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Deciphering the Role of ADAMTS6 in the Epithelial–Mesenchymal Transition of Lung Adenocarcinoma Cells

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Abstract

A disintegrin and metalloproteinase with thrombospondin motifs 6 (ADAMTS6) is an extracellular protease that promotes the invasion of lung adenocarcinoma (LUAD) cells. Herein, we investigate its role in epithelial-mesenchymal transition (EMT), a process that drives metastasis and drug resistance in LUAD. Re-analysis of microarray and RNA sequencing data from LUAD cells reveals that during EMT, TGF- β 1 increases *ADAMTS6* expression, presumably through the SMAD pathway, as SMAD2 loss completely blocks this effect. Moreover, it was shown that *ADAMTS6* occupies hub positions within TGF- β 1-associated gene networks. Using additional datasets, we found that *ADAMTS6* expression increases under other EMT-inducing conditions, including IL-1 β induction and acquired gefitinib resistance, but decreases upon knockdown of Twist1, a master regulator of EMT. This supports the involvement of *ADAMTS6* in EMT within a broader context. Our knockout experiments in A549 cells showed that *ADAMTS6* expression enhanced cell migration, invasion, colony formation, doxorubicin resistance, and expression of the mesenchymal markers N-cadherin, vimentin, and Twist1, but attenuated cell adhesion to the extracellular matrix and expression of the epithelial marker E-cadherin. Re-analysis of transcriptomic data from patient tumors demonstrates that high *ADAMTS6* expression correlates with the expression of EMT markers, further supporting the *ADAMTS6*–EMT link. Moreover, high *ADAMTS6* expression is associated with a worse survival prognosis. Overall, *ADAMTS6* promotes EMT in LUAD cells and may be considered a marker of this process, as well as a potential therapeutic target for its inhibition.

Keywords: ADAMTS; EMT; lung cancer; metastasis; drug resistance

1. Introduction

Lung cancer was the most common form of cancer worldwide in 2022, accounting for 2.48 million new cases, with about half being adenocarcinoma [1]. Lung adenocarcinoma (LUAD) often metastasizes to such organs as brain, bone, and liver even in its early stages, which complicates effective treatment [2]. Understanding the molecular mechanisms underlying metastasis offers valuable insights for monitoring and preventing the progression of LUAD.

Transforming growth factor beta (TGF- β) is a multifunctional cytokine with three isoforms, among which TGF- β 1 is the most widely distributed. Upon secretion into the extracellular matrix (ECM), it forms a large latent complex with other proteins. Cleavage of this complex by ECM proteases releases active TGF- β 1, which binds to the T β RI/T β RRII receptor, activating both the classical SMAD-dependent pathway and non-classical pathways like ERK, MAPK, and PI3K/AKT [3]. In LUAD cells, TGF- β 1 frequently promotes epithelial–mesenchymal transition (EMT), characterized by

the adoption of a spindle cell shape, cytoskeletal reorganization, secretion of ECM proteases, loss of epithelial markers, and the acquisition of mesenchymal markers [4–6]. These changes collectively enable tumor cells to penetrate the basement membrane and disseminate from the primary tumor site during the early stages of the metastatic cascade [7,8]. In addition, EMT increases LUAD cell resistance to chemotherapeutic agents such as docetaxel, cisplatin, and doxorubicin [9–11].

ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) is a family of 19 structurally related extracellular proteases involved in both physiological and pathological processes. Notably, some ADAMTS proteases regulate cancer metastasis and EMT by modulating cell–ECM interactions and oncogenic signaling pathways [12]. Among these, ADAMTS6 was long considered an orphan protein, but recent studies have shown that it targets ECM-associated proteins such as fibrillin-1/2, fibronectin, and syndecan-4 [13,14]. These activities may contribute to ADAMTS6's promotion of invasiveness in breast and colon cancer cells, although a direct link has yet to be established [15,16]. Additionally, ADAMTS6 activates the AKT and NF- κ B pathways via an unspecified mechanism and the SMAD pathway by cleaving latent TGF- β binding proteins 1 and 3 (LTBP-1 and LTBP-3) to activate TGF- β 1 [13,16,17]. Consequently, ADAMTS6 influences EMT marker expression by suppressing E-cadherin and inducing N-cadherin, vimentin, and Snail in colon cancer cells [16]. Thus, ADAMTS6 plays a multifaceted role in the regulation of cancer-associated EMT, acting both as an ECM remodeling protease and a regulator of cell signaling.

Although ADAMTS6 is expressed at similar levels in both tumor and normal tissues, it significantly affects chemoresistance and invasiveness in LUAD [18–21]. While its role in EMT has been established in colon cancer [16], it remains unexplored in LUAD. However, several lines of evidence suggest that ADAMTS6 may regulate EMT in LUAD cells: (1) TGF- β 1 and tumor necrosis factor alpha (TNF- α) synergistically upregulate ADAMTS6 via a KDM6B (lysine demethylase 6B)-dependent mechanism [18]; (2) ADAMTS6 promotes invasion of TGF- β 1/TNF- α -induced A549 cells in transwell assays [18]; (3) ADAMTS6 is co-expressed with vimentin in lung tumors from patients [18]; and (4) related proteases ADAMTS1 and ADAMTS16 activate latent TGF- β 1 to induce EMT in LUAD cells [22,23].

Therefore, this study aimed to investigate the role of ADAMTS6 in regulating EMT, which is important for establishing its prognostic and therapeutic significance in LUAD. To this end, *ADAMTS6* expression and its position within the gene network of TGF- β 1-induced EMT were evaluated through analysis of previously published microarray data. Publicly available microarray, RNA-sequencing (RNA-seq), and single-cell RNA-sequencing (scRNA-seq) datasets of LUAD cells were used to verify ADAMTS6 as a marker of EMT. Patient data were also analyzed to further validate the association of *ADAMTS6* with classical EMT markers, clinicopathological characteristics, and survival outcomes. Finally, *ADAMTS6* was knocked out in A549 cells using CRISPR/Cas9 methodology to assess its effect on the acquisition of mesenchymal traits by the cells.

2. Results

2.1. Assessing the EMT Regulatory Potential of ADAMTS6 in LUAD Cells

A comprehensive bioinformatic analysis of publicly available transcriptomic data was performed to investigate the potential of *ADAMTS6* in regulating TGF- β 1-induced EMT in LUAD cells. This analysis aimed to identify changes in *ADAMTS6* expression during TGF- β 1 induction and to define its position within the EMT regulome.

First, we performed differential expression analysis comparing TGF- β 1-treated H358, A549, and HCC827 human LUAD cells to untreated controls using microarray datasets GSE79235, GSE49644, GSE114761, and GSE123031. H358 cells were treated with two TGF- β 1 regimens: a full dose of 4 ng/mL for 2 wk (yielding 1906 differentially expressed genes (DEGs)) and a half-dose of 2 ng/mL for 3 wk (yielding 1032 DEGs). Both regimens shared a core transcriptomic response of 643 DEGs, while 1263 and 389 DEGs were specific to the full dose and half-dose, respectively. For A549 cells, pooled samples from 24-, 48-, and 120-h time points with 5 ng/mL TGF- β 1 treatment were compared to pooled control samples, revealing 556 DEGs. HCC827 cells treated with 10 ng/mL TGF- β 1 for 72 h

exhibited 437 DEGs (Figure 1A). Notably, only 46 DEGs were shared among the three cell lines, highlighting the strong dependence of TGF-β1-induced transcriptomic response on cell context.

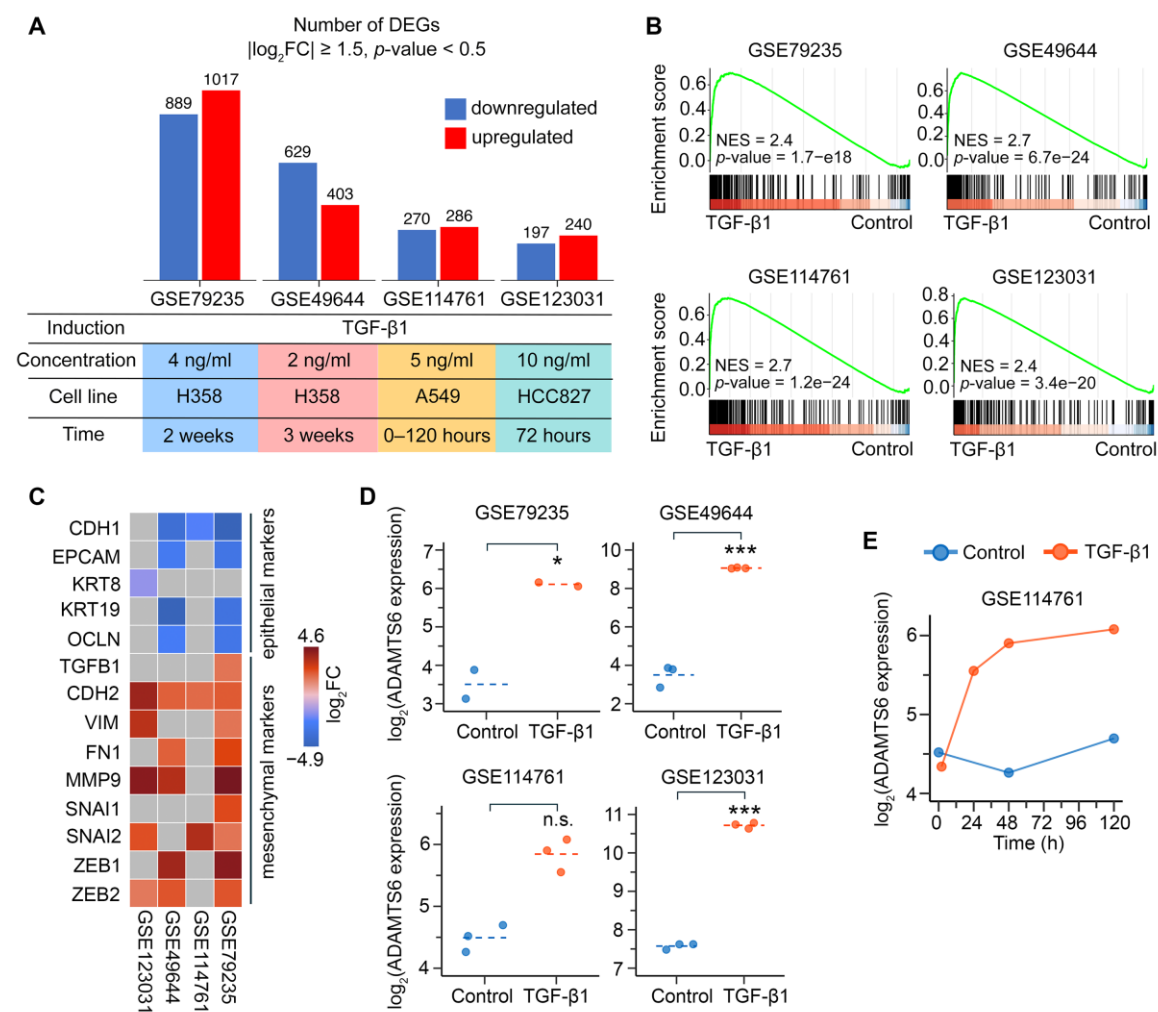


Figure 1. Dissecting the regulatory role of ADAMTS6 in TGF-β1-induced EMT of LUAD cells. (A) Differential expression analysis of TGF-β1-treated versus untreated human LUAD cells (top). The table reflects the experimental conditions used in each dataset (bottom). (B) Activation of EMT hallmark gene signature in the microarray datasets, as determined by GSEA. (C) Differential expression ($|\log_2(\text{fold change (FC)})| \geq 1.5$) of EMT markers between TGF-β1-induced and control cells in microarray datasets. Genes with non-significant changes (p -value ≥ 0.05) are indicated in gray. (D) Expression of *ADAMTS6* in control and TGF-β1-induced cells. * and *** indicate p -values of < 0.05 and < 0.001 , respectively, based on differential expression analysis. (E) Time-dependent changes in *ADAMTS6* expression in A549 cells following TGF-β1 treatment.

Next, we validated the induction of EMT in the analyzed datasets. Gene set enrichment analysis (GSEA) revealed that the EMT hallmark signature was enriched in TGF-β1-treated cells compared to controls across all four datasets (Figure 1B). While the specific set of differentially expressed EMT markers varied across datasets, a consistent pattern emerged: epithelial markers (e.g., *CDH1*, *EPCAM*, and *KRT19*) showed decreased expression, while mesenchymal markers (e.g., *CDH2*, *VIM*, and *FN1*) showed increased expression (Figure 1C). These data demonstrate that TGF-β1 induces EMT in LUAD cells, regardless of the treatment regimen, which is consistent with the original studies [24–26].

We then examined the changes in *ADAMTS6* expression across datasets. In H358 cells, TGF-β1 treatment at 4 ng/mL for 2 wk and at 2 ng/mL for 3 wk led to a 6.5- and an 8.6-fold upregulation of *ADAMTS6*, respectively. Similarly, *ADAMTS6* expression increased by 8.6-fold in HCC827 cells after 72 h of induction at 10 ng/mL. In contrast, TGF-β1 did not significantly regulate *ADAMTS6* in A549

cells, although a trend towards its increased expression was observed upon induction at 5 ng/mL (Figure 1D). Specifically, *ADAMTS6* expression increased by 30% at 24 h and rose slightly by another 6% at 48 h, before plateauing and remaining unchanged over the next 3 d of induction (Figure 1E). However, statistical validation was not feasible due to the use of a single biological replicate per time point. Despite this limitation, consistent results across four datasets suggest that *ADAMTS6* expression is induced during TGF- β 1-mediated EMT in LUAD cells.

To assess the regulatory potential of *ADAMTS6*, we performed a gene network analysis based on the assumption that genes acting as hubs within the EMT network are key regulators of this process [27]. We constructed four gene networks from DEGs obtained from four datasets using STRING database and visualized them in Cytoscape (Figure S1). Network topology was then analyzed using CytoHubba plugin, and genes belonging to the top 15% according to at least one of twelve centrality metrics were selected as hub candidates. Intersecting the candidate lists from all four networks revealed 50 hub genes that are central in at least three networks (Figure 2A). Among these, 23 were upregulated, 23 were downregulated, and four exhibited variable expression across the datasets following TGF- β 1 induction (Figure 2B).

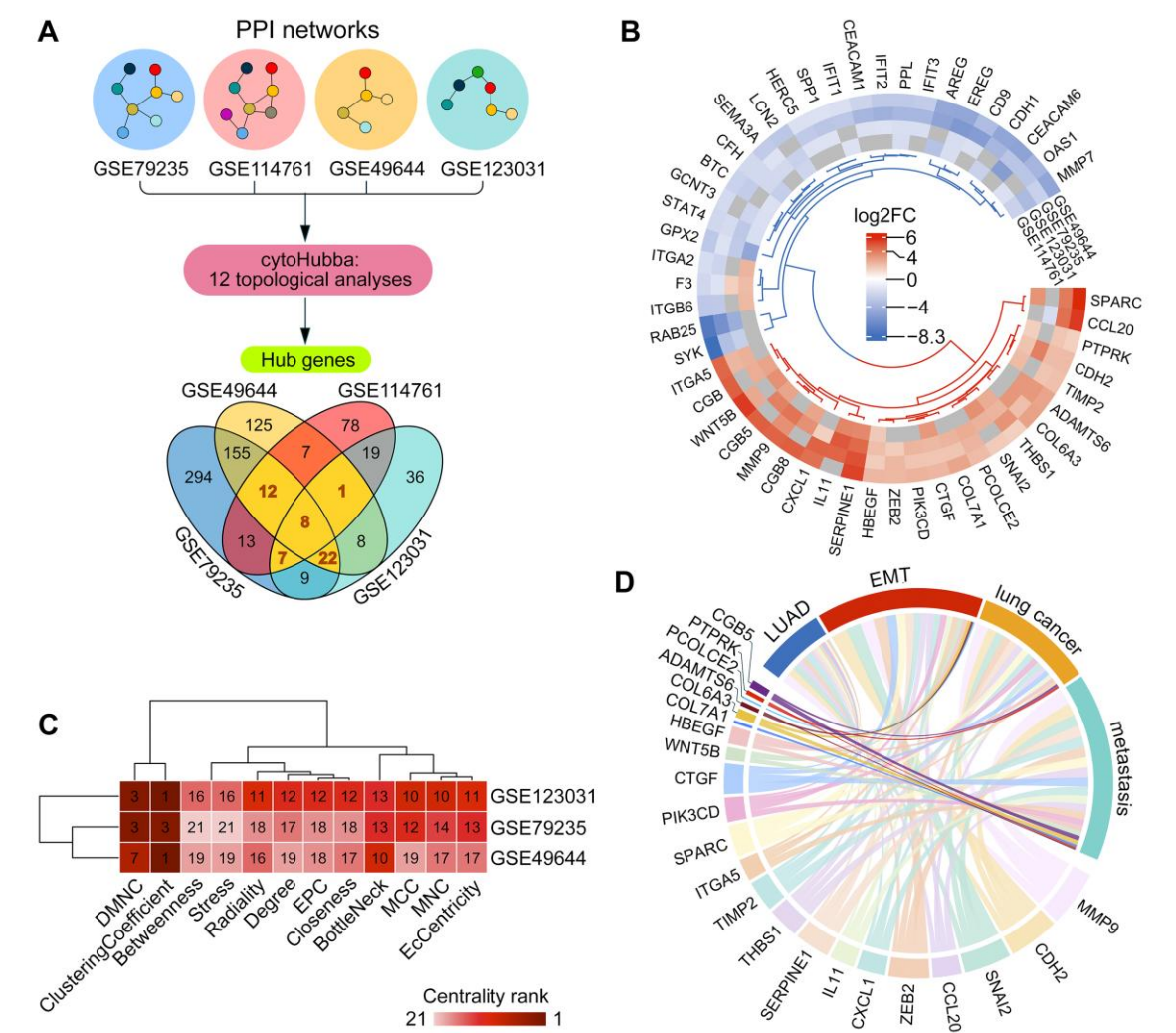


Figure 2. Gene network analysis. (A) Analysis workflow. Four gene networks were constructed from DEGs identified in each dataset by comparing TGF- β 1-treated and control cells using STRING plugin in Cytoscape, and visualized in Figure S1. Each network was analyzed with CytoHubba plugin, and genes ranked in the top 15% by at least one of twelve centrality criteria were classified as hub genes. The intersection of hub genes from four networks yielded 50 common hub genes. (B) Differential expression ($|\log_2FC| \geq 1.5$) of 50 common hub genes between TGF- β 1-induced and control cells in microarray datasets. Genes with non-significant changes (p -value ≥ 0.05) are indicated in gray. (C) Ranking of *ADAMTS6* among 23 upregulated hub genes according to 12

centrality measures computed by cytoHubba in gene networks. (D) Text mining summary. Associations of common hub genes with EMT, metastasis, LUAD, and lung cancer were extracted from PubMed Central. Line widths reflect the number of sentences in which a hub gene and term co-occur.

The downregulated hub genes included those with established roles in epithelial homeostasis and disease, such as E(epithelial)-cadherin (*CDH1*) and epidermal growth factor family proteins (*AREG*, *EREG*, and *BTC*) [28]. Additionally, genes linked to innate immunity were suppressed, including those involved in antiviral defense (*OAS1*, *HERC5*, and *IFIT1/2/3*), the antibacterial protein *LCN2*, and the complement regulator *CFH*, which reflects the central role of TGF- β 1 in coordinating immune evasion [29]. Consistently, the upregulated hub genes comprised well-studied mesenchymal markers, such as N(neural)-cadherin *CDH2*, matrix metalloproteinase *MMP9*, as well as transcription factors *SNAI2* and *ZEB2*, repressing *CDH1* expression [30].

Notably, *ADAMTS6* emerged as an upregulated hub gene. Despite its moderate centrality metrics, it ranked highly in DMNC and Clustering Coefficient (Figure 2C), suggesting a stable coordinating role within the analyzed networks.

Based on the identification of prominent EMT markers among the upregulated DEGs, we hypothesized that the remaining DEGs, including *ADAMTS6*, also play critical roles in EMT regulation. To test this hypothesis, we conducted a text mining analysis of Pubmed abstracts to examine the co-occurrence of DEG names with key terms such as “LUAD”, “lung cancer”, “EMT”, and “metastasis”. This approach identified 15 genes strongly associated with lung cancer (including 10 linked to LUAD) and 18 genes associated with metastasis, primarily connected to EMT (Figure 2D). Among these were previously mentioned *CDH2*, *MMP9*, *SNAI2* and *ZEB2*, as well as cytokines and chemokines shown to induce EMT, including *IL11* [31], *WNT5B* [32], *CCL20* [33], and *CXCL1* [34], and genes with established EMT-regulatory functions, such as *SERPINE1* [35], *THBS1* [36], and *TIMP2* [37]. The results of text mining confirm that our network analysis effectively identified key EMT regulatory genes in LUAD cells; therefore, *ADAMTS6* may also have regulatory potential, as it exhibits network connectivity similar or comparable to established EMT regulators. Additionally, text mining revealed that *ADAMTS6*, along with genes *CGB8*, *PCOLCE2*, *PTPRK*, and *COL7A1*, remains under-studied compared to established EMT regulators (Figure 2D). Overall, gene network analysis provided a strong rationale for further study of *ADAMTS6* function in EMT of LUAD cells.

2.2. Validation the Link Between *ADAMTS6* and EMT Induction in Pulmonary Epithelial Cells

To explore the connection between *ADAMTS6* and EMT induction in lung cells in greater detail, we formulated the following three questions: (i) Is *ADAMTS6* activation sustained during prolonged exposure of LUAD cells to TGF- β 1? (ii) Does *ADAMTS6* expression correlate with the EMT status of LUAD cells in scRNA-seq analysis? (iii) Is *ADAMTS6* activated upon EMT induction in other pulmonary epithelial cells, or is this relationship unique to LUAD?

Analysis of two independent transcriptomic datasets (Table S1) showed that a 2-d treatment of A549 cells with TGF- β 1 at 5–10 ng/mL increased *ADAMTS6* expression by more than 11-fold, which is consistent with the data described above. Chronic exposure of A549 cells to 2 ng/mL TGF- β 1 for 60 d produced an even more pronounced, 84.7-fold induction of *ADAMTS6* (Figure 3A). Interestingly, *ADAMTS6* upregulation was reduced 6.3-fold by a SMAD2 loss-of-function mutation but was not significantly affected by treatment with 40 mM sodium acetate (fold change = 1.2) (Figure 3A). These results suggest that TGF- β 1 induces *ADAMTS6* expression in both short-term and chronic regimens via a mechanism that is dependent on the SMAD2/3 pathway but independent of fatty acid metabolism.

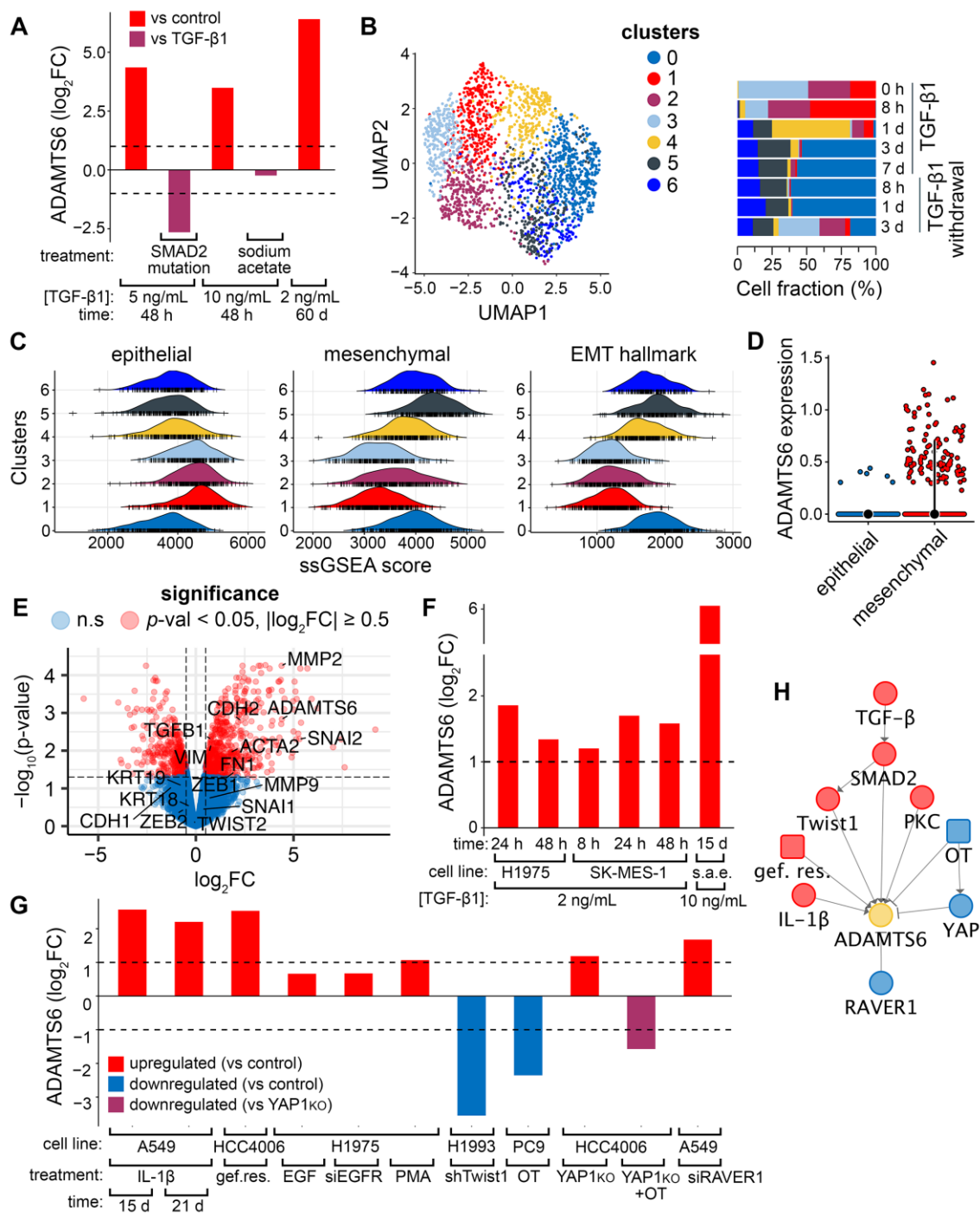


Figure 3. *ADAMTS6* as a marker of LUAD EMT. (A) Changes in *ADAMTS6* expression in A549 cells following treatment with TGF-β1 and sodium acetate at specified concentrations and time points, and after SMAD2 mutation. Comparisons were made to untreated controls (red) or TGF-β1-treated cells (dark pink). (B) UMAP embeddings of A549 cells (left) and a graph showing the relative representation of clusters under each experimental condition (right). (C) Enrichment of epithelial, mesenchymal, and hallmark EMT signatures in cell clusters evaluated by ssGSEA. (D) Normalized expression of *ADAMTS6* in epithelial and mesenchymal cells. (E) Volcano plot showing genes differentially expressed in mesenchymal cells, with *ADAMTS6* and classical EMT markers highlighted. (F) Changes in *ADAMTS6* expression in H1975 LUAD, SK-MES-1 SCC, and small airway epithelial (s.a.e.) cells following treatment with TGF-β1 at specified concentrations and time points, compared to untreated controls. (G) Changes in *ADAMTS6* expression in specified LUAD cells upon EMT induction by various stimuli. Red and blue indicate up- and downregulation compared to corresponding controls (untreated, DMSO-treated, siNC), while dark pink shows downregulation relative to YAP1 knockout. (H) Network summarizing the regulation of *ADAMTS6* by EMT-mediating genes and factors; red and blue indicate positive

and negative regulators of expression, respectively. Gef. res., gefitinib resistance; OT, osimertinib and trametinib treatment; siEGFR and siRAVER1, knockdown of EGFR and RAV1 by siRNA; shTwist1, Twist1 knockdown by shRNA; YAP1KO, CRISPR/Cas9-induced YAP1 knockout. Information on datasets, group comparisons, and treatment schemes is summarized in Table S1. Dashed lines in A, F, and G indicate significant expression changes ($|\log_2FC| \geq 1$).

To address the second question, we analyzed the scRNA-seq dataset GSE147405, which includes A549 cell samples collected from 0 h to 7 d during TGF- β 1 induction, as well as from 8 h to 3 d after TGF- β 1 withdrawal. Seven distinct cell clusters were identified: clusters 1, 2, 3, and 4 were present initially (time point 0 h), while clusters 0, 5, and 6 emerged following TGF- β 1 treatment (Figure 3B). We performed single-sample gene set enrichment analysis (ssGSEA) to evaluate the EMT status of these clusters. The epithelial gene signature was enriched in clusters 1, 2, and 3, whereas the mesenchymal gene signature and hallmark EMT signature were enriched in clusters 0, 4, 5, and 6 (Figure 3C). Accordingly, clusters 1, 2, and 3 were classified as epithelial-like, while clusters 0, 4, 5, and 6 were mesenchymal-like, which was consistent with the expression of classical EMT markers (Figure S2). *ADAMTS6* expression was higher in mesenchymal-like cells compared to that in epithelial like cells (Figure 3D), a finding further confirmed by pseudobulk analysis (Figure 3E). These results independently confirm the association between *ADAMTS6* and EMT at the single-cell level in LUAD cells.

Finally, to determine whether *ADAMTS6* activation during EMT is a common feature across pulmonary epithelial cells, we evaluated the effect of TGF- β 1 on *ADAMTS6* expression in H1975 LUAD cells, SK-MES-1 squamous cell carcinoma cells, and normal small airway epithelial cells (Figure 3F). Treatment of H1975 cells with TGF- β 1 at 2 ng/mL for 24 and 48 h increased *ADAMTS6* expression by 3.6- and 2.5-fold, respectively. In SK-MES-1 cells, TGF- β 1 at the same concentration induced a 2.3-fold upregulation of *ADAMTS6* at 6 h, rising to over 3-fold at 24 and 48 h (Figure 3F). Chronic exposure to 10 ng/mL TGF- β 1 for 15 d caused a pronounced 66.3-fold increase in *ADAMTS6* in normal small airway epithelial cells, similar to the chronic exposure effects observed in A549 cells (Figures 3A, 3F). These results indicate that TGF- β 1 induces *ADAMTS6* in a broad spectrum of pulmonary cell lines, despite differences in experimental incubation schemes. Additionally, findings in normal lung cells suggest a possible role of *ADAMTS6* in EMT during lung fibrosis, which requires further study.

2.3. Reconstruction of the EMT-Associated Upstream Regulatory Network for *ADAMTS6* in LUAD Cells

To reconstruct the upstream network controlling *ADAMTS6* activation during EMT, we supplemented our study with an analysis of transcriptomic data from LUAD cells undergoing EMT induced by factors other than TGF- β . We found that chronic exposure of A549 cells to interleukin 1 beta (IL-1 β) for 15 and 21 d increased *ADAMTS6* expression by 5.9-fold and 4.6-fold, respectively (Figure 3G). Surprisingly, another pro-inflammatory cytokine, TNF- α (20 ng/mL, 24 h), had no effect on *ADAMTS6* levels ($p > 0.05$; data not shown). Furthermore, a 5.8-fold activation of *ADAMTS6* was observed in HCC4006 cells rendered resistant to the EGFR inhibitor gefitinib (Figure 3G). Interestingly, EGFR signaling itself did not appear to play a major role in *ADAMTS6* regulation, as neither EGF treatment nor EGFR knockdown in LUAD cells induced *ADAMTS6* expression. Conversely, combined EGFR/MEK inhibition through a 2-wk treatment with 100 nM osimertinib and 30 nM trametinib (OT) induced EMT accompanied by a 5.1-fold downregulation of *ADAMTS6* [38]; this effect, however, may be attributable to MEK blockade.

Other upstream positive regulators of *ADAMTS6* included protein kinase C (PKC), induced by phorbol 12-myristate 13-acetate (PMA) in H1975 cells [39] and Twist1, the knockdown of which reduced *ADAMTS6* expression by 11.7-fold in H1993 cells (Figure 3G). In turn, the EMT repressor RAV1 and YAP1, a key component of the Hippo pathway, were identified as negative regulators of *ADAMTS6*. Their inhibition via siRNA and the CRISPR/Cas9 system, respectively, increased

ADAMTS6 levels in LUAD cells by 3.2-fold and 2.3-fold, respectively (Figure 3G). Notably, the addition of the OT combination to YAP1 knockout reduced this effect by 3-fold.

These findings (Figure 3G) were integrated into a final upstream regulatory network for *ADAMTS6*, depicted in Figure 3H. These results underscore the significance of *ADAMTS6* as an EMT marker in a broader context and support its further investigation.

2.4. The Development of a CRISP/Cas9 System Targeting *ADAMTS6*

Next, we investigated how *ADAMTS6* expression affects EMT in A549 cells using CRISPR/Cas9-induced knockout. The *ADAMTS6* gene consists of 31 exons and has multiple splice variants, with the main isoform containing 25 exons encoding a protein of approximately 125.3 kDa. To disrupt the *ADAMTS6* open reading frame (ORF), exon 4 was selected as the target region for CRISPR/Cas9-mediated editing due to its size (169 bp) and its position at the start of the transcript. The knockout strategy involved inducing two double-strand breaks (DSBs) simultaneously, one within intron 3 and another within exon 4 (Figure 4A).

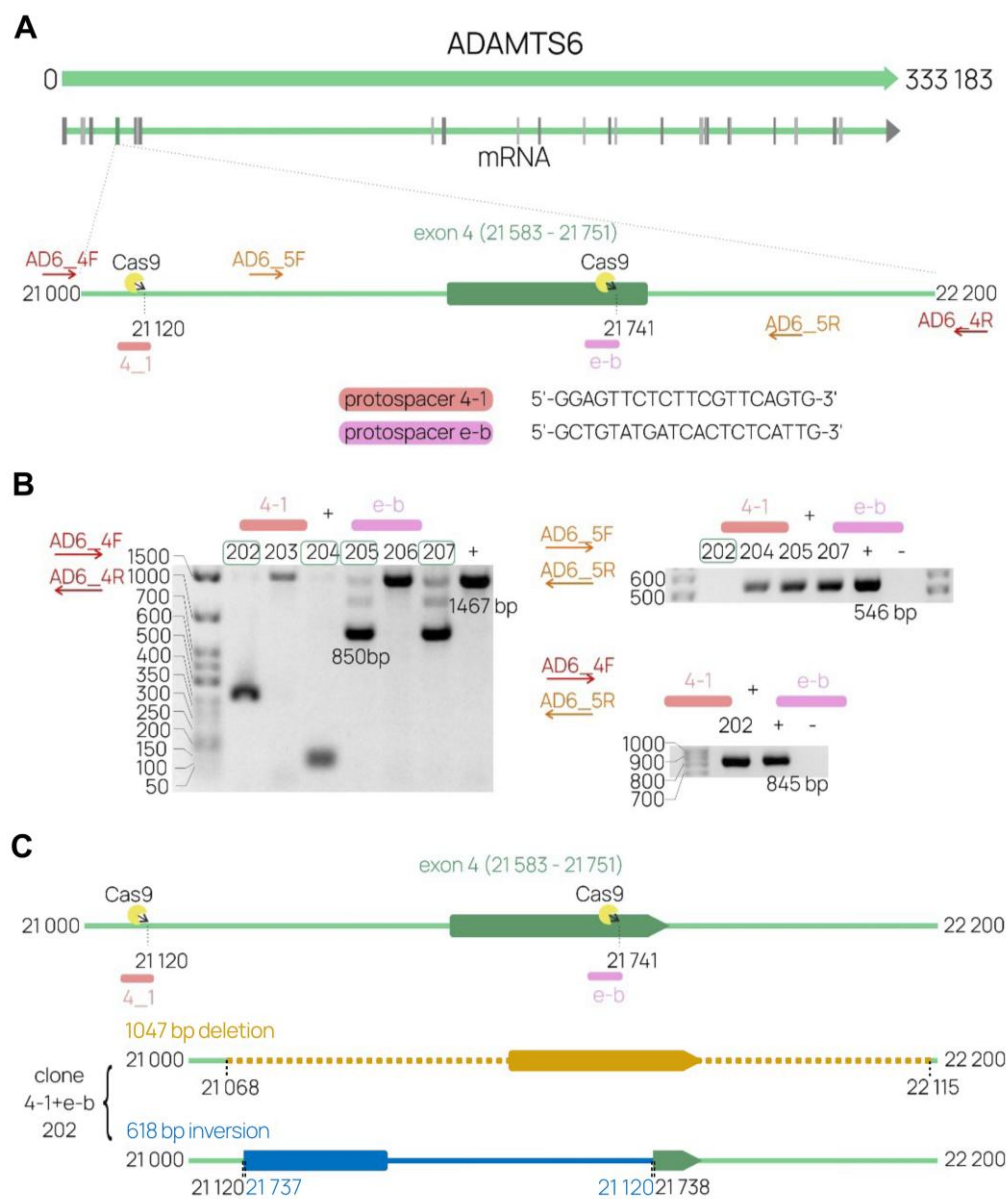


Figure 4. Generation of *ADAMTS6* knockout lines based on A549 human cells. (A) Editing strategy involving the usage of two CRISPR-induced DSBs with corresponding sequences of protospacers. (B) Clone selection based on PCR analysis of target genome region using various flanking primers. Products were analyzed in 1.5%

agarose gel. "+" denotes the control A549 cells used as a positive PCR control. (C) CRISPR-induced mutations were determined through Sanger sequencing (see supplementary data).

Target protospacer sequences identified via Benchling analysis demonstrated good efficiency/specificity ratios (Figure 4A). Constructs expressing CRISPR/Cas9 components were generated based on pX458 vector following a standard protocol [40]. A549 cells were transfected according to the proposed strategy. Subsequently, GFP-expressing cell fractions were sorted to obtain single-cell clones. Initial screening involved PCR analysis using primers flanking the expected deletion region. Several clones showed loss of genomic sequence between protospacers ("4-1" + "e-b") (Figure 4B). The size of deletions varied among clones and did not always match expectations; for example, clones 202 and 204 exhibited larger deletions. A second round of PCR, using primers closely flanking the exon, demonstrated the presence of a second *ADAMTS6* allele in all clones except one. The target region structure of clone 202 was further analyzed via Sanger sequencing of PCR products (Figure 4C). The results revealed two mutations: a 1047 bp deletion covering parts of exon 4 and introns 3 and 4, and an inversion between DSB sites that disrupts the exon structure (Supplementary results). Both mutations are predicted to result in the loss of functional *ADAMTS6* protein.

ADAMTS6 knockout was confirmed via RT-qPCR using primers located downstream of the established mutational sites. Clone 202 exhibited a 2.2-fold reduction in *ADAMTS6* mRNA level compared to the parental A549 line. Lachat et al. previously demonstrated that sequential incubation with 4 ng/mL TGF- β 1 for 3 d and 20 ng/mL TNF- α for 2 d significantly induces *ADAMTS6* expression [18]. Using this protocol, we observed a 2.6-fold increase in *ADAMTS6* mRNA level in control A549 cells. However, expression in clone 202 remained unchanged following TGF- β 1/TNF- α treatment and stayed stably suppressed relative to untreated control (Figure 5A).

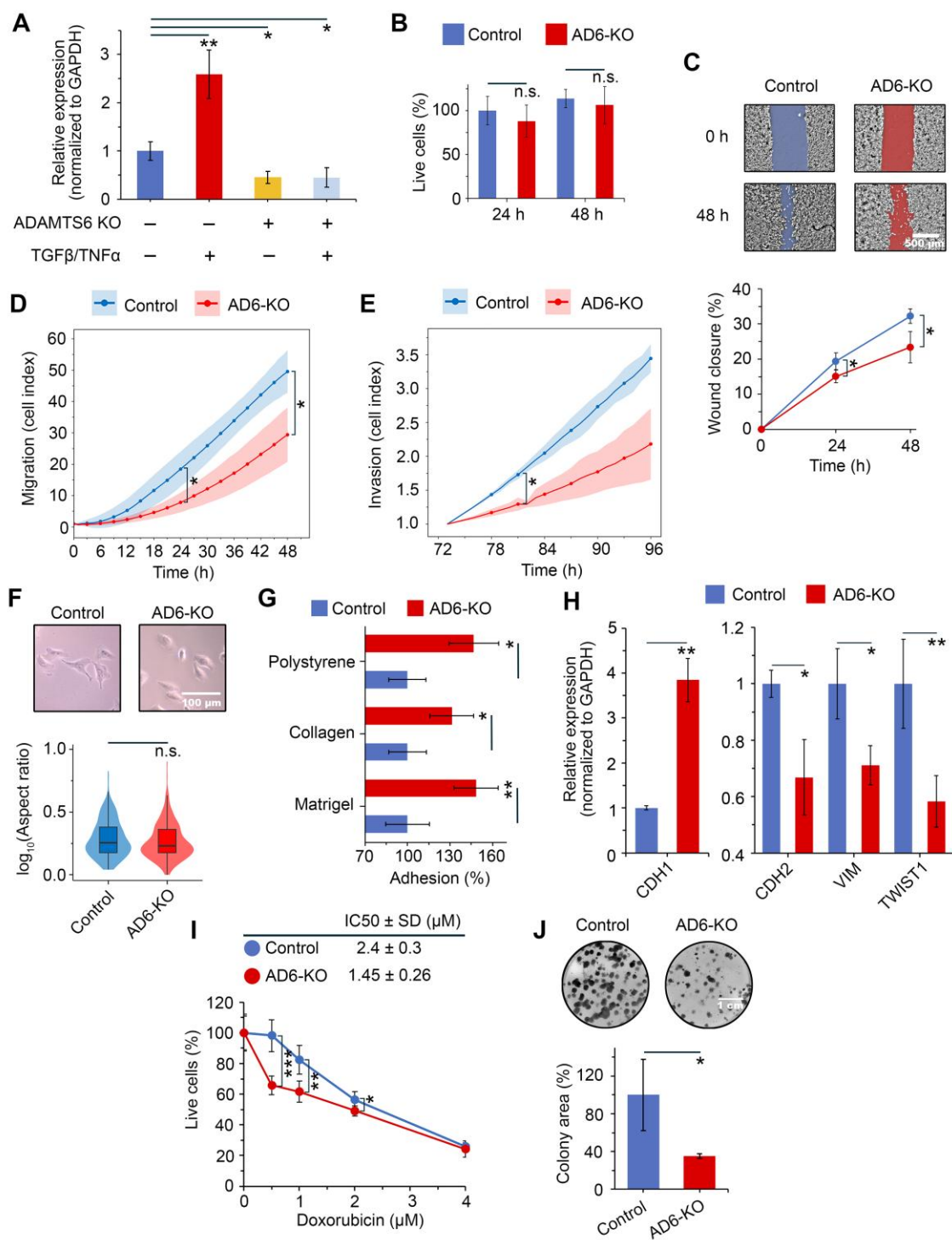


Figure 5. Comparison of EMT-associated phenotypic manifestations in parental A549 cells (control) versus cells with CRISPR/Cas9-mediated *ADAMTS6* knockout (AD6-KO). (A) Relative expression of *ADAMTS6* in control and AD6-KO cells incubated under regimens: (i) TGF-β (4 ng/ml) for 3 d + TNF-α (20 ng/ml) for 2 d, (ii) without induction (5 d). Assessment by RT-qPCR with normalization to the housekeeping gene *GAPDH*. (B) Viability of control and AD6-KO cells after 24 and 48 h of incubation evaluated by MTT assay. (C) Wound healing on cell monolayers (top). Quantification was performed by assessing the reduction of cell-free zone (bottom). (D) Real-time quantification of cell migration using xCelligence system. The cell index is normalized to the 1-h time point. (E) Real-time quantification of cell invasion through Matrigel using xCelligence system. The cell index is normalized to the 72-h time point. (F) Cell morphology (top). Quantification was performed by calculating aspect ratio, the ratio of the cell's long axis to its short axis (bottom). (G) Cell attachment to polystyrene, collagen, and Matrigel after 1 h of incubation, assessed by MTT assay after a triple wash step. (H) Relative expression of the epithelial marker *CDH1* and the mesenchymal markers *CDH2*, *VIM*, and *TWIST1* assessed via RT-qPCR and

normalized to the housekeeping gene *GAPDH*. (I) Doxorubicin cytotoxicity assessed by MTT after a 72-h incubation period. Table represents IC₅₀ quantification. (J) Colony formation after 10 d of incubation (top). Quantification was performed by calculating the total colony area (bottom). All experiments were conducted with a minimum of three biological replicates. Data are presented as mean $\pm$ SD. Statistical significance was determined by a two-tailed Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n.s., not statistically significant.

The obtained results showed that gene editing disrupted the *ADAMTS6* gene structure and abrogated its stable expression, presumably through nonsense-mediated decay [41]. Therefore, we used clone 202 to assess the influence of *ADAMTS6* on the EMT phenotype and hereafter refer to it as AD6-KO cell line.

2.5. Assessing the Effects of *ADAMTS6* Knockout on EMT Characteristics of Human LUAD Cells

To investigate the effects of *ADAMTS6* knockout on EMT-related processes, it was essential to distinguish these effects from potential impacts on cell viability caused by gene alterations. Therefore, we first evaluated the proliferative potential of the AD6-KO cell line compared to the parental A549 line using MTT staining. The results showed that AD6-KO cells had viable cell counts similar to A549 cells after 24–48 h of incubation (Figure 5B), confirming applicability for their use in experiments on the EMT phenotype.

EMT promotes tumor cell migration from the primary tumor site during dissemination, the initial stage of metastasis [42,43]. Prior study by Lachat et al. demonstrated that *ADAMTS6* knockdown suppressed, with a non-significant trend, the invasion of TGF- β 1/TNF- α -treated A549 cells ($p = 0.059$) through Matrigel, which mimics ECM [18]. Based on these findings, we investigated cell motility during *ADAMTS6* suppression in greater detail.

To assess the impact of *ADAMTS6* suppression on A549 cell motility, we performed wound healing assays. These assays revealed that AD6-KO cells exhibited a 22.4% and 27.4% reduction in migration to the center of the wound compared to A549 cells at 24 and 48 h, respectively (Figure 5C). Transwell assays further supported these findings, showing reductions of 57.6% and 40.7% in directed migration toward 10% FBS across the membrane at 24 and 48 h, respectively (Figure 5D). To model cell migration through ECM in tumors, we coated the upper chambers of a transwell system with Matrigel and monitored cell invasion into the lower chambers containing 10% FBS. Matrigel markedly impeded cell invasion, with active movement detectable only by day 3. After 83 h, AD6-KO cells exhibited a statistically significant decrease of 29.6% in their invasion capacity relative to A549 cells. This trend persisted through day 4, although increased intra-group variability diminished the statistical significance at later time points (Figure 5E). Thus, using three test systems, we confirmed the observation of Lachat et al. that *ADAMTS6* mediates LUAD cell movement.

Since cell motility relies on specialized cytoskeletal organization and adhesive interactions with the ECM substrate [43], we conducted targeted experiments to investigate whether *ADAMTS6* affects these migration-related factors. The shape of cells indirectly reflects cytoskeletal organization, as actively migrating cells are typically more elongated due to the formation of actin stress fibers and the anterior–posterior distribution of adhesive molecules [4,44]. However, microscopic examination revealed no significant differences in morphology between AD6-KO and A549 cells. Notably, the proportion of elongated cells with an aspect ratio exceeding 2 remained over 36% in AD6-KO cells, suggesting that cytoskeletal structures associated with active cell migration are not regulated by *ADAMTS6* (Figure 5F). In contrast, we observed a significant enhancement in cell adhesion upon *ADAMTS6* suppression. To evaluate this, cells were placed in culture plates coated with Matrigel or rat tail collagen, or in uncoated plates, and allowed to adhere for 1 h. Non-adherent cells were washed out, and the number of adherent cells was assessed using the MTT assay. The number of AD6-KO cells adherent to plastic, collagen, and Matrigel was 46.8%, 31.3%, and 48.6% higher, respectively, compared to the corresponding number of A549 cells (Figure 5G). These findings led us to conclude that *ADAMTS6* positively regulates A549 cell motility, likely by weakening cell adhesion to the

substrate. Supporting this conclusion, prior studies have established an inverse relationship between excessive adhesion and effective cell migration [45–47].

Both literature data and our transcriptomic analysis demonstrate that EMT is characterized by the loss of epithelial markers and the acquisition of mesenchymal lineage markers [5,6] (Figures 1C, S2). To further investigate this, we conducted RT-qPCR analysis to measure mRNA levels of epithelial markers (*KRT18*, *KRT19*, and *CDH1*) and mesenchymal markers (*FN1*, *VIM*, and *CDH2*) in A549 cells following *ADAMTS6* suppression. While the expression of the cytokeratin markers *KRT18* and *KRT19* remained unchanged (Figure S3), *ADAMTS6* knockout is accompanied by a significant increase of epithelial cadherin *CDH1* expression by 3.8-fold (Figures 5H). In contrast, mesenchymal markers *CDH2*, *VIM*, and *TWIST1* showed decreased expression by 1.5, 1.4, and 1.7 times, respectively, in AD6-KO cells compared to A549 cells (Figure 5H). These findings suggest that *ADAMTS6* facilitates the phenotypic switch to a mesenchymal phenotype during EMT.

In addition to enhanced motility, mesenchymal or hybrid E/M phenotypes generated during EMT are strongly associated with increased resistance to cancer therapy [48]. Specifically, LUAD cells have been shown to exhibit reduced sensitivity to topoisomerase II inhibitor doxorubicin following EMT induction [11,49]. In our study, *ADAMTS6* silencing decreased the doxorubicin IC₅₀ from 2.4 μ M to 1.45 μ M, with a significant increase in toxicity observed at concentration points of 0.5, 1, and 1 μ M in AD6-KO cells compared to A549 cells (Figure 5I). These findings suggest that *ADAMTS6* enhances tumor cell resistance to doxorubicin, likely through the activation of EMT.

EMT drives tumor cell intravasation into blood vessels as single cells or small cell clusters. At later stages, these disseminated cells colonize distant organ, where their ability to proliferate becomes a critical property for metastasis [42]. To model metastatic spread, we seeded cells at low density in culture plates and assessed colony formation after 2 wk. Our results showed that AD6-KO exhibited a 2.9-fold reduction in colony area compared to parental A549 cells, indicating significantly suppressed metastatic potential (Figure 5J). These findings suggest that, in addition to its role in EMT regulation, *ADAMTS6* may also contribute to metastatic outgrowth.

Collectively, our findings demonstrate that *ADAMTS6* may participate in metastatic spread of LUAD cells at two critical stages: dissemination and colonization. During the first stage, *ADAMTS6* regulates EMT by enhancing cell migration and invasion, reducing cell adhesiveness, altering marker expression, and increasing chemoresistance. The molecular mechanism underlying the role of *ADAMTS6* in the second stage, metastatic colonization, requires further investigation.

2.6. Validating Associations of *ADAMTS6* with EMT in Patient Cohort

As the final step of our study, we evaluated the extent to which findings on *ADAMTS6* involvement in EMT, obtained from cell culture models, can be applied to human tumors. We re-analyzed gene expression data from the TCGA-LUAD, GSE31210, and GSE72094 datasets, which include tumor sections from 516, 226, and 442 LUAD patients. Correlation analysis revealed a significant positive correlation between *ADAMTS6* expression and mesenchymal markers (*CDH2*, *VIM*, and *FN1*) as well as EMT-related transcription factors (*SNAI1*, *SNAI2*, *ZEB1*, and *ZEB2*), alongside a negative correlation with epithelial markers (*CDH1*, *EPCAM*, *OCLN*, and *KRT19*) (Figure 6A). Next, we selected the top 20 and bottom 20 tumor samples based on *ADAMTS6* expression in each dataset (Figure S4A) and performed GSEA, which demonstrated enrichment of the EMT hallmark signature in the “*ADAMTS6* high” groups across all three datasets (Figure 6B). Collectively, these results confirm the previously established positive association between *ADAMTS6* and EMT induction.

To further elucidate how *ADAMTS6* may regulate EMT in LUAD tumors, we analyzed the enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology molecular function (GO-MF) pathways in “*ADAMTS6* high” and “*ADAMTS6* low” groups. Beyond dataset-specific enrichment (Figures S4B, S4C), we identified two KEGG (“ECM-receptor interaction”, “focal adhesion”) and five GO-MF (“metallopeptidase activity”, “metalloendopeptidase activity”, “hormone activity”, “extracellular matrix structural constituent”, “extracellular matrix structural

constituent conferring tensile strength”) terms common to all three datasets (Figure 6C). These findings suggest that *ADAMTS6* may influence EMT not only through its metallopeptidase activity on ECM components but also by modulating focal adhesion and ligand-receptor interactions, particularly ECM-receptor interaction.

We analyzed TCGA-LUAD patients stratified by various clinicopathological characteristics and found no association between *ADAMTS6* expression and patient age, gender, pathological stage, and TNM stage (Figures S5A–F). However, *ADAMTS6* was significantly upregulated in tumor samples compared to normal lung tissues (Figure 6D), which was also confirmed using UALCAN and OncoDB databases (Figures S5G, S5H). The impact of *ADAMTS6* on the survival of LUAD patients was evaluated using Kaplan–Meier analysis. GEPIA2, utilizing RNA-Seq data from TCGA-LUAD, revealed a negative association between *ADAMTS6* expression and disease-free survival, while overall survival showed a similar trend but did not reach statistical significance in the log-rank test (Figure 6E). In contrast, an integrative analysis of 17 microarray datasets comprising 672 LUAD patients using KMplotter platform demonstrated a significant decrease in both progression-free and overall survival in patients with high *ADAMTS6* levels (Figure 6F).

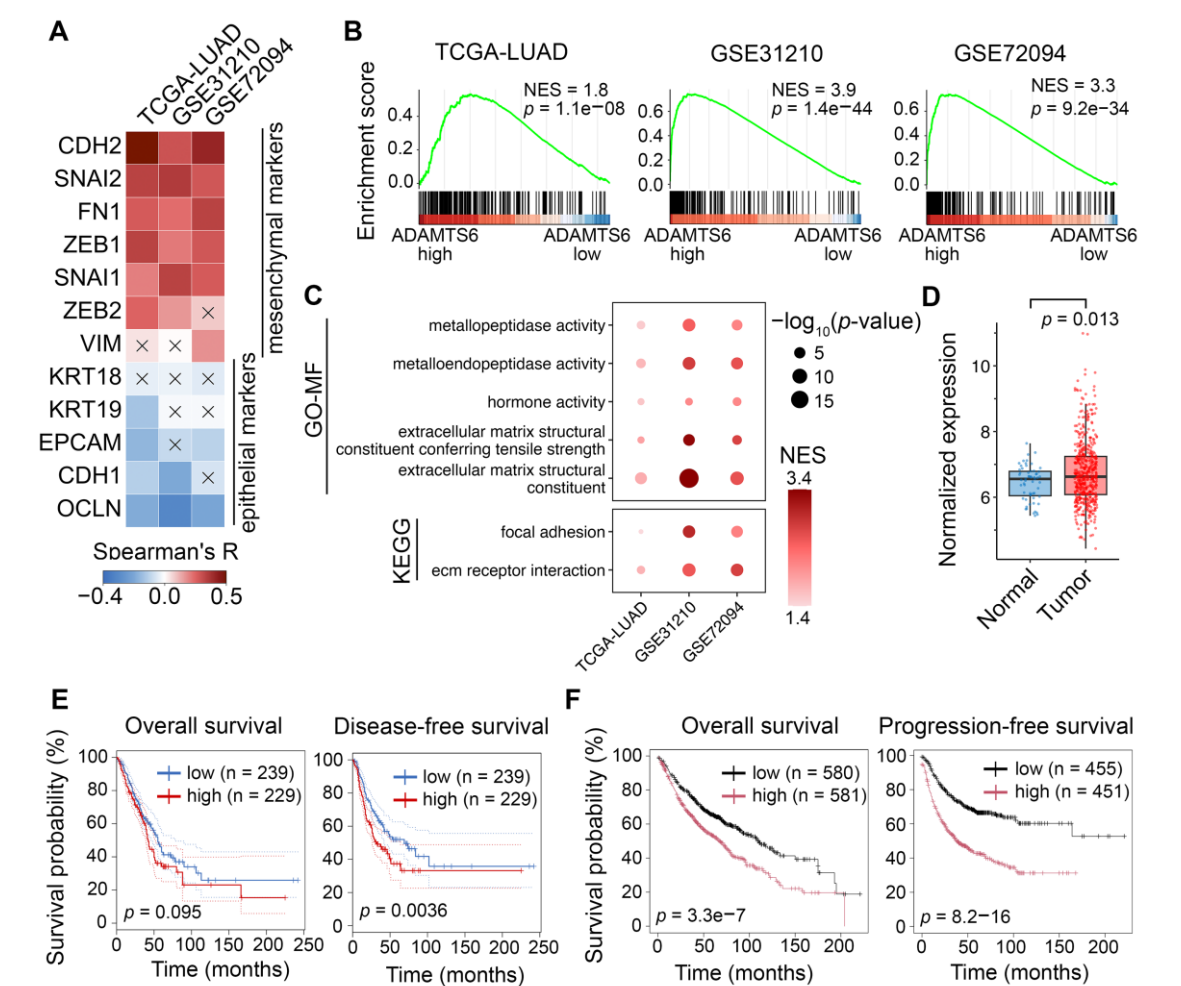


Figure 6. Correlations of *ADAMTS6* expression with EMT and clinicopathological features in LUAD patient cohorts (TCGA-LUAD, GSE31210, and GSE72094). (A) Heatmap showing the correlation between expression of *ADAMTS6* and EMT markers in patient tumors. Non-significant correlations ($p\text{-value} \geq 0.05$) are indicated by a cross. (B) Activation of the EMT hallmark gene signature in tumors with high *ADAMTS6* expression, as determined by GSEA. (C) KEGG and GO-MF terms enriched in three clinical datasets when comparing tumors with high versus low *ADAMTS6* expression. (D) *ADAMTS6* expression in normal lung tissues and tumors from TCGA-LUAD dataset. Statistical significance was determined by a two-tailed Student’s t-test. (E, F) Kaplan–

Meier survival analysis of ADAMTS6 in LUAD patients stratified by median expression, performed using GEPIA2 (E) and KMplotter (F).

Taken together, the clinical data support the involvement of *ADAMTS6* in the EMT of LUAD cells. According to *in silico* analysis, *ADAMTS6* may regulate focal adhesion and cell receptors through its metallopeptidase activity. Additionally, *ADAMTS6* expression is elevated in tumor tissues compared to normal lung tissues and is associated with worse patient survival.

3. Discussion

Metastasis is a life-threatening complication of cancer, particularly LUAD, and investigating its molecular mechanisms is crucial for developing improved methods of prognosis and treatment [2]. Recent reports show that EMT plays a key role in the early stages of LUAD metastasis by promoting cancer cell migration and invasion [4–8]. During EMT, cancer cells actively secrete extracellular proteases, among which matrix metalloproteinases (MMPs) are the most extensively studied and are increasingly recognized as promising therapeutic targets in LUAD [50]. Specifically, MMPs 2 and 9 (gelatinases) facilitate tumor cell invasion through the basement membrane by degrading its key components [51,52]. Furthermore, they support EMT by the release of TGF- β 1 from its latent complex and cleaving E-cadherin [53–55]. Consequently, MMP2/9 promote migration and invasion and upregulate the expression of N-cadherin, vimentin, and Snail in LUAD cells [55–57].

Nevertheless, clinical trials of MMP inhibitors for treating LUAD have been unsuccessful, partly because MMPs play a crucial role in maintaining normal tissue homeostasis, and their inhibition can cause severe side effects [50]. For this reason, the search for tumor-specific proteases is of great importance. ADAMTS proteases offer a promising therapeutic avenue, as they exhibit activities primarily detected in tumors, including promoting growth, drug resistance, angiogenesis, and metastasis [12,19,22,23,58–60]. Different ADAMTS family members have contrasting effects on LUAD prognosis: some, such as ADAMTS4, ADAMTS5, and ADAMTS16, are associated with poorer outcomes [22,59,61], while others, including ADAMTS1, ADAMTS8, and ADAMTS18, are linked to improved patient survival [62–64]. Therefore, it is crucial to develop inhibitors that selectively target specific ADAMTS members that are implicated in LUAD progression. For example, ADAMTS4 and ADAMTS5, which degrade aggrecan, have become targets for inhibitors currently in phase III clinical trials for osteoarthritis treatment [65]. This highlights the importance of in depth characterizing ADAMTS proteases in LUAD to better understand their prognostic significance and therapeutic potential.

Given the significant impact of metastasis on LUAD prognosis, the discovery that certain ADAMTS proteases can induce EMT and thereby promote metastasis in animal models is especially important. For example, ADAMTS4 promotes EMT in A549 and NCI-H1299 cells via c-Myc-dependent activation of the MAPK pathway [59]. ADAMTS16 and TGF- β 1 form a positive feedback loop that enhances EMT in A549 and H1975 cells: TGF- β 1 induces ADAMTS16 via the SMAD/SOX4 axis, while ADAMTS16 activates latent TGF- β 1. Knockdown of ADAMTS16 significantly reduces metastasis of A549 cells in nude mice [22]. Similarly, Hu et al. reported that ADAMTS1 promotes EMT in A549 and H226 cells and metastasis in nude mice by facilitating TGF- β 1 maturation [23]. However, Lee et al. found contradictory results, showing that ADAMTS1 suppresses EMT, as its knockdown in A549 cells increased migration, mesenchymal marker expression, and metastasis in nude mice [64]. Interestingly, ADAMTS5 enhances the motility of A549 cells while promoting a more epithelial-like phenotype, as indicated by E-cadherin and vimentin expression [61].

ADAMTS6, which targets fibrillin-1/2, fibronectin, LTBP-1/3, and syndecan-4, has emerged as a key factor in cardiovascular, musculoskeletal, and various cancer diseases [14]. The relationship between ADAMTS6 and EMT depends on the type of cancer. For example, ADAMTS6 facilitates EMT in colon cancer cells via the AKT and NF- κ B signaling pathways [16]. Immunohistochemical (IHC) studies have also shown that ADAMTS6 is co-expressed with the EMT master regulator Twist1 in esophageal squamous cell carcinoma tumors [66]. In contrast, ADAMTS6 inhibits EMT in epithelial

ovarian cancer [67]. In breast cancer, the effect of ADAMTS6 on EMT is cell line-dependent. While ADAMTS6 suppresses migration, invasion, and xenograft tumor growth in MCF7 and MDA-MB-468 cells by inhibiting the EGFR/ERK1/2 pathway, it acts downstream of the calcium channel TRPV6 to promote migration in BT549 cells [15,68].

Previously, Lachat et al. found that the histone demethylase KDM6B paradoxically induces EMT in A549 cells, whether overexpressed or inhibited. They also identified *ADAMTS6* as a direct KDM6B target that is critical for invasion triggered by co-treatment with TGF- β and TNF- α . Additionally, IHC analysis showed co-expression of ADAMTS6 with the mesenchymal marker vimentin in 30 patient tumors [18]. Based on these findings and ADAMTS6's established role in EMT across various tumor types [15,16,66–68], we propose that ADAMTS6 plays a significant regulatory role in LUAD EMT. This study supports the hypothesis by revealing that ADAMTS6 (1) shows increased expression during EMT induced by various stimuli, (2) occupies a central position in the EMT regulatory network, (3) influences EMT-associated traits, including migration, invasion, marker gene expression, chemoresistance, and colony formation, (4) correlates with clinicopathological characteristics and survival in LUAD patients.

Differential expression analysis revealed that *ADAMTS6* is significantly upregulated during TGF- β 1-induced EMT in A549, H358, HCC827, and H1975 LUAD cells (Figures 1D, 1E, 3A, 3F). ScRNA-seq of TGF- β 1-stimulated A549 cells confirmed higher *ADAMTS6* expression in mesenchymal-like clusters than in epithelial-like clusters (Figures 3D, 3E). These results are consistent with a previously published report showing that TGF- β induces *ADAMTS6* in primary human keratinocytes [69]. Moreover, ADAMTS6 activates latent TGF- β 1 by cleaving LTBP-1/3, thereby establishing a positive feedback loop [17].

Both this study and Lachat et al. demonstrated that TNF- α markedly enhances TGF- β 1-induced *ADAMTS6* expression in A549 cells (Figure 5A) [18], likely reflecting crosstalk between the SMAD and NF- κ B signaling pathways during EMT induction [70,71]. Consistently, the loss of SMAD2 or Twist1, a downstream transcription factor, suppressed *ADAMTS6* expression in TGF- β 1-induced A549 cells (Figures 3A, 3G). While TNF- α induces *ADAMTS6* in keratinocytes and retinal pigment epithelial cells [69,72], microarray analysis did not show this effect in LUAD, likely due to testing a single concentration and time point (20 ng/ml, 24 hours).

Broad analysis confirms *ADAMTS6* as an EMT marker in various conditions, including IL-1 β treatment, desensitization of LUAD cells to gefitinib, RAVER1 depletion, and PKC activation (Figure 3G). IL-1 and EGF induce *ADAMTS6* in keratinocytes [69]. In LUAD cells, IL-1 β significantly increases *ADAMTS6* expression, while the EGF/EGFR axis has little effect (Figure 3G). Conversely, ADAMTS6 inhibits EGFR activation without altering its expression in breast cancer cells [68]. Notably, resistance to the EGFR inhibitor gefitinib strongly elevates *ADAMTS6* expression in LUAD cells (Figure 3G). This resistance is associated with activation of the MEK/ERK pathway [73]. Dual inhibition of EGFR and MEK by OT reduced *ADAMTS6* expression, suggesting that MEK acts as an upstream regulator of ADAMTS6 (Figure 3G). Supporting this, nicotinamide N-methyltransferase (NNMT) decreases phosphatase PP2A methylation, which induces EMT via MEK activation in breast cancer cells [74] and promotes migration while increasing *ADAMTS6* expression in renal cancer cells [75]. RAVER1 regulates alternative splicing, and its depletion promotes TGF- β -induced EMT. The upregulation of ADAMTS6 following RAVER1 knockdown may be due to upregulation of TGF- β 1 and its receptors, T β RI and T β RII [76]. Similarly, the effect of PKC might be mediated through TGF- β 1 by inducing its secretion [77] and enhancing the expression of downstream transcription factors Snail and Twist [78]. Interestingly, YAP1 is negatively associated with *ADAMTS6* expression (Figure 3G). YAP1 mediates EMT induced by EGFR/MEK inhibition [38] or mechanical stress [79]. Since ADAMTS6 activates YAP in TC28a/2 chondrocytes [17], its downregulation by YAP1 may represent a regulatory negative feedback loop.

Gene network analysis combined with PubMed text mining identified *ADAMTS6* as a key hub in the EMT regulome (Figure 2). This approach, previously used to reveal EMT regulators such as CRTC2 [80], MDK [81], and POLR3G [82], is further validated here by its identification of well-known

EMT regulators, including *CDH2*, *SNAI2*, *ZEB2*, and *MMP9* [30,80–83]. Notably, all these genes displayed gene network interconnectivity comparable to that of *ADAMTS6*, highlighting its significant regulatory role (Figure 2C).

Using CRISPR/Cas9-induced knockout, we verified that *ADAMTS6* positively regulates EMT in LUAD. *ADAMTS6* promoted A549 cell migration and invasion by reducing cell-ECM adhesion (Figure 5C–E, 5G). This effect likely stems from *ADAMTS6* cleaving syndecan-4 and inhibiting focal adhesions, as shown in normal epithelial cells [13]. Transcriptomic analysis showed that patient tumors with high *ADAMTS6* expression were enriched for focal adhesion gene signatures (Figure 6C), possibly reflecting a compensatory response to restore cell adhesion. By cleaving syndecan-4, *ADAMTS6* reduces activation of focal adhesion kinase (FAK) [13]. To compensate, cells may upregulate Pyk2, a structurally similar kinase, or FAK-related non-kinase (FRNK), both of which can partially substitute for FAK functions [84,85]. The role of the *ADAMTS6*/syndecan-4/FAK axis in LUAD EMT requires further investigation.

We demonstrated that *ADAMTS6* regulates EMT markers at the transcriptomic level by increasing N-cadherin (*CDH2*), vimentin (*VIM*), and Twist1 (*TWIST1*), while decreasing E-cadherin (*CDH1*) in A549 cells (Figure 5H). Similar associations were observed in LUAD patient tumors, where high *ADAMTS6* expression correlated with enrichment of the EMT hallmark gene signature (Figures 6A, 6B). These findings support the previously reported link between *ADAMTS6* and vimentin expression in patient lung tumors [18]. Similar effects on N-cadherin, vimentin, and E-cadherin were reported in colon cancer cells [16], and *ADAMTS6* expression was also correlated with Twist in esophageal squamous cell carcinoma tumors [66].

EMT enhances the resistance of LUAD to various chemotherapy drugs [9–11]. Specifically, tumor cells that have undergone EMT exhibit reduced sensitivity to doxorubicin, a DNA intercalating agent [11]. In our study, doxorubicin demonstrated a pronounced cytotoxic effect on A549 with suppressed *ADAMTS6*, which we suspect is related to the role of *ADAMTS6* as an EMT inducer (Figure 5I). In contrast, *ADAMTS6* increases the sensitivity of PC-9 LUAD cells to gefitinib, an EGFR inhibitor, by forming a negative feedback loop with AGR2 [19]. Interestingly, AGR2 inhibits EMT in A549 and H1299 cells: upregulates the epithelial marker E-cadherin, downregulates the mesenchymal markers vimentin, N-cadherin, ZEB1 and Slug, reduces cell invasion, enhances cell adhesion to plastic and Matrigel, and strengthens cell-cell contacts [86]. These effects contrast with the *ADAMTS6* activity demonstrated here (Figure 5). Based on these observations, we hypothesize that the *ADAMTS6*/AGR2 axis may have opposing effects on gefitinib resistance and EMT, which requires further investigation.

We propose two mechanisms by which *ADAMTS6* may induce EMT, based on enriched terms from GSEA of patient LUAD tumors.

- One of the enriched terms is “ECM-receptor interactions” (Figure 6C). *ADAMTS6* cleaves the ECM-interacting receptor syndecan-4, as well as ECM components such as fibronectin and fibrillin-1 [13]. Syndecan-4, upregulated by TGF- β 1 or delivered via extracellular vesicles, induces EMT in A549 cells [87,88]. Although syndecan-4 shedding does not directly drive EMT [87], it may influence EMT indirectly, as syndecan-4 binds TGF- β 1 and attenuates SMAD3 activation [89]. Fibronectin proteolysis affects EMT in multiple ways, depending on the specific proteins released from its binding: the release of integrin α 5 β 1 inhibits FAK activation and cell invasion [90], whereas the release of latent TGF- β 1 activates the SMAD2/3 pathway in breast cancer cells [91]. Fibrillin-1 likely has little influence on EMT, acting downstream of syndecan-4 and fibronectin. Notably, fibronectin replaces syndecan-4 as the key regulator of fibrillin-1 deposition during TGF- β 1-induced EMT [92]. Fibrillin-1 knockdown does not affect fibronectin expression or EMT-associated processes, including migration, invasion, and marker expression, in breast cancer cells [93].
- Another enriched term is “extracellular matrix structural constituent conferring tensile strength” (Figure 6C). *ADAMTS6*-mediated ECM cleavage may alter matrix stiffness, inducing EMT in LUAD cells through mechanical forces. Consistently, *ADAMTS6* increases mechanotension in

TC28a/2 chondrocytes, promoting YAP/TAZ nuclear translocation [17], a hallmark of EMT triggered by mechanical stress [79]. Together, latent TGF- β 1, syndecan-4, fibronectin, and ECM stiffness are potential ADAMTS6 targets regulating EMT in LUAD cells and require further investigation.

Although *ADAMTS6* did not affect the viability of A549 cells under normal growth conditions (Figure 5B), it significantly enhanced their survival at low density, suggesting a role in metastatic outgrowth (Figure 5J). Other EMT-inducing ADAMTS proteases, ADAMTS1 and ADAMTS16, have also been shown to promote lung cancer metastasis: ADAMTS1 accelerates spontaneous pulmonary metastasis of Lewis lung carcinoma in C57BL/6 mice, while ADAMTS16 enhances pulmonary metastasis of A549 cells following tail vein injection in nude mice [22,94]. In contrast, ADAMTS8 suppresses colony formation in A549 and H460 cells, consistent with its association with improved patient survival [58,63]. Future studies should investigate whether ADAMTS6 promotes metastatic nodule growth in animal models and clarify the mechanisms involved.

By analyzing clinicopathological parameters, we found that ADAMTS6 expression did not vary in LUAD tumors from patients of different ages, genders, tumor stages, and TNM stages (Figure S5). However, ADAMTS6 levels were elevated in LUAD tumors compared to normal lung tissues (Figure 6D). Interestingly, this finding contradicts previous studies that reported no difference between normal and tumor tissues [20,21]. To validate our results, we cross-checked the data using the UALCAN and OncoDb databases [95,96] (Figures S5G, S5H). Additionally, we observed that high ADAMTS6 expression was associated with reduced overall, disease-free, and progression-free survival in LUAD patients (Figures 6E, F). Therefore, like ADAMTS4 [59], ADAMTS5 [61], and ADAMTS16 [22], ADAMTS6 may serve as a negative prognostic marker for LUAD.

This study has limitations. Although *ADAMTS6* knockout reduced both cell adhesion and invasion, further experiments are needed to establish a causal relationship between these effects (Figures 5C–E, 5G). Overexpression studies are also necessary to complement knockout results (Figure 5). Additionally, evaluating the impact of *ADAMTS6* on LUAD metastasis in mouse models would be valuable. Our analysis of molecular mechanism relied on tumor RNA sequencing data, which may be confounded by patient heterogeneity and the presence of non-tumor cell types within the tumors. To address this, we analyzed three independent clinical datasets to identify common pathways (Figure 6C); however, these findings require confirmation through biochemical and genetic validation in cell models.

4. Materials and Methods

4.1. Data Acquisition

Microarray data from TGF- β 1-induced H358 cells (GSE79235, GSE49644), A549 cells (GSE114761), HCC827 cells (GSE123031), and patient LUAD tumors (GSE31210 and GSE72094), along with scRNA-seq data from TGF- β 1-treated A549 cells (GSE147405) and several verification datasets (Table S1), were retrieved from Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>) with accession dates of 22 September 2022 (cell data), 23 August 2025 (patient data), 24 February 2025 (scRNA-seq), and 4 October 2025 (verification data). The Cancer Genome Atlas Lung Adenocarcinoma (TCGA-LUAD) RNA-Seq dataset was sourced from Genomic Data Commons (GDC) data portal (<https://portal.gdc.cancer.gov/>, accessed on 23 August 2025).

4.2. Microarray Analysis

Expression data were normalized using log normalization. Differential expression analysis of the cell culture datasets GSE79235, GSE49644, GSE114761, and GSE123031 was performed using GEO2R tool, applying a threshold of $|\log_2FC| \geq 1.5$ and a Benjamini-Hochberg corrected p -value < 0.05 . In the verification datasets, changes in *ADAMTS6* expression during EMT in LUAD, lung squamous carcinoma, and small airway epithelium cells were analyzed using GEO2R, with significant regulation defined as $|\log_2FC| \geq 1$ and a Benjamini-Hochberg corrected p -value < 0.05 . The design of these comparisons is presented in Table S1. For clinical datasets GSE31210 and GSE72094,

differential expression analysis was conducted by selecting the top 20 and bottom 20 samples based on *ADAMTS6* expression and comparing these groups using limma package (v3.62.2). GSEA was performed on DEGs to assess the enrichment of the hallmark EMT gene set, as well as gene sets from Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology molecular function (GO-MF).

4.3. Gene Set Enrichment Analysis

All gene sets were obtained from Molecular Signatures Database (MSigDB) using msigdb package (v25.1.1). GSEA was performed on DEGs ranked by log₂FC using clusterProfiler package (v4.14.6) with the following parameters: gene set sizes ranging from 25 to 500, multiple testing correction using the Benjamini-Hochberg method, and a *p*-value cutoff of 0.05.

4.4. Gene Network Analysis

Gene networks were constructed using DEGs from GSE79235, GSE49644, GSE114761, and GSE123031 with STRING plugin (v2.2.0) in Cytoscape platform (v3.10.3), applying a confidence score > 0.7 and no additional interactions as cutoff criteria. Gene centrality was assessed using twelve topological criteria from cytoHubba plugin (v0.1).

4.5. Text Mining

The co-occurrence of hub gene names with the terms “lung adenocarcinoma OR LUAD”, “epithelial-mesenchymal transition OR EMT”, “lung cancer”, and “metastasis” within the same sentence in the MEDLINE database was evaluated using GenCLiP 3.0 (<http://cismu.net/genclip3/analysis.php>, accessed on 6 March 2025). A gene was considered linked to a term if more than five articles mentioning both were found.

4.6. scRNA-Seq Analysis

scRNA-seq analysis was performed using Seurat package (v5.3.0). High-quality cells were included in the analysis if they had more 200 but less than 5000 detected genes, over 1000 UMI counts, and less than 5% mitochondrial gene content. Raw data were log-normalized, and expression values of all genes were scaled. Dimensionality reduction was conducted using principal component (PC) analysis on the top 2000 variable genes. Cells were clustered using the top 50 PCs and a resolution of 1 via FindNeighbors and FindClusters functions. To determine whether cell clusters were epithelial-like or mesenchymal-like, ssGSEA was performed using irGSEA package (v3.3.2) with default settings, assessing enrichment of the hallmark EMT set from MSigDB, along with epithelial and mesenchymal gene sets identified by Cook et al. [97]. Clusters were then merged into epithelial-like and mesenchymal-like groups, and pseudobulk differential expression analysis was performed using edgeR algorithm from DElegate package (v1.2.1), applying a threshold of $|\log_2FC| \geq 0.5$ and a false discovery rate (FDR)-adjusted *p*-value < 0.05.

4.7. sgRNA Design and Cloning Strategy

sgRNAs targeting intron 3 (“4-1”) and exon 4 (“e-b”) of the *ADAMTS6* gene were designed using Benchling (<https://www.benchling.com>, accessed on 25 October 2022) (Table S2). Top and bottom strands of protospacers were annealed and cloned into CRISPR/Cas9-expressing vector pSpCas9(BB)-2A-GFP (pX458; Addgene, #48138) as described previously [40]. The vector was pre-digested with *Bst*VI restriction endonuclease (SibEnzyme, Novosibirsk, Russia), and ligation was performed using T4 DNA ligase (Thermo Fisher Scientific, Waltham, MA, USA). Electrocompetent *E. coli* TOP10 cells were transformed using MicroPulser (Bio-Rad, Hercules, CA, USA). Plasmids were isolated using Plasmid-mini (Biolabmix Ltd., Novosibirsk, Russia) and HiPure Plasmid EF Maxi (Magen, China) kits. DNA concentration and quality were assessed using a Nano-500 spectrophotometer (Allsheng Instruments Co., Ltd, Hangzhou, China). Insertions into plasmids were verified by Sanger sequencing.

4.8. Cell Lines and Transfection

Human A549 LUAD cells were obtained from Russian Cell Culture Collection (St. Petersburg, Russia) and maintained in DMEM (Sigma-Aldrich Inc., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Dia-M, Moscow, Russia), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Central Drug House, New Delhi, India) at 37 °C in 5% CO₂. A549 cells (1×10^6) were plated in 6-well plates and, after 24 h, transfected with 2 µg of expression vectors containing the “4-1” and “e-b” inserts mixed at a 1:1 ratio using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. Cells transfected with pX458 plasmid lacking the spacer sgRNA region served as the transfection control. After 48 h, cells were sorted by FACS (S3e Cell Sorter, Bio-Rad, Hercules, CA, USA), and 72 h later, single GFP-positive cells were plated in 96-well plates at a density of 0.5 cells per well. After 10–14 d of growth in full DMEM/F12, single-cell clones were divided into two 96-well plates for maintenance and mutation verification, respectively.

4.9. Verification of CRISP/Cas9-Generated Mutation

DNA was extracted from clones using 100 µL of Fast lysis buffer (Biolabmix Ltd., Novosibirsk, Russia) supplemented with Proteinase K, following the manufacturer’s instructions. Routine PCR amplification was performed using BioMaster HS-TaQ PCR-Color mix (Biolabmix Ltd., Novosibirsk, Russia) and primers listed in Table S2. Mutations were identified by analyzing PCR products separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

Clones harboring mutations were selected for Sanger sequencing using 4F/4R primers (Table S2). Samples preparation involved isolating genomic DNA using D-cells kit (Biolabmix Ltd., Novosibirsk, Russia), followed by PCR amplification and purification of the resulting amplicons using DR kit (Biolabmix Ltd., Novosibirsk, Russia).

4.10. Sanger Sequencing

Sequencing was performed using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). Reaction products were purified on Sephadex G-50 columns (Cytiva, Sweden) and analyzed using an ABI 3130XL Genetic Analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at SB RAS Genomics Core Facility.

4.11. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

To study ADAMTS6 expression, cells were seeded in 12-well plates for 24 h, then cultured in DMEM with 5% FBS and treated with 4 ng/mL TGF-β (ProSpec-Tany Technogene Ltd., Ness-Ziona, Israel) for 3 d followed by 20 ng/mL TNF-α (Sci-Store, Skolkovo, Russia) for 2 d. To evaluate EMT marker expression, cells were cultured in DMEM with 10% FBS for 24 h. After the end of incubation periods, total RNA was isolated from cells using TRIzol Reagent (Ambion, Austin, TX, USA) according to the manufacturer’s protocol. First strand cDNA was synthesized using 4 µg total RNA, 0.1 µM random hexaprimer, 5000 U/mL M-MuLV-RH revertase, and RT buffer mix (Biolabmix, Novosibirsk, Russia). RT-qPCR was performed using gene specific primers (Table S3) and BioMaster SYBR Blue reagent kit (Biolabmix, Novosibirsk, Russia). Relative expression was determined by normalizing to the housekeeping gene *GAPDH* using the $2^{-\Delta\Delta CT}$ method.

4.12. Cell Viability Analysis

Cells (10^4) were cultured in 96-well plates in DMEM supplemented with 10% FBS for 24 and 48 h. Then, MTT dye was added at a concentration of 0.5 mg/mL and incubated for 3 h. After incubation, the medium was removed, formazan crystals were dissolved in DMSO, and the optical density was measured at 570 nm using Multiscan RC plate reader (Thermo LabSystems, Helsinki, Finland).

4.13. Wound Healing Assay

Cells (1.3×10^5) were seeded in 24-well plates and incubated for 24 h to reach a confluent monolayer. Wounds were created on the monolayers using 10 μ L pipette tip, and detached cells were removed by washing with PBS. The cells were then cultured in serum-free DMEM for 24 and 48 h, and their migration toward the wounds was captured using EVOS XL Core microscope with a built-in camera (Thermo Fisher Scientific, Waltham, MA, USA). Wound closure was quantified by measuring the wound area at each time point using ImageJ (v2.14.0) and normalizing it to the wound area at 0 h.

4.14. Transwell Assays

For migration assays, 2×10^4 cells suspended in serum-free DMEM were placed in the upper chamber of CIM-plate, while DMEM with 10% FBS was added to the lower chamber. The invasion assay followed a similar protocol except that the bottom of the upper chamber was coated with a 1:5 dilution of Matrigel (BD Biosciences, Bedford, MA, USA) in DMEM for 1 h before adding the cells. Cell migration and invasion to the lower chamber were monitored using xCELLigence RTCA DP system (ACEA Biosciences Inc., San Diego, CA, USA).

4.15. Evaluation of Cell Morphology

Cells (2500) were plated in 96-well plates for 24 h. Next, images were captured using EVOS XL Core microscope and 200 cells from five random fields of view were quantified for shape (aspect ratio, AR) and size using ImageJ.

4.16. Adhesion Assay

Cells (5×10^4) were seeded in 96-well plates coated with Matrigel, rat tail collagen (Cell Applications Inc., San Diego, CA, USA), or left uncoated. After 1 h, non-adherent cells were removed by washing three times with PBS, and adherent cells were quantified by MTT assay.

4.17. Colony Formation

Cells were plated at low density (300 cells per well) in 6-well plates and incubated for 10 d, with medium refreshed every 5 d with 10% FBS-supplemented DMEM. Colonies were fixed with 4% formaldehyde and stained with 0.1% (w/v) crystal violet dye. Images were captured using iBright 1500 Imaging System (Thermo Fisher, Waltham, MA, USA), and colony area was measured using ImageJ.

4.18. Bulk RNA-Seq Analysis

Expression data from the TCGA-LUAD dataset were normalized using variance stabilizing transformation (VST) method. Subsequently, the top 20 and bottom 20 samples were selected based on *ADAMTS6* expression levels. Raw counts were then extracted for these selected samples, and differential expression analysis comparing high and low *ADAMTS6* expression groups was performed using DESeq2 package (v1.46.0). Subsequently, GSEA was performed on DEGs to assess the enrichment of the hallmark EMT gene set, as well as gene sets from Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology molecular function (GO-MF).

4.19. Survival Analysis

The impact of *ADAMTS6* on LUAD patient survival was assessed using GEPIA2 (<http://gepia2.cancer-pku.cn/>, accessed on 25 June 2025) for RNA-Seq TCGA data and KMplotter (<https://kmplot.com/analysis/>, accessed on 25 June 2025) for 17 TCGA and GEO microarray datasets (JetSet best probe set). Patient were divided based on median *ADAMTS6* expression, and overall, progression-free, and disease-free survival were analyzed using Kaplan–Meier curves and the log-rank test, using p -value < 0.05 as a significance threshold.

4.20. Statistical Analysis and Visualization

Data analysis and visualization was conducted using R v4.4.2 and RStudio v.2024.12.1. Pairwise comparisons were conducted using a two-tailed Student’s t-test from the stats package (v4.4.2). Spearman correlation analysis was performed, with results visualized using Hmisc (v5.2.3) and corrrplot (v0.95) packages. Additional visualization tools included ggplot2 (v4.0.0), pheatmap (v1.0.13), circlize (v0.4.16), and ggvenn (v0.1.10) packages.

5. Conclusions

This study demonstrates that the extracellular protease *ADAMTS6* plays a regulatory role in the acquisition of the mesenchymal phenotype during EMT in LUAD cells. Suppression of *ADAMTS6* reduces migration, invasion, mesenchymal marker expression, and colony formation, while increasing epithelial E-cadherin expression and improving doxorubicin sensitivity in A549 cells. Along with its established association with poorer patient outcomes, these findings show the importance of *ADAMTS6* in LUAD progression and suggests its potential as a prognostic marker and therapeutic target.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Figure S1: EMT gene networks; Figure S2: Expression of EMT cell markers in epithelial-like and mesenchymal-like clusters of A549 cells; Figure S3: Relative expression of the cadherins *KRT18* and *KRT19*; Figure S4: Molecular pathways associated with high *ADAMTS6* in tumors; Figure S5: Expression of *ADAMTS6* in TCGA-LUAD tumors; Table S1: Design of differential expression analysis in verification datasets of EMT in LUAD; Table S2: Protospacer and PCR primer sequences; Table S3: RT-qPCR primer sequences; Supplementary results: Sanger sequencing of *ADAMTS6* alleles. References [38,39,76,98–108] are cited in the supplementary materials.

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