE50081, GSE72094, GSE31210, GSE3141, GSE26939, GSE30219, GSE20913, and GSE166720. Data preprocessing and normalization were conducted as described in previous research [23, 24]. Subsequently, we integrated the normalized bulk transcriptomic data with scRNA-seq reference data and applied the ssGSEA algorithm to analyze the bulk RNA-seq data, enabling the inference and definition of CAF subtype-specific expression profiles.

Histopathological analyses

Lung adenocarcinoma tissue microarrays were purchased from Outdo Biotech (Shanghai, China).

For immunohistochemical (IHC) staining, tissue sections were first deparaffinized in xylene and rehydrated through a graded series of alcohols. Antigen retrieval was performed using EDTA buffer (pH 8.0). Non-specific binding was blocked by incubating the sections with PBS containing 5% bovine serum albumin (BSA) for 30 min at room temperature. The sections were then incubated with a primary antibody overnight at 4°C. After thorough washing, the sections were incubated with the appropriate secondary antibodies for 1 h at room temperature. Visualization was achieved using DAB (Servicebio, Wuhan, China), followed by counterstaining with hematoxylin. Additionally, Hematoxylin-eosin (H&E) and TUNEL staining were performed using the Hematoxylin-eosin HD constant dye kit (Servicebio, Wuhan, China) and the TUNEL Cell Apoptosis Detection Kit (Servicebio, Wuhan, China) according to the manufacturer's instructions. Bright-field images were captured using an ECLIPSE E100 microscope (Nikon, Tokyo, Japan), and digital scanning was performed with a Panoramic MIDI (3DHISTECH, Budapest, Hungary).

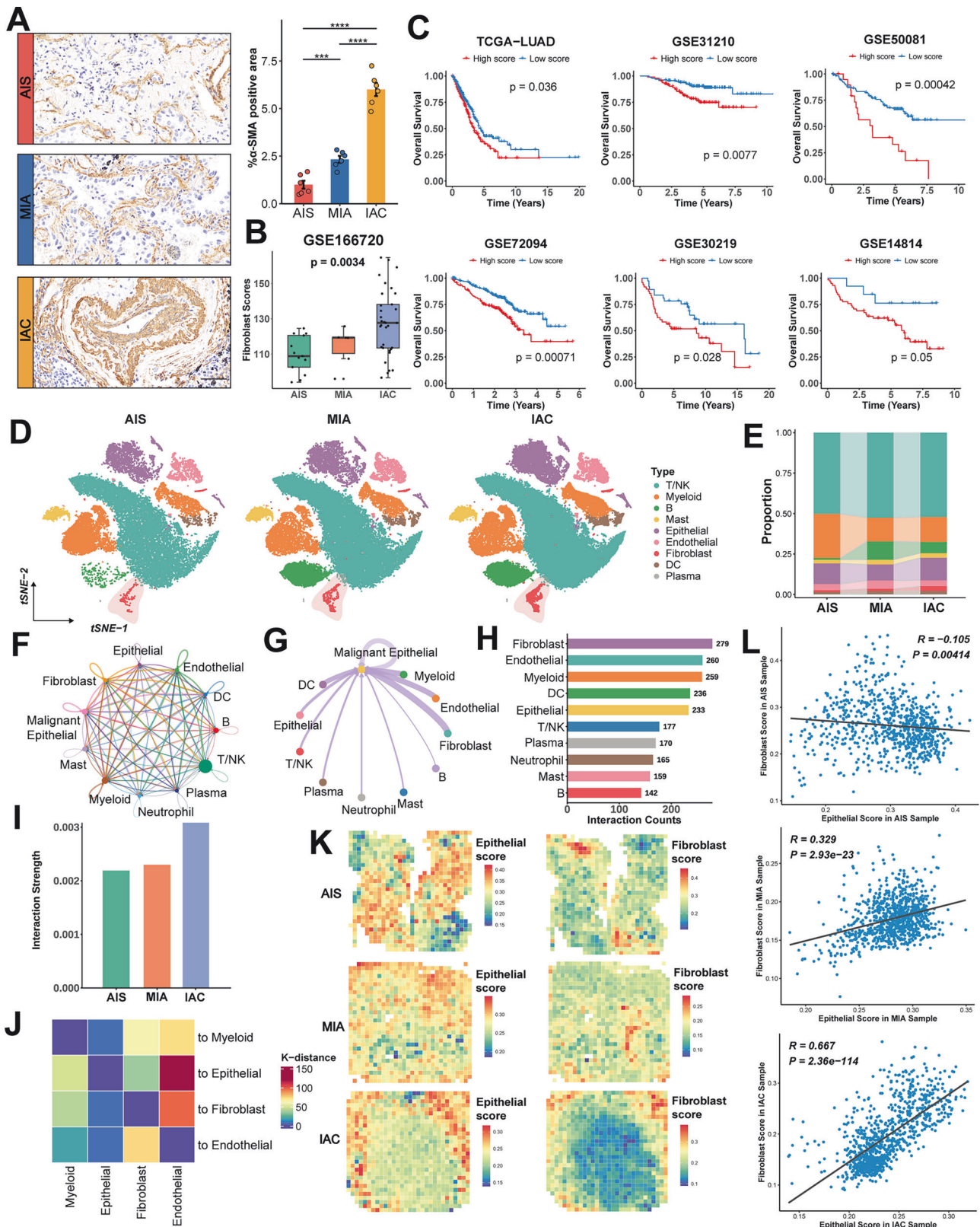
For multiplex immunohistochemistry (mIHC), slides were initially deparaffinized and rehydrated, followed by antigen retrieval using microwave treatment. Endogenous peroxidase activity and non-specific binding sites were blocked. Primary antibodies were then applied, followed by secondary HRP-conjugated polymers from the TSA fluorescent staining kit. The covalent reaction was followed by additional antigen retrieval to eliminate any background signal before proceeding to the next step. After several cycles of labeling, nuclear counterstaining with DAPI was performed to complete the staining procedure. Color images were acquired using the ZEISS AXIOSCAN slide scanning system (ZEISS, Oberkochen, Germany). We employed the pathology analysis software Halo (version 3.5, Indica Labs, United States) to conduct single-cell-based quantitative statistical analysis. This involved segmenting tumor and stromal regions and quantifying multiple data types, including the number of positive cells and the positivity rate for both single and multiple marker combinations, using a relative quantification method.

Human and mouse CAFs

Mouse lung cancer tissues were obtained from orthotopic tumors established in mice using LLC cells. Tumor tissues, free from contamination, were washed several times with 5× trypsin PBS (Servicebio, Wuhan, China) until the PBS was clarified, removing adipose and necrotic tissue. The tissues were then cut into small pieces (~1 mm³) and digested with 1 mg/mL type IV collagenase (Thermo Scientific, Shanghai, China) at 37°C for 2 h. After centrifugation to remove the supernatant, the remaining tissue was filtered through a 200-mesh filter. The tissue was then resuspended in DMEM high-glucose culture medium supplemented with 10% fetal bovine serum and seeded into 6 cm culture dishes. After 72 h, the culture medium was replaced to remove non-adherent cells. Human pulmonary cancer-associated fibroblast cells were purchased from Pricella Company.

Preparation of recombinant protein

The cDNA sequence for mouse THBS2 was obtained from GenBank (NM_011581.3). The plasmid pQE-80L/mTHBS2 was constructed by Sangon Biotech (Shanghai, China). Recombinant mouse THBS2 (rmTHBS2) was expressed in *Escherichia coli* BL21(DE3) strains and induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) (Sangon Biotech, Shanghai, China) at 30°C for 6 h. rmTHBS2 was purified using nickel affinity chromatography (Sangon Biotech, Shanghai, China). Expression of rmTHBS2 was confirmed by SDS-PAGE (Supplementary Fig. 7) and Western blot analysis. Detailed antibody information is provided in Supplementary Table 1. Additionally, human THBS2 (hTHBS2) was successfully constructed in a similar manner and was used in our previously study [25].



Subcutaneous tumor model

Male BALB/c nude mice (4 weeks old) were purchased from Vital River Laboratory Animal Technology (Beijing, China). All animal procedures were approved by the Institutional Animal Care and Use Committee of Zhengzhou University. A total of 5×10^6 A549 cells mixed

with 5×10^6 CAF-NC or CAF-THBS2 cells were subcutaneously injected into the right axilla of each mouse. Tumor weight was recorded at the end of the experiment, and tumor volume was measured every 4 days using the following formula: tumor volume = $0.5 \times \text{length} \times \text{width}^2$.

Fig. 1 Fibroblasts exhibit stronger interactions and associations with LUAD cells in multimodal data. A Immunohistochemical (IHC) staining of α -SMA in LUAD samples from AIS ($n = 6$), MIA ($n = 6$), and IAC ($n = 6$) across different clinical stages. Scale bar 50 μ m. *** $P < 0.001$, **** $P < 0.0001$. **B** The level of fibroblast infiltration in LUAD samples, across AIS, MIA, and IAC stages, was assessed using the GSE166720 dataset. **C** Kaplan–Meier (KM) survival analysis was performed to assess the association between fibroblast infiltration and prognosis in LUAD patients across multiple datasets, including TCGA-LUAD, GSE31210, GSE50081, GSE72094, GSE30219, GSE14814. **D** Single-cell RNA sequencing was used to identify nine cell subpopulations in LUAD samples, including AIS, MIA, and IAC stages. **E** The proportion of nine cell subpopulations changes dynamically during the progression from AIS to MIA and IAC. **F–H** Analysis of cell-cell communication between tumor cells and other cell populations. The thickness of the lines represents the number of interactions between each cell type and tumor cells. **I** Interaction strength between tumor cells and fibroblasts during the progression from AIS to MIA and IAC. **J** Spatial distances between various cell types in lung adenocarcinoma spatial transcriptomics data. **K, L** The spatial distribution and correlation of fibroblast scores and epithelial scores in AIS, MIA, and IAC stages of lung adenocarcinoma spatial transcriptomics data.

Thbs2 conditional knockout mice and orthotopic lung tumor model

Wild-type C57BL/6J adult mice and C57BL/6J background Col1a2-CreER mice were sourced from Cyagen Biosciences (Suzhou, China). Thbs2-floxed mice, also on the C57BL/6J background, were created by Cyagen using the following approach: Two gRNAs targeting the promoter region and exon 3 flanking sequence of the Thbs2 gene were designed. Cas9, gRNAs, and a donor vector were co-injected into zygotes. Cas9, guided by the gRNAs, induced a double-strand break in the Thbs2 gene, which was repaired via homologous recombination using the donor vector, incorporating flox sites. The resulting F0 Thbs2^{flox/+} mice were further bred to obtain Thbs2^{flox/flox} mice, which were then crossed with Col1a2-CreER mice to generate tissue-specific Thbs2 knockout mice (THBS2^{flox/flox}; Col1a2-CreER). Genotyping confirmed the modifications in both control (Thbs2^{flox/flox}) and knockout (Thbs2^{flox/flox}; Col1a2-CreER, referred to as cKO) mice. Tamoxifen (Merck, Darmstadt, Germany) was dissolved in corn oil (Aladdin, Shanghai, China) at 10 mg/ml. Mice received tamoxifen 75mg/kg per day by intraperitoneal injection for 5 consecutive days. Experiments were performed 5 days after injection.

6–8-week-old Thbs2^{flox/flox} or Thbs2^{flox/flox}; Col1a2-CreER mice were induced with tamoxifen. After anesthesia, the left thorax of the recipient mice was shaved and disinfected. A 0.5 cm lateral abdominal incision was made to expose the intercostal muscles. Then, 1×10^6 LLC-luc cells were resuspended in 50 μ l PBS and injected into the left lung lobe within 10 s. Following the inoculation, the orthotopic experiments were conducted over two main observation periods. Tumor volume and in vivo fluorescence imaging were monitored over a period of 3–4 weeks. For the survival rate assessment, the experiment was conducted over 45 days. After the experiment, mice were humanely euthanized, and tissues were collected for analysis to assess the therapeutic effects.

Statistical analysis

All data processing, statistical analysis, and plotting were conducted in R 4.3.3 software. Correlations between two continuous variables were assessed via Pearson's correlation coefficient. Continuous variables were compared through the Wilcoxon rank-sum test or T test. The survminer package was used to determine the optimal cut-off value. Cox regression and Kaplan–Meier analyses were performed via the survival package. The signatures were scored in single-cell data using the AUCell algorithm. All statistical tests were two-sided. $P < 0.05$ was regarded as statistically significant.

RESULTS

Multimodal data identifies fibroblasts as facilitators of the invasive process in LUAD

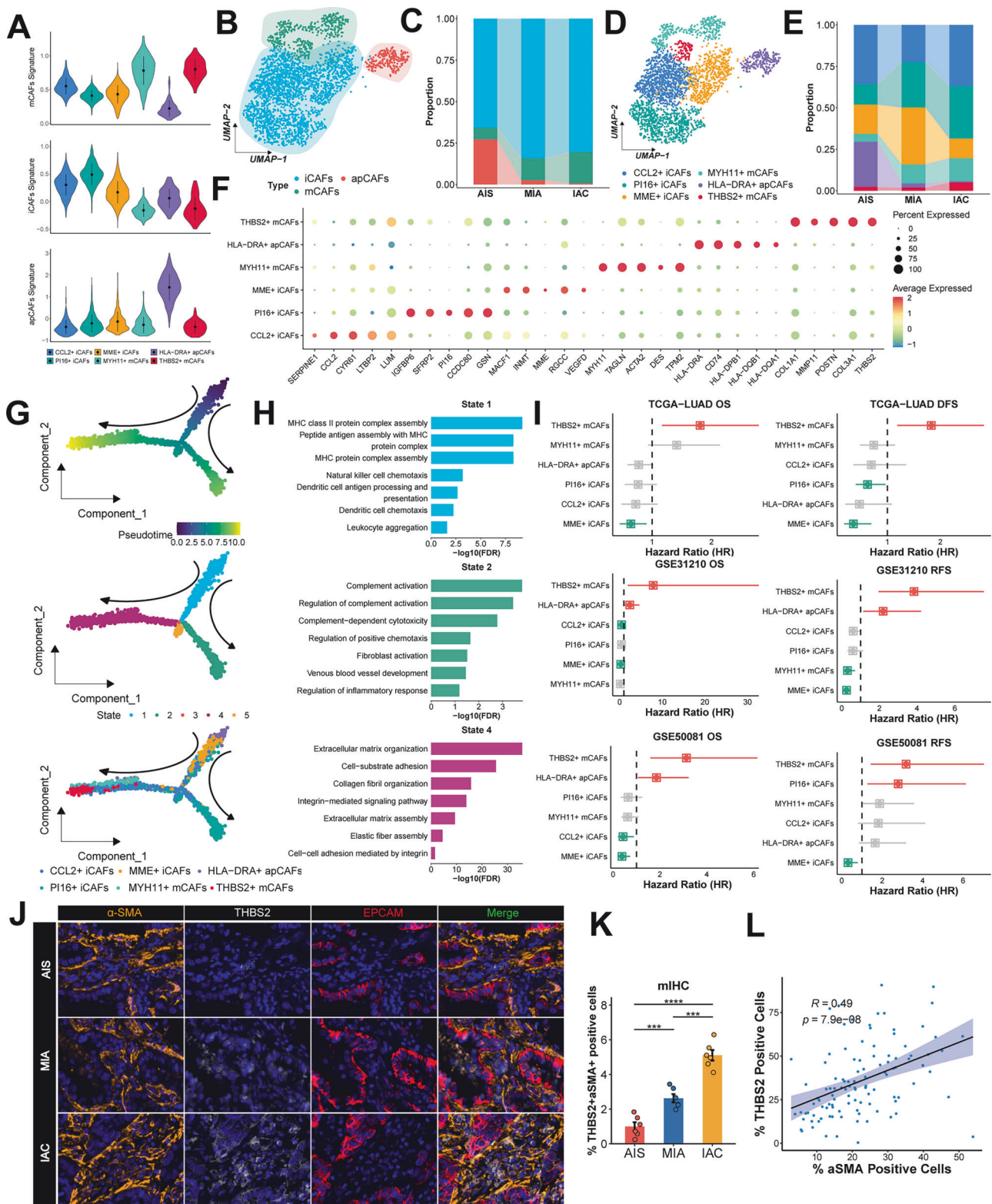
In tissue samples, we quantified fibroblast abundance using α -SMA and observed a progressive increase in their proportion with disease progression (Fig. 1A). This trend was corroborated by independent bulk RNA-seq dataset (RNA-seq) (Fig. 1B). Survival analysis further revealed that a high fibroblast content was associated with unfavorable prognosis (Fig. 1C). To explore this relationship at a higher resolution, we performed single-cell analysis based on GSE164789 dataset, which consistently demonstrated an increased fibroblast proportion as the disease progressed (Fig. 1D, E). These findings highlight the link between fibroblasts and the malignant progression of LUAD from AIS to IAC, as well as their association with unfavorable clinical outcomes.

Based on these observations, we hypothesized that fibroblasts might interact with tumor cells to promote malignant phenotypic transformation [26, 27]. CellChat analysis [28] supported this notion, revealing that the interaction between fibroblasts and tumor cells was the strongest among all cell types, with its intensity increasing alongside disease progression (Fig. 1F–I). To further elucidate this relationship in a spatial context, we applied robust cell type decomposition (RCTD) [29] to deconvolve single-cell spatial transcriptomic data from GSE189487 dataset across three tissue types. This analysis revealed that fibroblasts were in closest spatial proximity to epithelial cells (Fig. 1J), and as tissue invasiveness increased, regions of high fibroblast and epithelial cell density became increasingly co-localized (Fig. 1K). While spatial proximity alone does not confirm cell-cell communication, these results provide spatial support for the potential interactions inferred from single-cell analysis. Finally, correlation analysis revealed that the fibroblast-epithelial cell correlation coefficient shifted from -0.105 ($P = 0.00414$) in AIS to 0.329 ($P < 0.001$) in MIA, and further to 0.667 ($P < 0.001$) in IAC (Fig. 1L).

THBS2 + mCAFs represent a key subgroup within CAF heterogeneity and are associated with poor tumor prognosis

Fibroblasts were re-clustered into six distinct subpopulations. Using previously reported markers [30–33] (Supplementary Table 3), these subpopulations were categorized into three major subtypes: matrix cancer-associated fibroblasts (mCAFs), inflammatory cancer-associated fibroblasts (iCAFs), and antigen-presenting cancer-associated fibroblasts (apCAFs) (Fig. 2A, B). Notably, these subtypes exhibited distinct distribution patterns across different tissue types. From AIS to IAC, the abundance of apCAFs decreased as tissue invasiveness increased, while the proportion of mCAFs progressively increased alongside tumor progression (Fig. 2C).

We identified differentially expressed genes (DEGs) specific to each fibroblast subcluster to define and characterize the unique features of each subgroup (Fig. 2D–F, Supplementary Fig. 1A and Supplementary Table 4). Among mCAFs, subpopulations such as MYH11+ mCAFs and THBS2+ mCAFs were marked by high expression of matrix-associated gene signatures (Fig. 2A). MYH11+ mCAFs exhibited high levels of MYH11 and smooth muscle-related genes (e.g., ACTA2), while displaying low expression of POSTN (Supplementary Fig. 1B), consistent with previously described MYH11+ CAFs in non-small cell lung cancer [34]. THBS2+ mCAFs were characterized by high expression of extracellular matrix remodeling genes such as COL1A1, COL12A1, COL5A1 and MMP11, indicating their roles in contractility and matrix organization. Within the iCAF category, we identified subtypes such as CCL2+ iCAFs, PI16+ iCAFs, and MME+ iCAFs, all of which displayed elevated expression of pro-inflammatory cytokines (e.g., CXCL12) and complement components (e.g., C3 and C7) (Supplementary Fig. 1B), suggesting their active involvement in modulating the immune landscape. Notably, PI16+ iCAFs have been associated in prior studies with lymphocyte recruitment, implying a potential role in orchestrating immune cell infiltration within the TME [35]. Meanwhile, HLA-DRA+ apCAFs showed upregulation of several



MHC class II molecules (including HLA-DPA1, HLA-DRB1) and immunomodulatory genes such as CD74, indicating their possible capacity for antigen processing and presentation (Supplementary Fig. 1B). This population resembles apCAFs described in pancreatic and lung cancers, further supporting the immunoregulatory versatility of CAFs [36, 37]. These subpopulations exhibited distinct distributions across tissue types: the proportions of MYH11+

mCAFs, THBS2+ mCAFs and PI16+ mCAFs, increased progressively from AIS to IAC, whereas HLA-DRA+ apCAFs decreased (Fig. 2E).

Trajectory analysis using Monocle2 [20] revealed that THBS2+ mCAFs were located at the terminal end of the differentiation trajectory (Fig. 2G). Functional enrichment analysis along pseudo-time showed that early differentiation was predominantly associated with immune presentation, implying that fibroblasts

Fig. 2 CAF dynamics and functional heterogeneity in LUAD progression. **A** Previously established classic CAF signatures were used to characterize their relationship with the CAF subpopulation identified in this study. **B** Single-cell data was used to identify classical CAF subpopulations in LUAD. **C** Distribution characteristics of classic CAF subtypes across AIS, MIA, and IAC stages. **D** Six distinct CAF subpopulations were identified in LUAD samples. **E** The distribution of six CAF subtypes shifts during the progression from AIS to MIA and IAC stages. **F** Average expression of signature genes of six CAF subpopulations. **G** Differentiation trajectories of six CAF subpopulations visualized in a two-dimensional state-space using Monocle. Each point represents a single cell, with the differentiation pathways initiating from State1 based on pseudotime analysis. **H** Functional enrichment analysis of different states (State1, State2, and State4) in pseudotime analysis. **I** Univariate Cox analysis of six CAF subtypes across multiple bulk cohorts (TCGA-LUAD, GSE31210 and GSE50081), including overall survival (OS), disease-free survival (DFS), and recurrence-free survival (RFS). **J** Representative mIHC staining images of THBS2+ CAFs in LUAD tissues of AIS, MIA and IAC patients. DAPI (blue), THBS2 (white), α -SMA (orange), EPCAM (red) in individual and merged channels are shown. Scale bar, 50 μ m. **K** Percentage of THBS2 + α -SMA+ positive cells measured by mIHC in different groups: AIS ($n = 6$), MIA ($n = 6$), and IAC ($n = 6$). *** $P < 0.001$, **** $P < 0.0001$. **L** Spearman correlation analysis of THBS2+ cells and α -SMA+ cells.

might establish tumor immune evasion mechanisms during their initial transition into CAFs (Fig. 2H). Conversely, terminal states (state 2 and state 4) were primarily enriched for pathways related to ECM remodeling, angiogenesis, and metabolic regulation (Fig. 2H). Collectively, these findings highlight the heterogeneity of CAF subpopulations, with distinct functions during various stages of tumorigenesis and progression. **Prognostic evaluation of these CAFs in bulk cohorts demonstrated that THBS2+ mCAFs were associated with poor prognosis across all cohorts (Fig. 2I).** Multiplex immunohistochemistry (mIHC) analysis revealed a significant correlation between the presence of THBS2+ CAFs and malignant progression of LUAD (Fig. 2J–L), with increased THBS2+ α -SMA+ cells observed from AIS to IAC samples. Overall, these results indicate that THBS2+ mCAFs may drive the malignant progression and invasion of lung adenocarcinoma, contributing to adverse prognosis.

THBS2 is identified as a critical factor linking CAFs to LUAD progression

The signature genes of THBS2+ mCAFs were closely associated with ECM remodeling, T regulation, cell migration, and invasion. For example, genes such as COL12A1, VCAN, CTHRC1, MMP11, POSTN, and THBS2 are prominently involved (Fig. 3A). To identify the key drivers within the THBS2+ mCAFs that promote tumor progression and invasion, we performed differential expression analysis between normal tissues and LUAD tissues (Fig. 3B) and applied the Mfuzz [38] algorithm to pinpoint “invasive” genes whose expression gradually increased with tumor infiltration from AIS to IAC (Fig. 3C). Intersection analysis of THBS2+ mCAFs signature genes, tumor-upregulated genes, and the invasive genes yielded 34 shared genes (Fig. 3D). These genes were significantly enriched in pathways related to cell adhesion, ECM remodeling, Wnt signaling, and EMT (Fig. 3E). Moreover, protein-protein interaction (PPI) network analysis identified that THBS2, along with COL1A1, COL1A2, COL11A1, COL3A1, COL5A1, COL5A2, POSTN, and COL6A3, as central nodes that collectively drive ECM remodeling and regulating key processes such as tumor cell migration, invasion, and EMT (Fig. 3F).

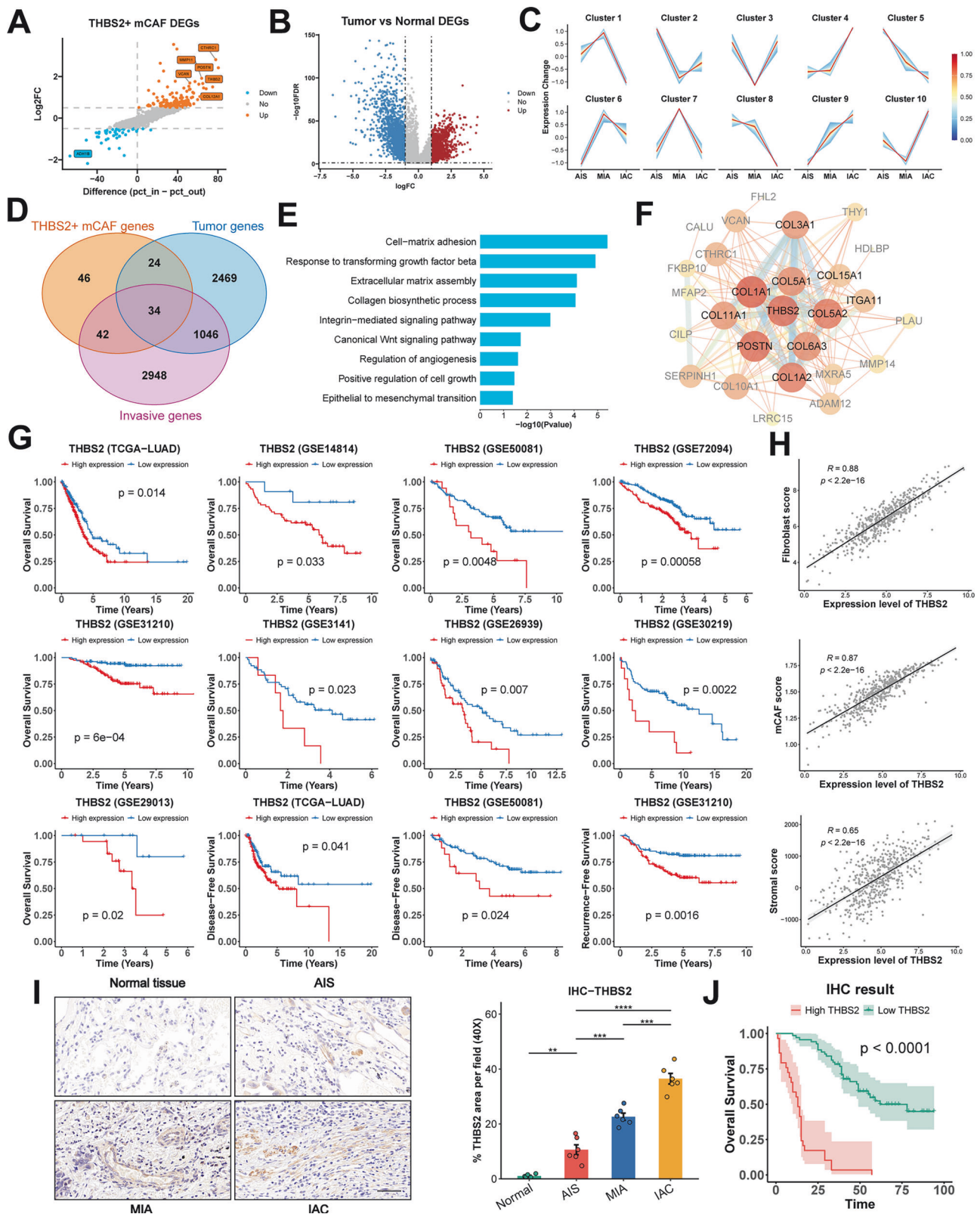
Previous studies have suggested that THBS2 may indirectly promote cancer cell migration and invasion by influencing ECM remodeling—specifically by regulating collagen structure to provide a “highway” for cancer cell migration [39, 40]. Based on our prior research, THBS2 is associated with tumor progression and chemotherapy resistance [41]. Therefore, we further focused on the role of THBS2 in the early stages of LUAD progression and malignant invasion. Prognostic analysis across multiple independent cohorts indicated that high THBS2 expression is significantly correlated with poorer overall survival (OS), disease-free survival (DFS), and recurrence-free survival (RFS) (Fig. 3G). Additionally, THBS2 expression in LUAD showed a positive correlation with stromal infiltration (Fig. 3H), suggesting that it is a stromal-derived molecule involved in the TME. IHC demonstrated that THBS2 expression progressively increases with LUAD invasion, progression and poor prognosis (Fig. 3I, J).

CAF-derived THBS2 promotes tumor progression and EMT in LUAD

Further investigation of THBS2 were conducted using the recombinant human THBS2 protein (rhTHBS2), recombinant mouse THBS2 protein (rmTHBS2), and CAFs stably overexpressing THBS2 (human CAF-THBS2 and mouse CAF-Thbs2). Consistent with its secretory role, Enzyme-linked immunosorbent assay (ELISA) analysis revealed significant secretion levels of THBS2 in culture supernatants (Supplementary Fig. 2A). Western blot (WB) and quantitative PCR (qPCR) analyses confirmed a significant upregulation of THBS2 in the CAF-THBS2 and CAF-Thbs2 groups compared to CAF-NC, demonstrating successful overexpression (Supplementary Fig. 2B–E). CCK-8 and EdU assays showed that THBS2 significantly promoted lung adenocarcinoma cell proliferation. Compared with the negative control group (NC group), rhTHBS2 or rmTHBS2, CAF-THBS2 conditioned medium (CM) or CAF-Thbs2 CM treatments all increased cell proliferation (Fig. 4A, B, F). Adhesion assays demonstrated that THBS2 significantly improved cell adhesion. Both recombinant THBS2-treated tumor cells and those exposed to CAF-THBS2 or CAF-Thbs2 exhibited stronger adhesion compared to their respective control groups (Fig. 4C, F), highlighting the role of THBS2 in cell-ECM interactions. Wound healing and transwell assays further showed that THBS2 enhanced cell migration and invasion (Fig. 4D–G and Supplementary Fig. 3A–C). In both experimental settings, THBS2 significantly promoted proliferation, adhesion, migration, and invasion, supporting its role in tumor progression and stromal remodeling. Additionally, WB assays confirmed that treatment with rhTHBS2, rmTHBS2, or CM from CAF-THBS2 and CAF-Thbs2 enhanced tumor EMT activity, as evidenced by upregulation of ZEB1, Vimentin, and N-cadherin, along with the downregulation of E-cadherin (Figs. 4H and 6H). Taken together, these results suggest the secretory protein THBS2 enhances oncogenic phenotypes and induces EMT activity.

THBS2 drives tumor progression in vivo models

To assess the role of THBS2 in tumor growth, we established subcutaneous tumor models by injecting a mixture of tumor cells and either CAF-THBS2 or CAF-NC. Tumors in the CAF-THBS2 group were visibly larger, with significantly higher volumes and weights compared to the CAF-NC group (Fig. 5A–C). We further evaluated the in vivo role of THBS2 using a conditional knockout (cKO) mouse model. The schematic of the THBS2 conditional knockout strategy is illustrated in Fig. 5D. In an orthotopic LUAD model, in vivo fluorescence imaging revealed reduced tumor burden in the cKO group compared to the wild-type (WT) group (Fig. 5E and Supplementary Fig. 4A). Hematoxylin and eosin (H&E) staining of mouse lung tissue confirmed that tumors from cKO mice were smaller compared to those from WT mice (Fig. 5F and Supplementary Fig. 4B). Moreover, survival curves showed that cKO mice had significantly prolonged survival compared to WT mice (Fig. 5G), suggesting that THBS2 deficiency inhibits tumor progression and improves survival outcomes. Histopathological analysis further supported these findings. H&E staining



demonstrated a decreased cellularity and nuclear atypia in cKO mice compared to WT mice (Fig. 5H). IHC staining confirmed reduced THBS2 expression in cKO mice, verifying successful knockdown (Fig. 5H and Supplementary Fig. 4C). Additionally, Ki67 staining, a marker for cell proliferation, showed decreased

proliferative activity in cKO tumors compared to WT tumor (Fig. 5H and Supplementary Fig. 4D). TUNEL staining indicated increased apoptosis in tumors from cKO mice compared to WT mice (Fig. 5H and Supplementary Fig. 4E). Collectively, these *in vivo* results demonstrate that THBS2 drives tumor progression.

Fig. 3 Identification of critical genes linking CAFs to LUAD progression. **A** Differential gene expression analysis between THBS2+ mCAFs and other CAF subpopulations. Genes are labeled if they meet the following criteria: log-fold change >1, percentage difference >50%, and an adjusted P -value < 0.05 from the Wilcoxon rank-sum test. **B** Volcano plot of differential gene expression analysis between tumor and normal tissues in lung adenocarcinoma. **C** Mfuzz analysis identified 10 clusters in AIS to IAC tissues, among which gene expression in cluster 4 and cluster 9 increased with the progression of tissue invasiveness. **D** Venn diagram of THBS2+ mCAF signature genes, LUAD tumor upregulated genes, and genes upregulated from AIS to IAC, identifying a total of 34 overlapping genes. **E** Functional enrichment analysis of the 34 overlapping genes. **F** Protein-protein interaction (PPI) network analysis of the overlapping genes. **G** KM survival analysis was performed to assess the association between THBS2 expression and prognosis in LUAD patients across multiple datasets, including OS, DFS, and RFS. **H** Correlation of THBS2 expression with fibroblast, mCAF and stromal scores. **I** IHC staining of THBS2 in LUAD samples across different clinical stages, including normal ($n = 6$), AIS ($n = 6$), MIA ($n = 6$) and IAC ($n = 6$). Scale bar 50 μm . $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. **J** KM survival analysis was performed to assess the association between THBS2 expression and prognosis in LUAD patients across IHC cohort ($n = 98$).

THBS2 enhances tumor growth and invasion through SDC4 interaction

Cell-cell communication analysis [28] revealed that the THBS2-SDC4 ligand-receptor pair exhibited the strongest interaction between THBS2+ mCAFs and malignant tumor cells (Fig. 6A). In line with this, SDC4 expression is highest in malignant epithelial cells (Fig. 6B). Spatial transcriptomics data further supported the proximity of THBS2 and SDC4 expression regions in tumor tissues. The correlation between THBS2 and SDC4 at the spatial transcriptomics level gradually increases from AIS to IAC, consistent with single-cell analysis (Fig. 6C). mlHC assays demonstrated co-localization of THBS2 and SDC4 in tumor cells, with the strongest co-localization observed in IAC tissues (Fig. 6D and Supplementary Fig. 5A). Additionally, co-immunoprecipitation (Co-IP) experiments confirmed the physical binding of THBS2 to SDC4 at the protein level (Fig. 6E). Consistently, THBS2 expression was positively correlated with SDC4 in the LUAD bulk RNA-seq cohort (Supplementary Fig. 5B).

To assess the functional relevance of the THBS2-SDC4 interaction, we conducted phenotypic rescue experiments in A549 and LLC cells using recombinant THBS2 treatment alongside SDC4 knockdown (siSDC4). qPCR and WB confirmed efficient knockdown of SDC4 in the siSDC4 group compared to siNC (Fig. 6H and Supplementary Fig. 5B, C). CCK-8 and EdU assays demonstrated that the effect of recombinant THBS2 on cell proliferation was significantly reduced following SDC4 gene knockdown (Fig. 6F, G and Supplementary Fig. 5D). Similarly, adhesion assays, along with transwell invasion and wound healing assays, revealed that the THBS2-induced enhancements in adhesion, invasion, and migration were significantly diminished when SDC4 was knocked down (Fig. 6F–G and Supplementary Fig. 5E, F). Collectively, these results indicate that SDC4 is essential for mediating the tumor-promoting effects of THBS2.

THBS2-SDC4 axis drives tumor progression via EMT signaling activation

To further elucidate the mechanism by which THBS2 promotes tumor progression, we performed transcriptomic sequencing on cellular models treated with rmTHBS2 or control. In rmTHBS2-treated LLC cells, genes such as *Dsp*, *Lcp2*, *Prkcz*, *Mkn2os*, *Ceacam19*, *Tssk2*, *Ncf2*, *Pappa2*, and *Cbe1*—implicated in proliferation, migration, and invasion—were significantly upregulated (Fig. 7A). Enrichment analysis revealed significant activation of the EMT signaling pathway in rmTHBS2-treated tumor cells (Fig. 7B, C). In LUAD tissues, THBS2 expression was positively correlated with the EMT pathway scores ($R = 0.82$, $P < 0.0001$) and EMT-associated markers, such as mesenchymal markers *VIM* (Vimentin; $R = 0.37$, $P < 0.0001$), *CDH2* (N-cadherin; $R = 0.51$, $P < 0.0001$), and the EMT-transcription factor *ZEB1* ($R = 0.58$, $P < 0.0001$) (Fig. 7D–G). IHC results further demonstrated a protein-level association between THBS2 and key EMT molecules (Fig. 7H and Supplementary Fig. 6A, D). Additionally, WB analysis confirmed that rhTHBS2 treatment enhanced EMT signaling, accompanied by increased expression of *ZEB1*, N-cadherin, and Vimentin, while E-cadherin expression was reduced. These changes were reversed in the siSDC4 group, suggesting that the co-

expression of THBS2 and SDC4 activated key molecules in the EMT signaling pathway (Fig. 6H).

In summary, our findings highlighted the pro-tumor characteristics of THBS2+ mCAF in the TME (Fig. 7I). The increased infiltration of THBS2+ mCAFs contributed to the enhanced malignant infiltration in LUAD. THBS2+ mCAFs remodeled the stromal microenvironment by secreting THBS2, a protein associated with poor prognosis in LUAD patients. Through transcriptomic data, as well as in vitro and in vivo experiments, we demonstrated the key role of THBS2 and its receptor, along with the related pathways. Specifically, the THBS2 protein interacted with the SDC4 molecule expressed on tumor cells, activating the EMT signaling pathway, which promoted malignant invasive features and drove the progression of LUAD.

DISCUSSION

LUAD exhibits significant prognostic differences across its various stages, including pre-cancerous, pre-invasive, and invasive lesions. Despite early radical resection of the primary tumor, a subset of patients experiences high rates of tumor recurrence, leading to reduced survival rate [4]. Therefore, understanding the cellular and molecular mechanisms underlying LUAD progression is critical for predicting recurrence, identifying biomarkers, and developing potential therapeutic targets. Here, we identified THBS2+ mCAFs as key drivers of LUAD initiation and invasion, spanning AIS and MIA to IAC, through high THBS2 expression, ECM remodeling, and direct interactions with tumor cells. THBS2 binds to its receptor SDC4 on tumor cells, which we identified for the first time as a novel receptor for THBS2, activating the EMT pathway and driving invasive phenotypes and poor clinical outcomes. These findings underscored the THBS2/SDC4/EMT axis as a critical mechanism in LUAD progression and a potential therapeutic target.

The dense fibers at the tumor-stroma interface form a barrier around the tumor mass, restricting tumor cells from interacting with other cells within the TME [42]. However, CAF subtypes, as key architects of this barrier, maintain closer communication with tumor cells. mCAFs have been widely recognized for their central role in ECM remodeling [9, 43, 44]. Our study further confirmed the supportive role of mCAF subtypes in malignant invasion of LUAD and their association with poor prognosis. Specifically, we focused on a distinct THBS2+ mCAFs that not only exhibits superior ECM remodeling capabilities but also directly participates in tumor-stroma interactions by secreting THBS2. Prior works have primarily focused on how CAFs regulate the TME to influence cancer cell stemness, thereby promoting drug resistance and metastatic dissemination [45, 46]. However, prior to our study, the specific interactions between CAF subtypes and LUAD cells, particularly through THBS2 signaling, had not been thoroughly investigated. Unlike previous studies identifying CD36 and CD47 as known receptors of THBS2 [47], we have for the first time discovered that THBS2 can directly bind to SDC4, forming the THBS2-SDC4 complex. This complex significantly promotes LUAD progression by activating the EMT pathway. Targeting this interaction could therefore inhibit this oncogenic signaling

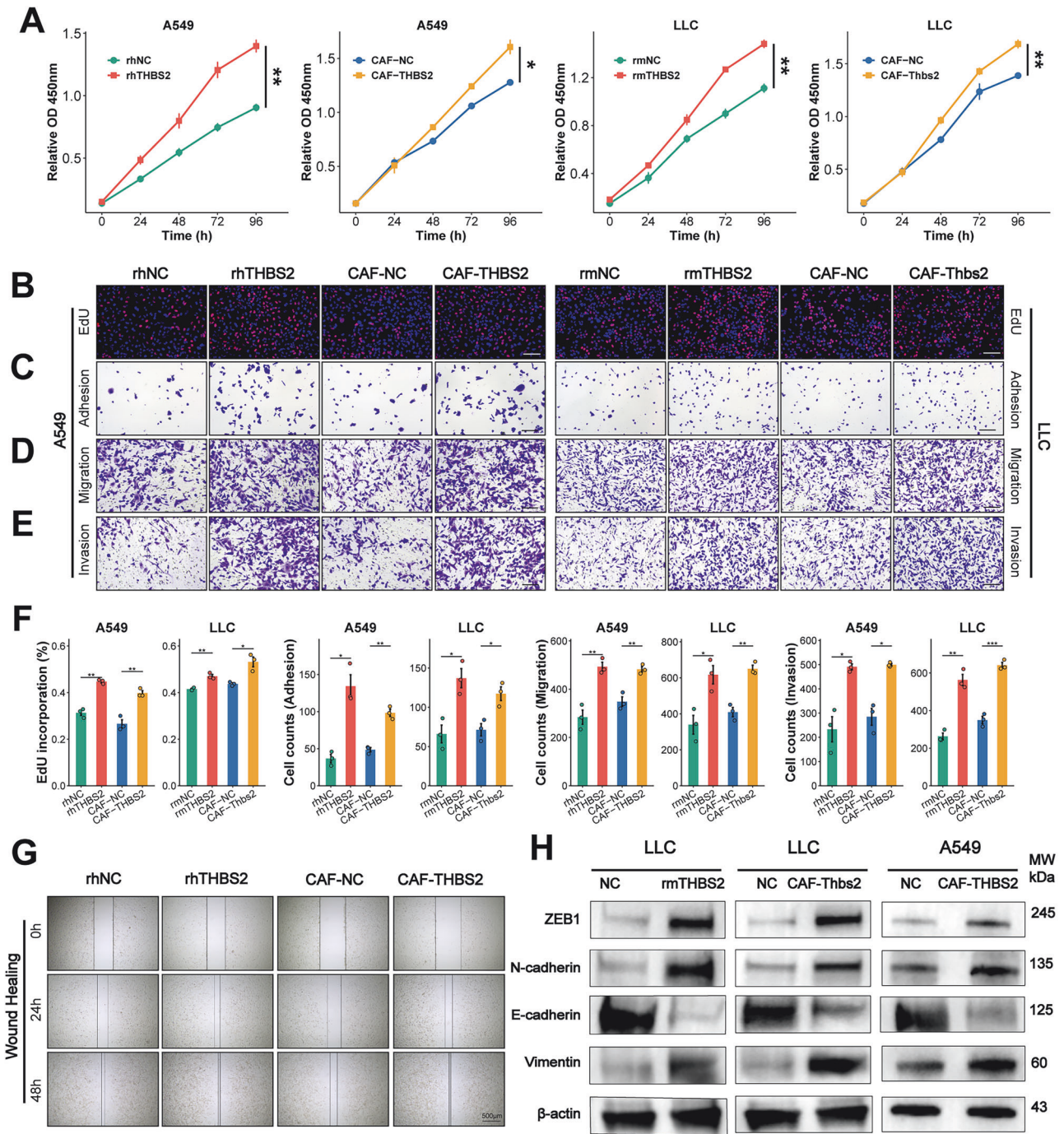


Fig. 4 THBS2 enhances oncogenic phenotypes and induces EMT in LUAD cells. **A** Proliferation assay measuring OD values of tumor cells (A549 and LLC) treated with THBS2 recombinant proteins or conditioned medium (CM) from CAF cell lines, with three biological replicates per group. **B–F** Representative images and quantitative analysis of EdU incorporation assays (**B**), adhesion assays (**C**), transwell migration assays (**D**), and transwell invasion assays (**E**) for tumor cells (A549 or LLC) treated with recombinant proteins or CAF CM with three biological replicates per group. Scale bars, 200 μm (black and white). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **G** Representative images of wound healing assays for tumor cells (A549 and LLC) treated with recombinant proteins or CAF CM. Scale bars, 500 μm. **H** Western blot analysis of ZEB1, N-cadherin, E-cadherin, Vimentin and β-actin levels in A549 and LLC cells treated with recombinant proteins or CAF CM.

cascade, reducing tumor proliferation and invasion potential. Previous studies have demonstrated that members of the SDC family are critical prognostic indicators and therapeutic targets in malignant tumors [48–50]. For instance, monoclonal antibodies or antibody-drug conjugates targeting SDCs have shown promising efficacy in studies on melanoma, triple-negative breast cancer, and multiple myeloma [51]. Additionally, Heng Yang et al. found that bufalin can specifically bind to SDC4, suppressing HCC cell

proliferation and invasion [52]. These studies further highlight the druggable potential of SDC4. Based on our identification of the oncogenic role of the THBS2-SDC4 complex in LUAD, targeting SDC4 or its associated signaling pathways may offer a novel therapeutic strategy for LUAD patients.

Although our analysis highlights the critical role of THBS2+mCAFs in the invasive progression of LUAD, their potential as effective biomarkers for identifying high-risk LUAD populations

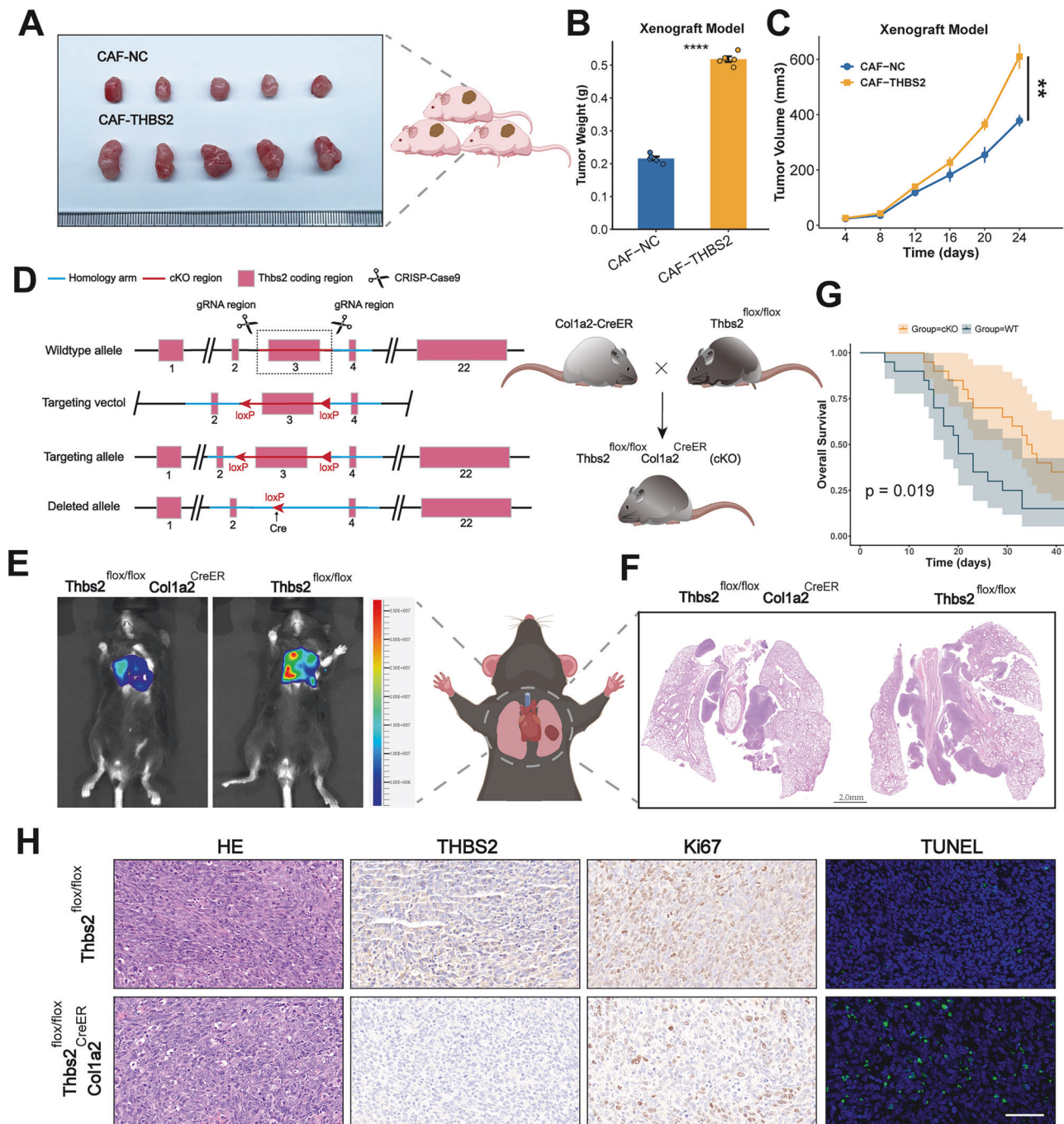


Fig. 5 THBS2 promotes tumor growth in vivo. **A** Representative images of subcutaneous xenografts model from A549 cells pre-cultured with CAF-THBS2 CM ($n = 5$) and CAF-NC CM ($n = 5$). **B**, **C** Tumor weights and volumes of subcutaneous xenografts model. The CAF-THBS2 CM treatment group displayed larger tumor weight, markedly accelerating tumor growth in vivo setting, with 5 mice per group. $**P < 0.01$, $****P < 0.0001$. **D** Schematic representation of the genetic engineering strategy used to generate fibroblast-specific *Thbs2* knockout mice. **E** Representative bioluminescence images of orthotopic tumor models from *Thbs2*^{flx/flx} (WT) and fibroblast-specific knockout mice (cKO). **F** Representative H&E staining images of orthotopic tumor models comparing tumor size between WT and cKO. Scale bars, 2 mm. **G** KM survival analysis was performed to assess the survival of cKO ($n = 20$) and WT group ($n = 20$). **H** Representative H&E staining of orthotopic tumors from WT ($n = 6$) and cKO mice ($n = 6$) revealed reduced cellular density and nuclear atypia in cKO tumors. Immunohistochemical analysis of THBS2 and Ki67 shows reduced proliferation in cKO tumors, while TUNEL staining indicates increased apoptosis. Scale bars, 50 μ m.

requires validation in larger, independent cohorts. Additionally, the therapeutic potential of the THBS2-SDC4-EMT axis in LUAD needs to be further investigated through large-scale clinical trials. Moreover, given the growing evidence that CAFs can regulate immune components within the TME to promote tumor progression, the interactions between THBS2+ mCAFs and other components of the TME, such as immune cells, remain largely

unexplored and thus warrant further investigation in the future. Understanding how CAFs influence immune cell recruitment, polarization, and function could provide valuable insights into the immune microenvironment's role in tumor progression. Future studies could focus on these complex intercellular interactions to unravel the full extent of the TME's involvement in LUAD, with particular emphasis on the dynamic crosstalk

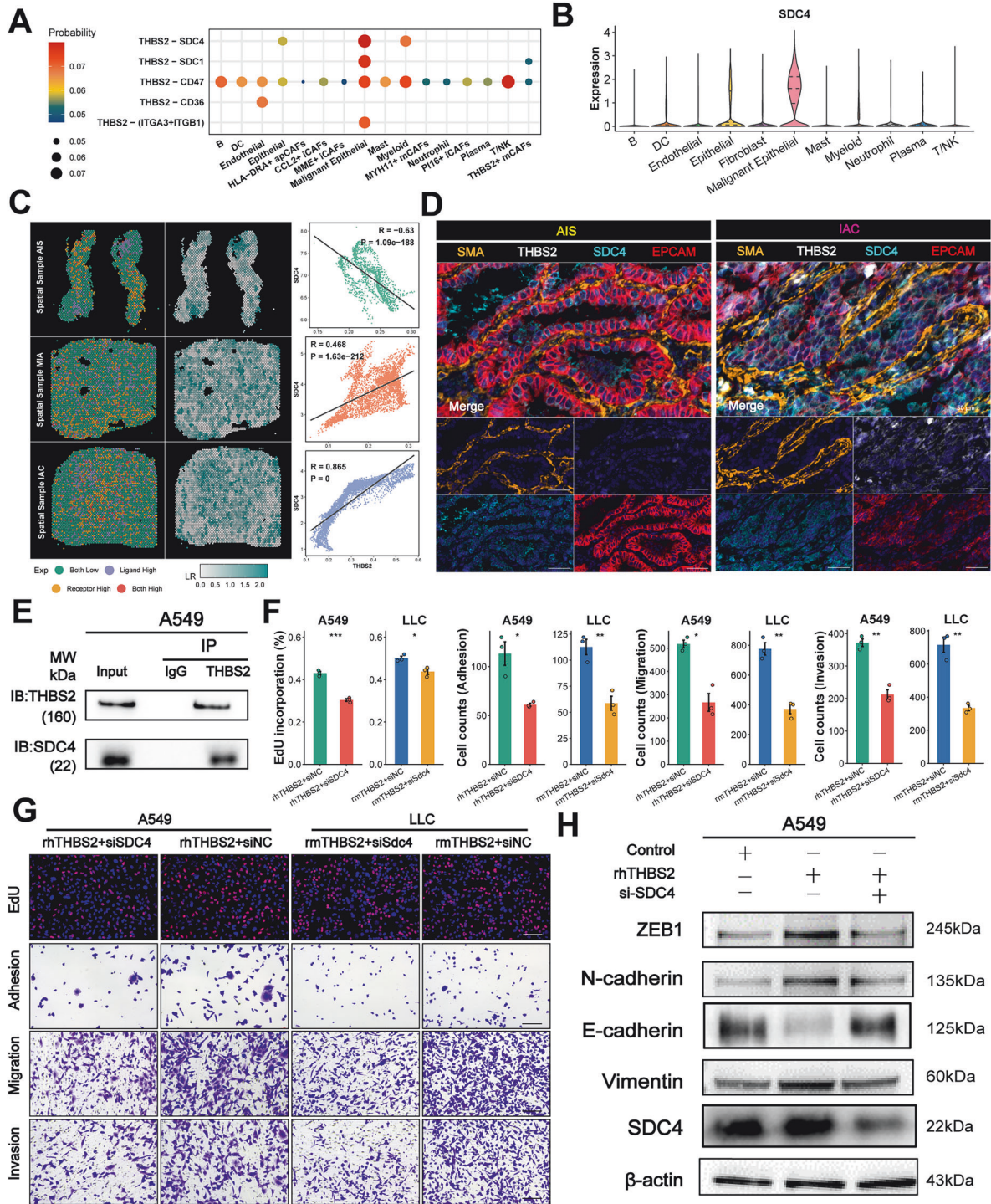


Fig. 6 THBS2 promotes malignant phenotype of LUAD through Binding to SDC4. **A** CellChat analysis identifying potential interactions between THBS2+ mCAFs and other cells in all samples. **B** The expression level of SDC4 across all cell types in single-cell transcriptomics. **C** Co-localization and correlation of the THBS2-SDC4 receptor pair at the spatial transcriptomics level in AIS, MIA, and IAC samples. **D** Representative mIHC staining images of tumor cells and CAFs in LUAD tumor tissues of AIS and IAC patients. EPCAM (red), SDC4 (blue), THBS2 (white), α-SMA (orange) in individual and merged channels are shown. Scale bar, 50 μm. **E** Co-immunoprecipitation (Co-IP) validating the direct physical interaction between THBS2 and SDC4. **F** Quantification of tumor cell proliferation, migration, invasion, and adhesion after siSDC4 treatment in A549 and LLC cells following rhTHBS2 or rmTHBS2 treatment, with three biological replicates per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. **G** Representative images of EdU incorporation assays, adhesion assay, transwell migration assays, and transwell invasion assays for tumor cells (A549 and LLC). Scale bars, 200 μm (black and white). **H** WB analysis of EMT pathway components (ZEB1, N-cadherin, E-cadherin, and Vimentin) in cells treated with rhTHBS2, siSDC4, or control.

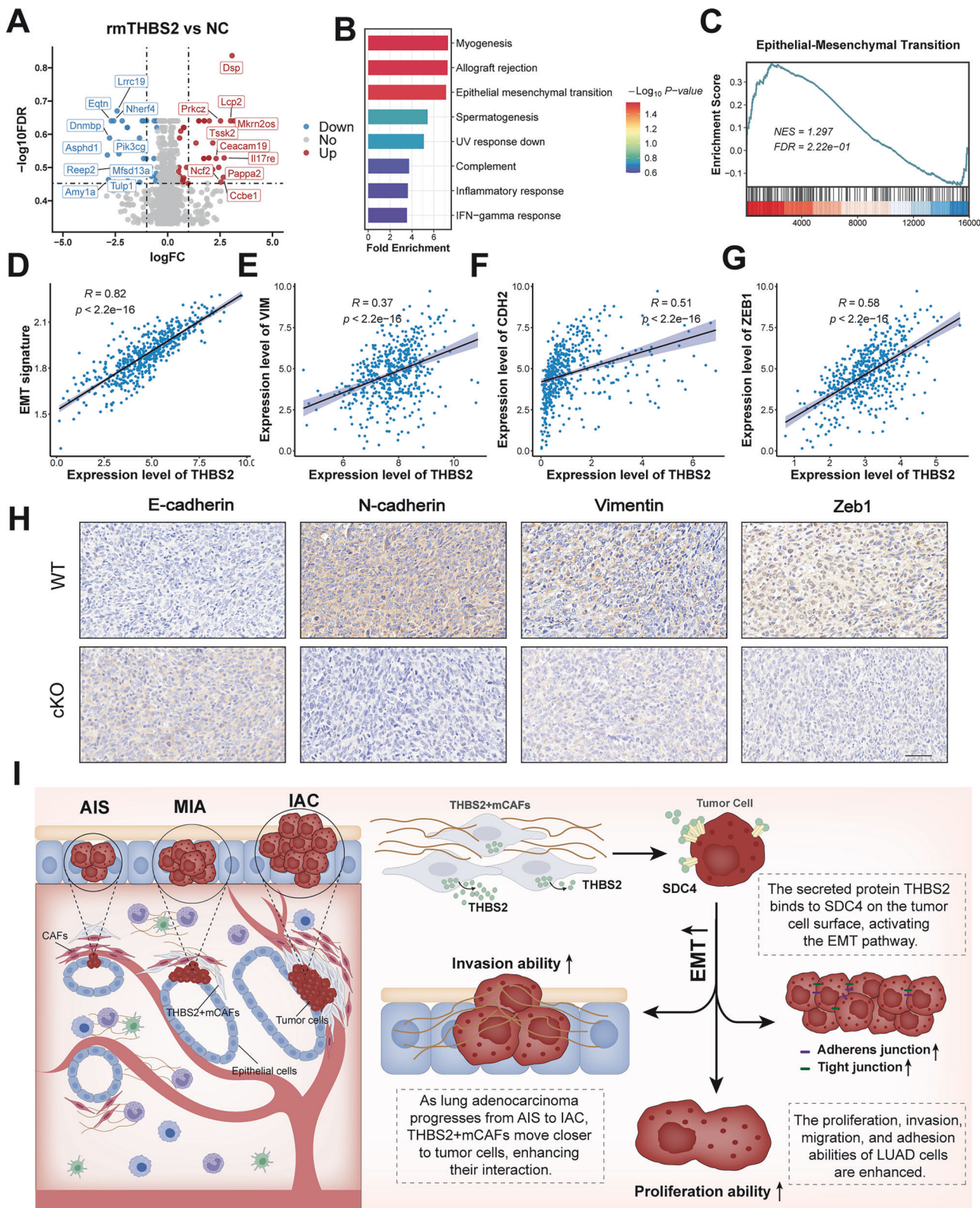


Fig. 7 THBS2-SDC4 axis promotes tumor progression through the activation of EMT signaling. **A** Volcano plot of differential gene expression analysis between rmTHBS2-treated LLC cells and control groups. **B** Transcriptomic sequencing showing enrichment of the EMT signaling pathway in rmTHBS2-treated LLC cells. **C** Functional enrichment analysis of the EMT signaling pathway in rmTHBS2-treated tumor cells. **D–G** Correlation analysis of THBS2 expression with EMT-related genes in LUAD tumor tissues. **H** IHC staining of orthotopic tumors from WT and cKO mice for E-cadherin, N-cadherin, Vimentin, and Zeb1. Scale bars, 50 μ m. **I** Schematic illustration of pro-tumor roles of THBS2+mCAFs in the tumor microenvironment of LUAD.

between CAFs, immune cells, and tumor cells. Although our study provides valuable insights into the tumor-stromal interactions during the early stages of LUAD progression, the function and presence of CAFs at metastatic sites remain poorly understood. Given that CAFs have been shown to facilitate metastasis through modulation of the extracellular matrix and interaction with immune cells, investigating their role in metastatic sites is crucial. The dynamic changes in CAF populations during tumor dissemination and their potential contribution to metastatic niche formation could provide new insights into therapeutic targeting strategies aimed at limiting metastatic spread.

In conclusion, we identified THBS2+ mCAFs as a critical subgroup involved in the dynamic process of malignant invasion in LUAD, progressing from AIS to IAC. This subgroup promotes LUAD invasion and progression by interacting with tumor cells through the secretion of THBS2, which binds to SDC4 and activates the EMT pathway. Our findings highlighted the therapeutic potential of targeting tumors with high stromal involvement and provide a potential therapeutic target for intervention in LUAD.

DATA AVAILABILITY

All scRNA-seq datasets used in this study are publicly available. The datasets can be accessed via the following links: GSE189357 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE189357>) and GSE164789 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE164789>). The bulk transcriptome data was downloaded from the TCGA (<https://portal.gdc.cancer.gov/>) and Gene Expression Omnibus database, including GSE14814 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14814>), GSE50081 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50081>), GSE72094 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72094>), GSE31210 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31210>), GSE3141 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE3141>), GSE26939 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26939>), GSE30219 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30219>), and GSE20913 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20913>). Additionally, the 10X Visium Spatial transcriptome data were collected from GSE189487 dataset (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE189487>).

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AUTHOR CONTRIBUTIONS

ZQL conceived, designed, and supervised the research. XWH and DDZ provided project guidance and funding support. YQR and RJM conducted the bioinformatics analysis. ANZ, YHB, STL and HX performed the experiments, with ANZ conducting the statistical analysis of experimental trials. YQR, RJM, and DDZ wrote the manuscript. RJM, PL and QC offered research guidance. YYZ, YY, SYW, JHD, TP, and YKC collected the data. ZQL, RJM and DDZ revised the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All human tissue samples were obtained with written informed consent from each patient and approved by the Ethics Committee (Approval Nos. HLugA120PG01: 2020-0930; HLugA030PG04-2: 2021-0215; HLugA180Su07: 2022-0401). All animal experiments were approved by the Institutional Animal Care and Use Committee of Zhengzhou University (Ethical Approval No. 2019-KY-383) and conducted in strict accordance with the National Institutes of Health (NIH) guidelines for animal care and use.

ADDITIONAL INFORMATION

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