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Original Article

CASZ1 promotes migration, invasion, and metastasis of lung cancer cells by controlling expression of ITGAV

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Abstract: CASZ1, a zinc finger transcription factor with two isoforms, is known to play important roles in cardiac and neural development. The abnormal expression of CASZ1 is also frequently found in a variety of tumors but has different effects on different tumors; for example, it acts as a tumor suppressor in neuroblastoma but promotes cancer metastasis in ovarian cancer. However, the effect of CASZ1 in lung cancer, the most lethal cancer, remains unclear. Here, we found that the expression of CASZ1 in lung cancer is positively associated with cancer metastasis and poor prognosis. The overexpression of CASZ1b promotes lung cancer cell migration, invasion, and epithelial-mesenchymal transition and is associated with poor prognosis in lung cancer patients. The knockdown of CASZ1 resulted in the suppression of epithelial-mesenchymal transition, migration, and invasion of lung cancer cells and reduced metastasis in vivo. The results of an RNA-sequencing analysis of CASZ1-silenced cells showed that CASZ1 considerably affected the integrin-mediated pathways. CASZ1 bound to the *ITGAV* promoter and transcriptionally regulated ITGAV expression. Our findings demonstrate that CASZ1 plays an oncogenic role in lung cancer and that CASZ1 promotes lung cancer migration, invasion and metastasis is mediated by ITGAV.

Keywords: Lung cancer, epithelial-mesenchymal transition, metastasis, integrin alpha V (ITGAV)

Introduction

Lung cancer is the leading cause of cancer-related death globally. With more than 2.2 million new cases and 1.8 million deaths reported in 2020, it is the most diagnosed cancer, with the highest rates in European and Asian countries, followed by Turkey [1, 2]. Non-small-cell lung cancer (NSCLC) accounts for approximately 85% of lung cancers, and the most common NSCLC types are lung squamous cell carcinoma, large cell carcinoma, and lung adenocarcinoma (LUAD). It is more frequently diagnosed in men than women and is more fatal in men. The higher mortality rate observed in lung cancer patients is due to metastasis, whereby the cancer readily metastasizes to the livers, lungs, bones and brains of the patients, resulting in poor survival [3]. Epithelial-mesenchymal transition (EMT) is a biological process that occurs in several tissues and developmental stages and is one of the important cancer cell metastasis mechanisms. During different cancer metastasis processes, including the invasion of cancer cells at the primary tumor site and the intravasation and extravasation of tumor cells, cancer cells frequently exhibit an EMT state or the converse condition [4]. Cancer starts, develops, and progresses due to dysregulated gene expression, which results in aberrant cell growth and development. The key process responsible for initiating tumor progression and metastasis is transcriptional regulation [5]. Therefore, identifying the novel driver genes associated with lung cancer progression is important for understanding the underlying molecular mechanisms to overcome this disease and develop new cancer therapies.

CASZ1, known as castor zinc finger 1, is a transcription factor that was originally characterized as a neural fate determinant in *Drosophila*

[6]. Human CASZ1 is composed of two major isoforms, CASZ1a and CASZ1b. CASZ1a comprises 1759 amino acids with 11 zinc finger domains; CASZ1b comprises 1166 amino acids with five zinc finger domains and lacks six zinc fingers at the C-terminus compared with CASZ1a [7, 8]. CASZ1 is highly expressed in the lung, pancreas, heart, stomach, small intestine and skeletal muscle. CASZ1 is known to be critical for vasculature development and the differentiation of many cell types, including neuroblasts, cardiomyocytes and lymphocytes [9-11]. Although CASZ1 acts as a tumor suppressor in some tumors such as neuroblastoma and liver cancer [12, 13], it plays a role in promoting cancer metastasis in ovarian cancer [14]. However, the role of CASZ1 in lung cancer, especially in cancer metastasis, remains unclear. Considering its function as a transcriptional factor, how it affects cancer metastasis by regulating the expression of genes that are important for cell adhesion, migration and invasion needs further investigation.

Integrins are members of the heterodimeric transmembrane protein family that establish tight bonds between the interior and exterior environments of cells at extracellular junctions to regulate cell adhesion, proliferation, migration, and differentiation. They are heterodimerically assembled from 18 α and 8 β subunits to form 24 distinct transmembrane receptors, each of which is connected to one or more extracellular matrix (ECM) ligands [15]. They activate intracellular signaling pathways, particularly those between integrins and growth factor receptors, and these cross-talks govern the expansion and invasion of tumor cells [16]. Various oncogenes or growth receptors have been shown to require particular integrins to promote cancer initiation and progression. *ITGAV*, which encodes integrin alpha V, is overexpressed in the tumors of patients who have evolved metastatic disease [17, 18]. Most alpha subunits normally only dimerize with one specific beta subunit, but integrin alpha V can bind to five different beta subunits, and the resulting heterodimers recognize a variety of ECM ligands, including vitronectin and fibronectin [19]. In addition, integrin alpha V is an important target for the development of therapeutic strategies to combat solid and hematological malignancies [20, 21].

Using our RNA sequencing (RNA-seq) data, we discovered that CASZ1 contributes to the migration, invasion, and metastasis of lung cancer cells by regulating the integrin pathway and EMT status. This study sheds light on the biological significance of CASZ1 in lung cancer through its transcriptional regulation of ITGAV expression.

Materials and methods

Cell lines and culture conditions

The human LUAD cell lines with progressive levels of invasiveness (CL1-0, CL1-1, CL1-2, and CL1-3) were established and characterized as previously described [22]. All the cell lines were maintained in RPMI1640 media supplemented with 10% fetal bovine serum (FBS) along with 1% penicillin and streptomycin and incubated at 37°C in a humidified atmosphere with 5% CO_2.

Antibodies and reagents

The primary antibodies used in the study including anti-CASZ1 (Santa Cruz Biotechnology), anti-ITGAV (Abcam), anti-vimentin (Cell Signaling), anti-E-cadherin and anti-N-cadherin (BD Biosciences), and anti-β-actin (GeneTex). We purchased ECL™ Western Blotting Detection Reagents from GE Healthcare Life Sciences (Piscataway, NJ), Protease Inhibitor Cocktail EDTA-free tablets from Roche Diagnostics (Basel, Switzerland), and Invitrogen™ Lipofectamine 2000 from Thermo Fisher Scientific.

shRNA transfection and lentiviral infection

The cells were transfected with CASZ1b and ITGAV overexpressing plasmids by using Lipofectamine 2000 (molar ratio of 1:3). *CASZ1* and *ITGAV* were constructed in the pCMV-Tag2B and pCMV3-HA vectors, respectively. We purchased shRNAs (sh21 and sh755) and lentiviral vectors for CASZ1 knockdown from National RNAi Core Facility (Academia Sinica, Taiwan). HEK293T cells were cotransfected with pLKO.1 shRNA, pCMV-R8.91, and pMD.G according to the method described in the previous paper [23]. After 24 h of transfection, we collected the virus-containing medium to generate the stable silenced cell clones. We infected the CL1-3 cells with the shRNA-expressing lentivirus and added polybrene to increase the effi-

ciency. After 24 h of infection, puromycin was added to the cells to select for resistant cells.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted using an RNeasy Micro Kit (Qiagen, Valencia, CA). We reverse-transcribed 1 μg of RNA into cDNA with M-MLV Reverse Transcriptase and Oligo(dT)15 primer (Promega, San Luis Obispo, CA). The qRT-PCR was performed with Fast SYBR Green Master Mix on a StepOne Real-Time PCR System (Applied Biosystems Foster City, CA) with the specific primers. The sequences of the primers are shown below. We normalized the mRNA expression level with that of GAPDH and calculated the relative expression levels with the -ΔCT method.

The primer sequences were as follows: CASZ1a Forward, 5'-GGATGCTGAGACAGATGAGTGC-3'; CASZ1a Reverse, 5'-CTGTCGGCATAGAGATGGTGTT-3'; CASZ1b Forward, 5'-TCCCTCCGAGCCTCCGTAT-3'; CASZ1b Reverse, 5'-GGGTCCCTTCCACCCAAGA-3'; ITGAV Forward, 5'-AACACACCAGTGGCAAACAA-3'; ITGAV Reverse, 5'-TAGCAGGAGTCCCGAGAGAA-3'.

Western blotting analysis

The cell lysates were prepared with RIPA lysis buffer supplemented with a protease inhibitor cocktail. The quantification of the protein concentrations was analyzed using a BCA Protein Assay Kit (Thermo Scientific). We ran the protein samples on 10% SDS-PAGE and transferred them to polyvinylidene membranes (Millipore, Billerica, MA). Then, we incubated the membranes with primary antibodies overnight. The next day, we washed the membranes with TBST and incubated the horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour. The protein bands were visualized by using ECL™ Western Blotting Detection Reagents.

Wound healing assay

8×10^4 cells were seeded in culture inserts (Ibidi, Munich, Germany). When the cells developed a single monolayer, the inserts were removed to create a wound. We took photographs of the wound area at 0 h and then at the different indicated time intervals. We analyzed the wound areas using ImageJ software (NIH, Bethesda, MD).

In vitro transwell invasion and migration assay

Transwell chambers with 8-μm pore membrane inserts (Corning Costar, Cambridge, MA) were pre-coated with Matrigel (BD, San Jose, CA) for an invasion assay and without Matrigel for a migration assay. In the upper chamber, we seeded the cells with a culture medium containing 1% FBS and filled the bottom chamber with a culture medium containing 20% FBS. The cells were incubated for the indicated times, after the cells on the upper surface of the membrane were removed with a cotton swab, and the penetrating cells were fixed in methanol and stained with Giemsa's solution for 30 min. The migrated or invaded cells were counted in five fields of view under a microscope at 100× magnification.

Cell adhesion assay

We seeded the 96-well plate coated with fibronectin (R&D systems) with 2.5×10^4 cells and incubated them at 37°C for 1 h, then, we washed them with PBS/0.1% BSA to remove the unbound cells. We fixed the cells for 10 min with 4% paraformaldehyde and washed them with PBS, and then stained them with crystal violet (5 mg/ml in 2% ethanol) at room temperature for 10 min.

Chromatin immunoprecipitation (ChIP) assay

We carried out ChIP in CL1-3 cells according to the Abcam Cross-linking Chromatin Immunoprecipitation (X-ChIP) protocol. We performed ChIP with normal mouse IgG (negative control) and anti-CASZ1 (sc-398303, Santa Cruz Biotechnology) mAbs. Immunoprecipitation reactions were incubated overnight at 4°C with rotation. DNA was eluted with a ChIP Elution Buffer/RNase A mixture and purified with spin columns. The purified DNA was used to perform qPCR and analyzed the interesting sequence that was amplified by specific primers.

Animal studies

The Institutional Animal Care and Use Committee (IACUC), NCKU approved all the animal studies. We injected SCID mice (6 weeks old) with 5×10^5 shCASZ1 (21)/shLacZ-transduced

CL1-3 cells suspended in 200 μL of Hank's balanced salt solution (HBSS) via tail vein injection. After 7 weeks, we euthanized the mice, resected the lung tumor tissues, and fixed them in a 10% formalin solution. We counted the tumor colonies and embedded them in paraffin for 4-μm thick sections that were stained with hematoxylin and eosin (H&E) for histological studies.

Statistical analysis

All the experimental data were performed in triplicates and expressed as mean ± SD or mean ± SEM. The unpaired two-sided Student's *t*-test was used to compare the difference between two groups for continuous variables and the Wilcoxon signed-rank test was used to draw comparisons between the tumor samples. The Spearman correlation test was used to analyze the correlation between the two gene expressions because of the small sample size. The statistical analyses were performed using GraphPad Prism 9 software. Kaplan-Meier estimations and the two-sided log-rank test were used to compare the survival rates of the patient groups. All statistical data analysis was considered significant with *p* values, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$.

Results

CASZ1b upregulation promotes cancer cell EMT, migration, and invasion and is associated with poor prognosis in lung adenocarcinoma

We first studied the expression level and regulation of CASZ1 in lung cancer cell lines in vitro. We monitored the mRNA expression levels of the CASZ1 isoforms a and b in lung adenocarcinoma cell lines CL1-0-CL1-3 with the same genetic background. As shown in **Figure 1A**, the CASZ1b mRNA levels increased with the metastatic ability of lung cancer cells, and the most metastatic lung cancer cell line (CL1-3) had the highest expression level. As shown in **Figure 1B**, the CASZ1b protein expression levels also increased in the highly metastatic cell lines. Next, we checked the expression of EMT markers after CASZ1b overexpression in CL1-0 cells, where we found that the levels of epithelial marker E-cadherin decreased whereas the levels of mesenchymal markers N-cadherin and vimentin increased, suggesting that they underwent an EMT shift (**Figure 1C**). Furthermore, to confirm the effect of CASZ1b overexpression on cell invasion and migration, we performed transwell invasion and wound healing assays. CASZ1b overexpression significantly enhanced invasion and migration of lung cancer cells (**Figure 1D**, **1E**), and this effect was not due to the growth promotion of CASZ1b, because CASZ1b overexpression did not significantly affect the growth rate of lung cancer cells within 72 hours (**Figure 1F**). In particular, Kaplan-Meier survival curves showed that higher CASZ1 expressions were associated with poor overall survival in lung adenocarcinoma patients (**Figure 1G**), demonstrating that CASZ1b overexpression may cause lung cancer metastasis and reduces the overall survival.

CASZ1 knockdown suppresses lung cancer cell invasion and migration

We next examined the effect of CASZ1 silencing on lung cancer cells, CL1-3 cells were infected with lentiviral-based shRNAs targeting CASZ1 exons 6 and 7 or a control LacZ gene. CASZ1b mRNA and protein levels were efficiently knocked down in the CL1-3/sh21 and CL1-3/sh755 cells (**Figure 2A**, **2B**). We found that the epithelial marker E-cadherin levels increased in the CASZ1-silenced cells, whereas the levels of the mesenchymal marker N-cadherin decreased, demonstrating that the reduced CASZ1 level suppressed the EMT shift (**Figure 2C**). CASZ1 silencing slightly inhibited cell growth rate, a significant difference until culture day 5. (**Figure 2D**, **2E**). The results of the wound healing and transwell invasion assays showed that CASZ1 knockdown inhibited cancer cell migration and invasion (**Figure 2F**, **2G**). Furthermore, CASZ1b silencing converted the elongated mesenchymal shape of the CL1-3 cells into a cubic epithelial structure (**Figure 2H**). The results of a time-lapse cell tracking analysis also showed that the mean path length and line length were considerably reduced in the shCASZ1 cells (**Figure 2I-K**). These data confirmed that CASZ1b inhibition in CL1-3 cells reverses the epithelial-to-mesenchymal transition of metastatic cells and reduces cell motility and invasion.

CASZ1 knockdown suppresses lung cancer metastasis in vivo

In accordance with the finding of our in vitro studies, we postulated that CASZ1 expression

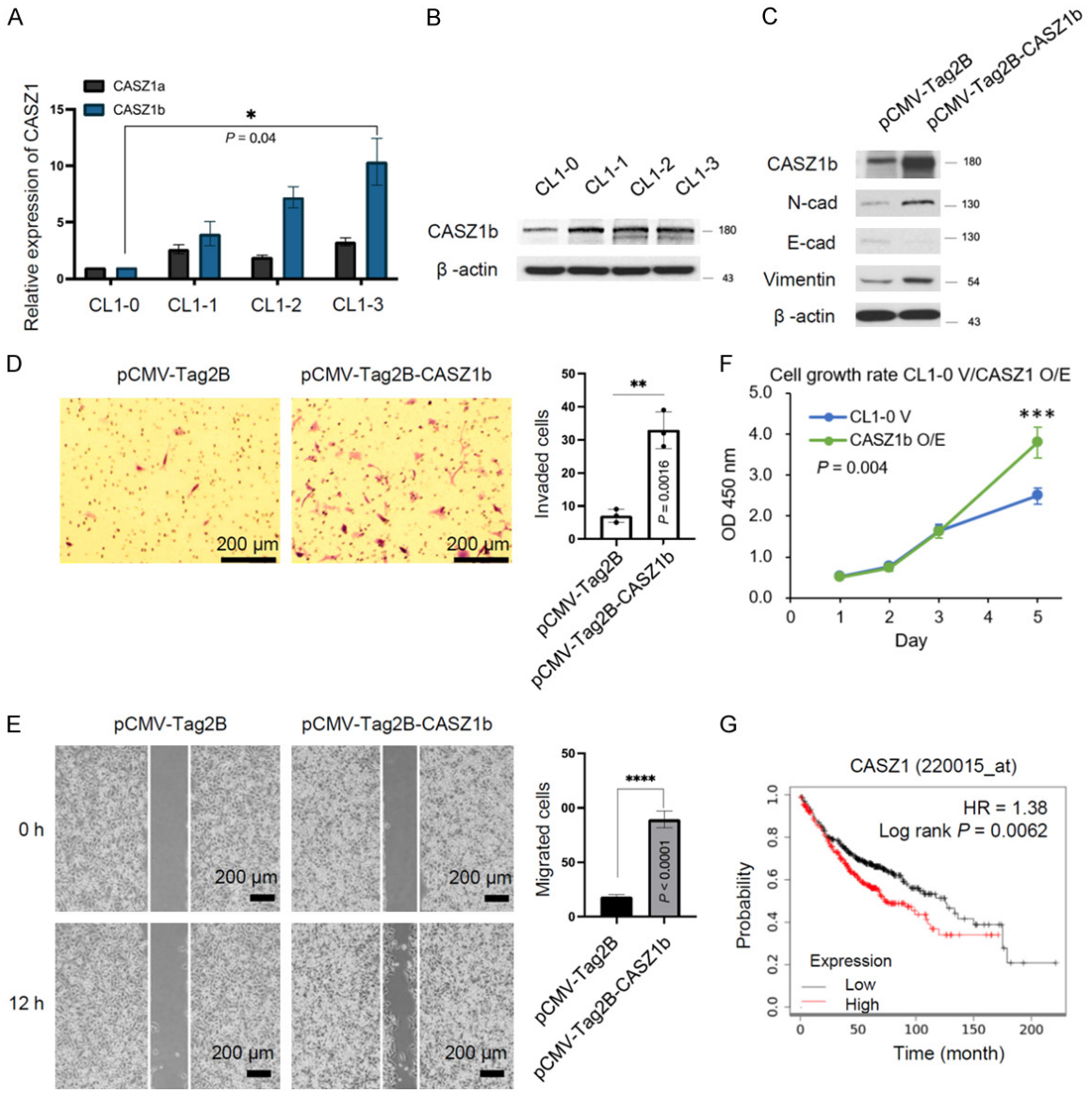

Figure 1. CASZ1 upregulation promotes cancer cell EMT, migration, and invasion and is associated with poor prognosis in lung adenocarcinoma. A. The mRNA expression levels of CASZ1a and CASZ1b isoforms were determined using qRT-PCR in various lung cancer cell lines. B. The protein expression levels of CASZ1b were examined with immunoblot assays, using β-actin as the loading control. C. The expression of EMT markers in CL1-0 cells with or without CASZ1 overexpression was determined using a Western blotting. D, E. The invasive and migrative abilities of CASZ1b-overexpressed cells were determined using transwell invasion and wound healing assays, respectively. F. Cell viability was measured using a WST-1 assay. The data are represented as mean ± SD (n = 4). G. Lung adenocarcinoma patients with high CASZ1 expression have poor Kaplan-Meier overall survival. The patients were divided into high and low groups based on the median expression value of the gene in the cohort. The results are based on the Mantel-Cox log rank test. The data are presented as the means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001, two-tailed Student's t test.

is associated with cancer metastasis in CL1-3 cells. To further determine the effect of CASZ1 expression on metastatic colonization in vivo, CASZ1-knockdown CL1-3 cells (sh21) or control shLacZ cells were injected into severe combined immunodeficiency (SCID) mice via a tail vein injection. We monitored the body weight of each mouse weekly after the injection (**Figure 3A**). After 7 weeks, we euthanized the mice, removed their lungs, and counted the tumor nodules (**Figure 3B**). The results showed that the number of lung nodules colonized by the CASZ1-knockdown cancer cells was substantially reduced by 60% compared with the con-

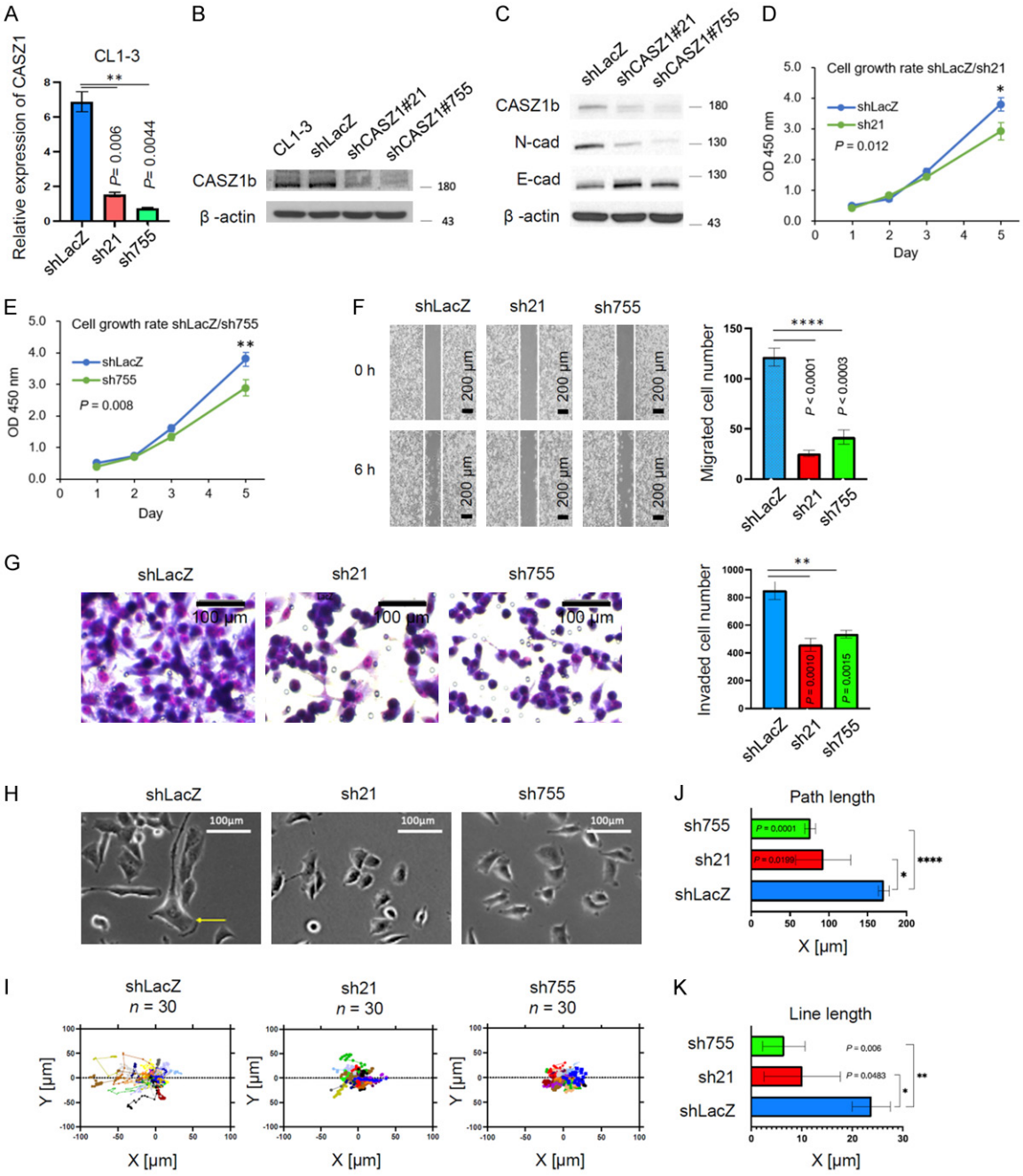

Figure 2. CASZ1 knockdown leads to suppression of lung cancer cell migration/invasion. A, B. CL1-3 cells were infected with lentiviruses carrying CASZ1 shRNAs (sh21 and sh755) or LacZ shRNA (shLacZ). The levels of mRNA and protein were determined using qPCR and immunoblotting. β-actin served as an internal control. C. The expression of EMT markers in shLacZ- and shCASZ1-infected CL1-3 cells was determined using Western blotting. D, E. Cell viability was measured using WST-1 assay. Data are represented as mean ± SD (n = 4). F, G. The migrative and invasive abilities of shLacZ- and shCASZ1-infected CL1-3 cells were determined using wound healing and transwell invasion assays, respectively. Scale bars = 200 μm, 100 μm; data are represented as mean ± SD (n = 3), $^{**}P < 0.01$; $^{****}P < 0.0001$, two-tailed Student's t test. H. Observation of cell morphology in shLacZ- and shCASZ1-infected CL1-3 cells, scale bars = 100 μm. I-K. Time lapse cell tracking analysis through XY position plots determining the cell motility in the shCASZ1/shLacZ cells. The average path length and line length were analyzed in 30 cells in each group. Data are represented as mean ± SD (n = 3), two-tailed Student's t test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$.

trol cells (**Figure 3C**). Next, through H&E staining of the lung tissue, we determined the size of the tumor and found that the volume of the CASZ1-knockdown tumor tissue was signifi-

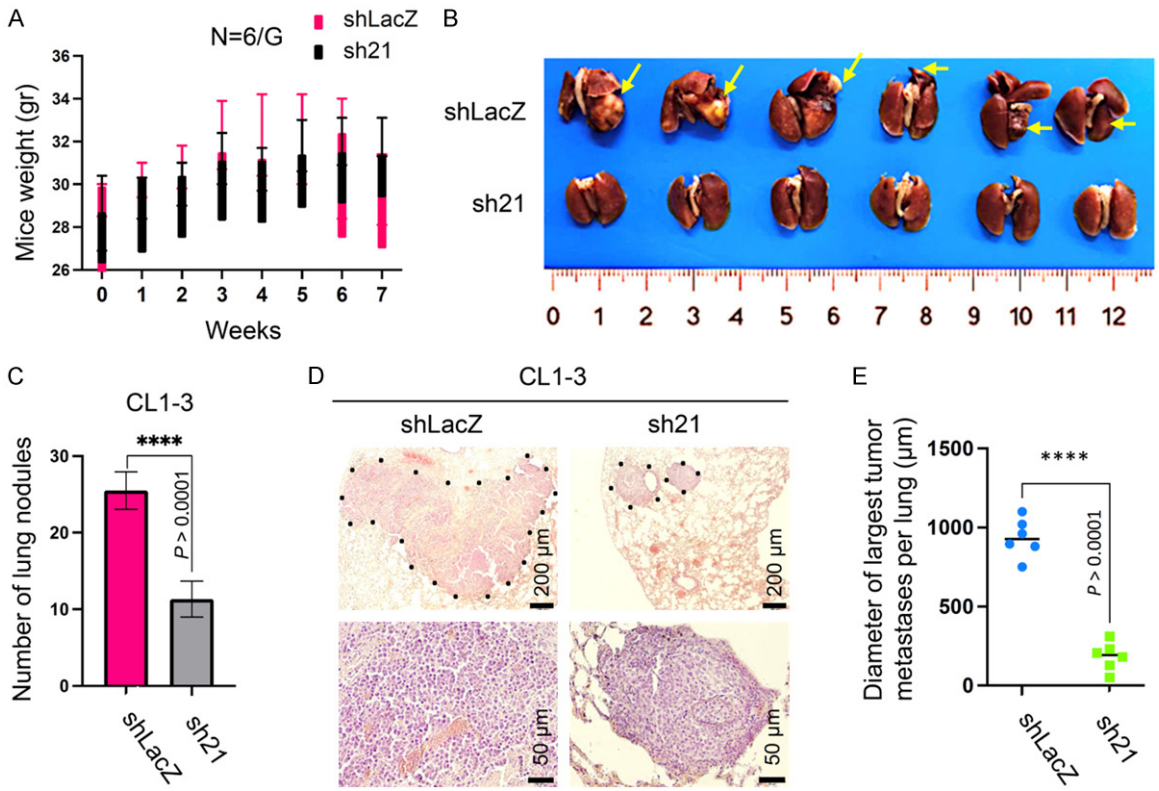

Figure 3. CASZ1 knockdown decreases lung cancer metastasis in vivo. A. Mice body weights were monitored for 7 weeks after tail vein injection of shCASZ1-21/LacZ CL1-3 cells. B. Photographs of lung nodules after mice were sacrificed. C. The number of lung nodules in mice injected with shCASZ1-21 and shLacZ CL1-3 cells. Data are represented as mean ± SEM, two-tailed Student's *t* test, ****$P < 0.0001$. D. H&E staining of lung tissues. Tumor tissues are circled by black dots, and magnified images are shown in the lower panels. E. The diameter of the largest tumor in the lung of each mouse. Data are represented as mean ± SD ($n = 6$), two-tailed Student's *t*-test, ****$P < 0.0001$.

cantly smaller compared with that of the control group (**Figure 3D**). Finally, we assessed the diameters of the biggest tumors in each lung in the shLacZ and sh21 groups and found that these dimensions in the CASZ1-knockdown group were drastically smaller (**Figure 3E**). These data confirmed that CASZ1 knockdown leads to the suppression of lung cancer metastasis in vivo.

GO molecular pathway analysis reveals that integrin-associated pathways mediate CASZ1 cellular function

To further discover the novel factors associated with CASZ1 promoting cancer metastasis, we performed RNA-sequencing (RNA-seq) to compare the differentially expressed genes (DEGs) in CL1-3 with shCASZ1 #21 versus the control cells. By using a Gene Ontology (GO) analysis, we found that most of the down-regulated genes in the sh21 cells were mainly enriched in integrin-related pathways, such as in adherent junctions, focal adhesion, and cell-substrate adherent junctions, etc. (**Figure 4A**). The results of cellular component analysis demonstrated that the DEGs in the sh21 cells were highly associated with the growth factor, integrin, cell adhesion molecule, and extracellular matrix bindings, etc. (**Figure 4B**). Furthermore, exploring the protein-protein interaction network, we found that highly associated networks were all integrin-mediated, including the integrin-mediated signaling pathway and the ECM binding, focal adhesion, and ECM-receptor interaction networks (**Figure 4C**). The ECM usually comprises physical and biochemical properties that promote migration, invasion and signaling in the tumor microenvironment. Integrins are cell adhesion molecules that play multiple roles in signal transduction, mechanical stimulations, and cell migration leading to metastasis [15]. Our RNA-seq data showed that ITGAV expression was substantially reduced in the CASZ1b-

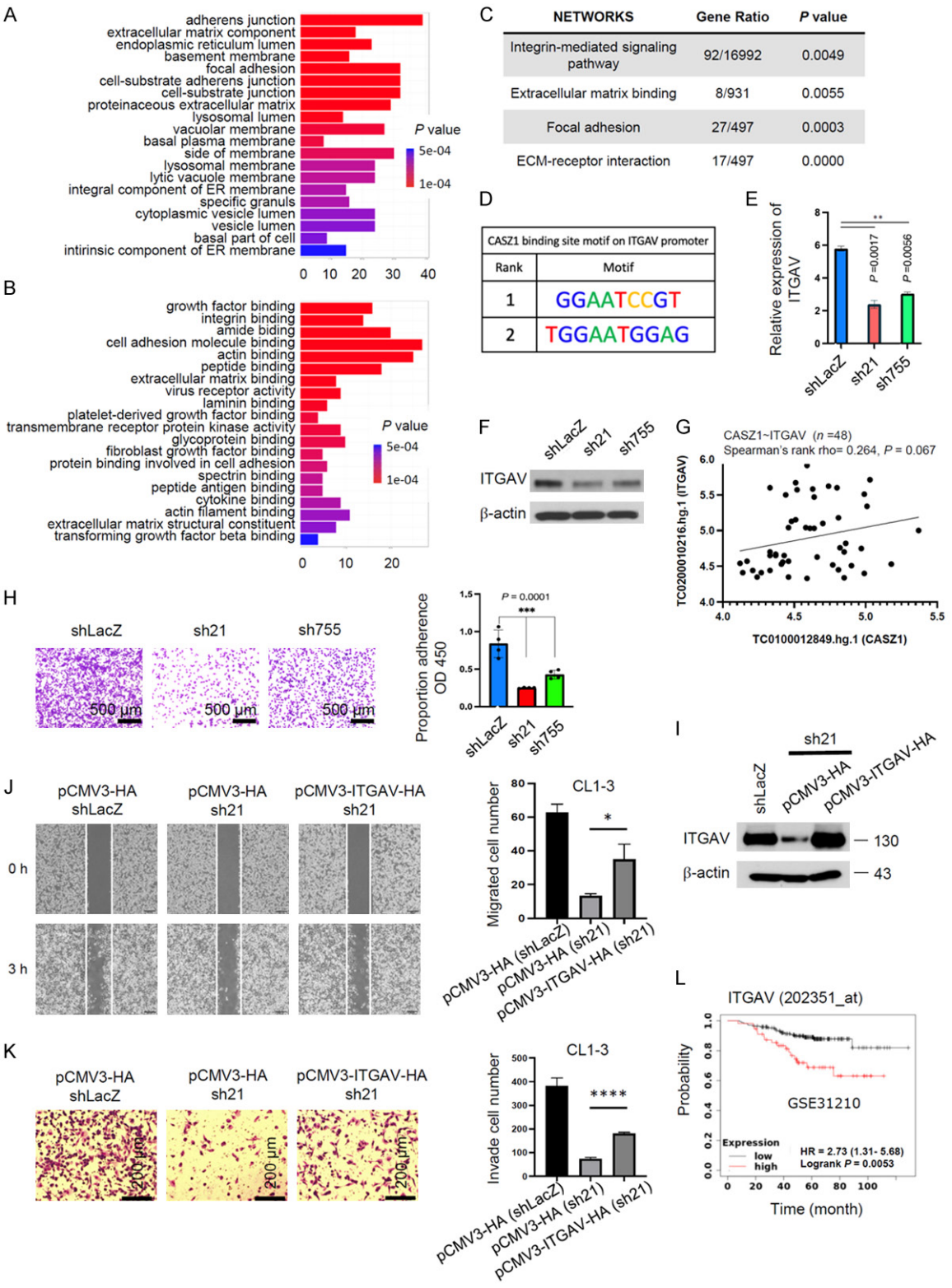

Figure 4. GO molecular pathway analysis reveals that integrin-associated pathways mediate CASZ1 cellular function. Differentially expressed genes (DEGs) in shCASZ1 versus shLacZ cells were analyzed using RNA sequencing. Gene Ontology (GO) analysis of DEGs determined the molecular function (A) ($P < 0.0002$, $n = 20$ of 41 entries) and the cellular component (B) ($P < 0.0002$, $n = 20$ of 133 entries). (C) MetaCore based analysis of DEGs determined the downregulated networks in shCASZ1 cells. (D) Putative CASZ1 binding motifs in *ITGAV* promoter. Homer de novo motif analysis of CASZ1 binding sequences in the *ITGAV* promoter region was performed. (E, F) The mRNA and pro-

tein expression levels of ITGAV in shCASZ1/shLacZ cells were analyzed using qRT-PCR and immunoblotting. β-actin acted as a loading control. (G) The expression correlation between *CASZ1* and *ITGAV* data obtained from a GEO dataset (GSE168466) was analysed using Spearman's correlation analysis. Spearman's rank ratio = 0.264. (H) Cell adhesion assay of shCASZ1/shLacZ CL1-3 cells was performed on a fibronectin-coated 96-well plate. Scale bar = 500 μm; data are represented as mean ± SD (n = 3), ***P < 0.001. (I) ITGAV overexpression in shCASZ1-21 CL1-3 cells. (J, K) ITGAV overexpression increased the migration and invasion capabilities of shCASZ1 CL1-3 cells. Scale bars = 200 μm; data are represented as mean ± SD (n = 3), two-tailed Student's t-test, *P < 0.05; ****P < 0.0001. (L) Kaplan-Meier survival analysis of lung cancer patients showed that high *ITGAV* expression resulted in poor overall survival rates. The patients were divided into high and low groups based on the median expression value of the gene in the cohort. The results are based on the results of a Mantel-Cox log rank test.

silenced cells. We also found two motifs in the *ITGAV* promoter that best matched the reported sequence of the CASZ1 binding site [24] (**Figure 4D**). ITGAV, a transmembrane glycoprotein involved in cell-matrix interactions, promotes tumor growth in multiple tumor types [25]. Furthermore, the role of ITGAV in the progression, metastasis, and overall survival of cancer patients with various cancer types is well understood [20, 26, 27]. We suggest that *ITGAV* is the downstream gene of CASZ1; therefore, we further verified the effect of CASZ1 on *ITGAV* expression with a qRT-PCR and Western blotting and found that the mRNA and protein levels of ITGAV were reduced in the shCASZ1 lung cancer cells (**Figure 4E**, **4F**). Spearman correlation analysis of *CASZ1* and *ITGAV* expression in the GEO lung cancer dataset (GSE168466) also identified a positive correlation (Spearman's rank ratio = 0.264; **Figure 4G**). ITGAV is the major cellular receptor for the ECM component fibronectin and plays an important role in cell adhesion, so we performed a cell adhesion assay to evaluate the effect of CASZ1 on cell adhesion to fibronectin. As shown in **Figure 4H**, CASZ1 knockdown significantly reduced cell adhesion to fibronectin compared with that in the control cells. Moreover, ITGAV overexpression effectively restored the shCASZ1-mediated inhibition of lung cancer cell migration and invasion (**Figure 4I-K**). In particular, the Kaplan-Meier survival curves showed that higher *ITGAV* expressions were associated with poorer survival rates in lung adenocarcinoma patients, whose data were taken from lung cancer datasets (**Figure 4L**). Taken together, these data suggested that ITGAV is a key mediator in the regulation of lung cancer metastasis by CASZ1.

CASZ1 transcriptionally upregulates expression of ITGAV

To further determine whether CASZ1b directly regulates ITGAV expression, we first overexpressed CASZ1b in CL1-0 cells using the pEGFP-C1-CASZ1b plasmid and found that CASZ1b localized in the nucleus (**Figure 5A**). Through Homer de novo motif scanning, we identified two putative CASZ1-binding sites on the *ITGAV* promoter (**Figures 4D**, **5B**). To understand whether CASZ1 is directly associated with ITGAV expression regulation, we cloned the promoter region (from -2030 to -1 upstream of the transcriptional start site of *ITGAV*) into the pGL4.17-luciferase reporter vector and generated three mutant constructs (**Figure 5B**, **5C**). We observed that the luciferase activity in the CASZ1-knockdown cells was significantly lower than that in the control cells (**Figure 5D**). Mutations in the binding site #2 (MUT#2) of CASZ1 resulted in a significantly reduced luciferase activity level, and mutations in both binding sites (MUT #1&2) had the strongest inhibitory effect (**Figure 5E**). Moreover, we used chromatin immunoprecipitation to demonstrate that CASZ1 bound to the *ITGAV* promoter (**Figure 5F**, **5G**) in CL1-3 cells, whereas the bindings were significantly reduced in the CASZ1-knockdown CL1-3 cells (**Figure 5H**). These results confirmed that CASZ1 transcriptionally regulates ITGAV expression in lung cancer cells.

Discussion

In this study, we identified that CASZ1 (especially CASZ1b) is a critical transcription factor involved in lung cancer metastasis by regulating two important cellular capabilities, including induction of EMT and promotion in ITGAV expression to support cancer cell migration and invasion in vitro and in vivo. CASZ1 contributes to the oncogenic program and causes a poor overall survival in lung adenocarcinoma patients. We demonstrated that CASZ1 directly regulates ITGAV expression, which increases the attachment of mesenchymal lung cancer cell to the vast fibronectin network in the extracellular matrix and leads to increased metastasis of lung cancer cells (**Figure 6**).

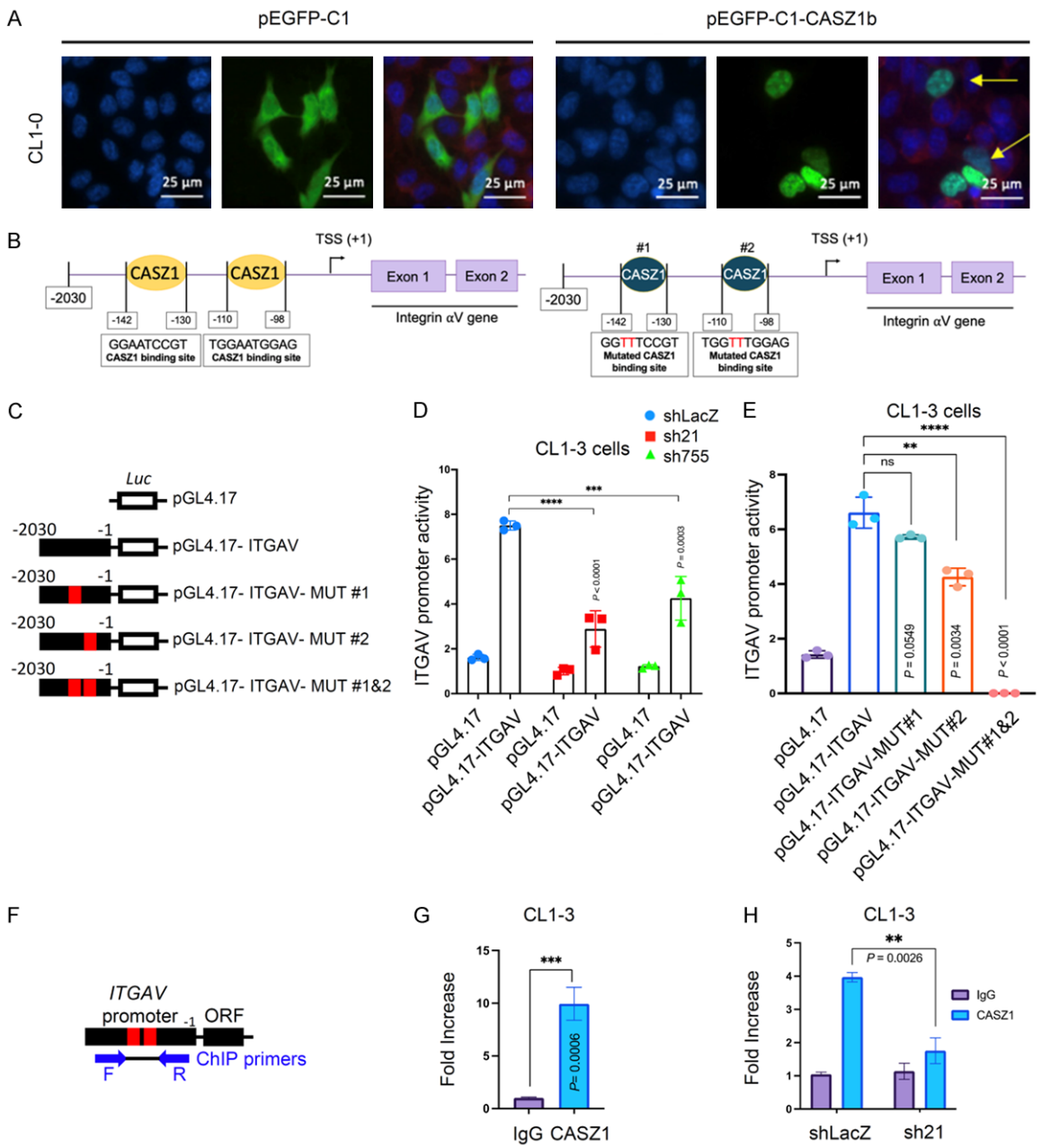

Figure 5. CASZ1 transcriptionally upregulates *ITGAV* expression. A. Nuclear localization of CASZ1 was determined using immunofluorescence. EGFP-tagged CASZ1 was overexpressed in CL1-0 cells. Green represents the EGFP-expressing regions, and blue represents DAPI-stained nuclei. Yellow arrows indicate the nuclear localization of CASZ1. B. Putative CASZ1 binding motifs in *ITGAV* promoter. Left, the sequences of two putative CASZ1 binding sites in *ITGAV* promoter. Right, the sequences of mutated putative CASZ1 binding sites in the *ITGAV* promoter. C. Diagrams of reporter constructs. D, E. Relative luciferase reporter activities driven by CASZ1 in *ITGAV* promoter regions. ShLacZ or shCASZ1 CL1-3 cells were cotransfected with phRL-CMV and 1 of the 5 plasmid vectors (pGL4.17, pGL4.17-ITGAV, pGL4.17-ITGAV-MUT#1, pGL4.17-ITGAV-MUT#2, and pGL4.17-ITGAV-MUT#1&2), cultured for 24 h and subjected to the assay for firefly and *Renilla* luciferase activities. Firefly luciferase activities were normalized to *Renilla* luciferase activities. Data are presented as the mean ± SD (n = 3), two-tailed Student's t-test, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. F. Diagram showing the region of *ITGAV* promoter amplified by ChIP primers used in this study. G, H. Results of ChIP assay determined endogenous CASZ1 bound to the *ITGAV* promoter. Chromatin was isolated from parental, shLacZ, or shCASZ1 CL1-3 cells and immunoprecipitated with IgG or anti-CASZ1 antibodies. The amount of immunopurified genomic fragments covering the CASZ1 binding sites was quantified with qPCR. Data are presented as the mean ± SD (n = 3), two-tailed Student's t-test, $^{**}P < 0.01$, $^{***}P < 0.001$.

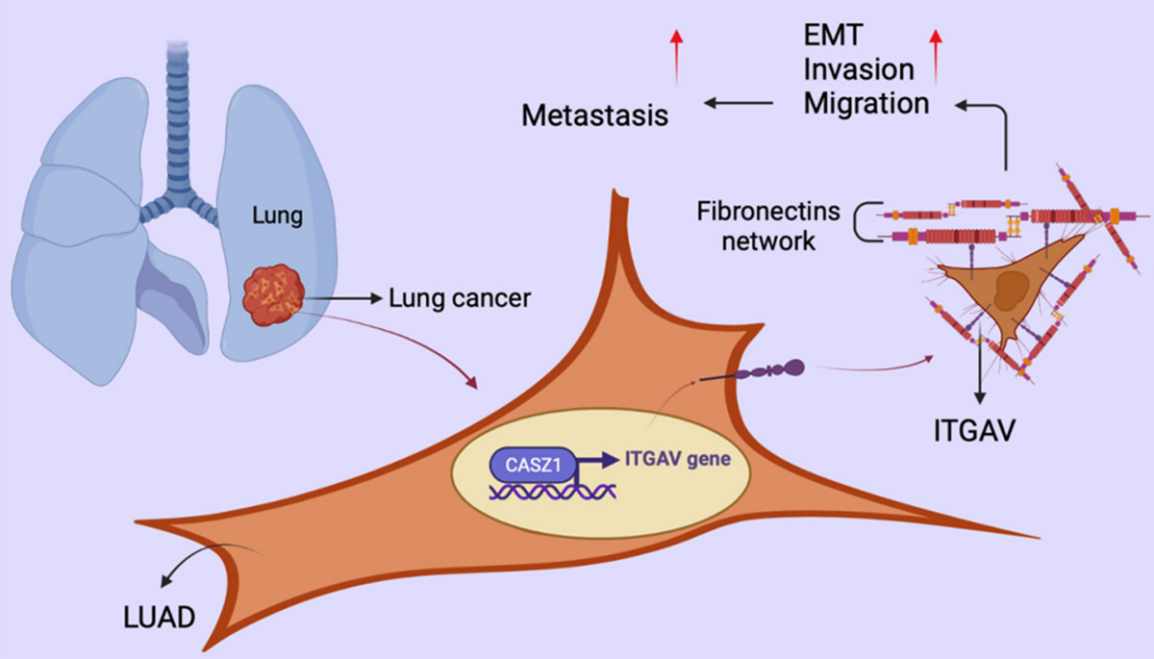

Figure 6. Diagram summarizes how CASZ1 enhances lung cancer metastasis.

An in silico data mining analysis using EST clustering data from the ECgene database (http://genome.ewha.ac.kr/ECgene/) revealed that *CASZ1* mRNA was up-regulated in cancer tissues derived from the lung, muscle, ovary, pancreas, large intestine, stomach, and skin, but downregulated in prostate and breast cancer tissues [14]. Some investigations have been conducted on the metastasis and mechanism of CASZ1 in various cancers. To acts as a tumor suppressor, CASZ1, a transcription factor, was reduced in aggressive stage tumors and in those with a poor prognosis in neuroblastoma and HCC [8, 13]. Additionally, CASZ1a or CASZ1b overexpression inhibited cell migration in the SH-SY5Y neuroblastoma cell line [14]. In contrast, CASZ1 expression increased in metastatic tumors of ovary epithelial carcinoma and was a metastasis promoter [14]. Furthermore, the prognosis of LUAD patients correlated with CASZ1 expression [28]. These findings suggested that CASZ1 has diverse tumor-specific functions in various tumor types. Herein, we proved that CASZ1 promotes cancer metastasis and causes poor survival in patients with lung adenocarcinoma.

EMT is a dynamic and reversible process that converts polarized, cobblestone-like epithelial cells into migratory, spindle-shaped mesenchymal cells. EMT cells exhibit molecular abnormalities such as the loss of epithelial markers such as E-cadherin, ZO-1, and occludin and the increase in the levels of mesenchymal markers such as N-cadherin, vimentin, and fibronectin, in addition to exhibiting morphological changes [29]. The most important action of EMT is its involvement in cytoskeleton remodeling, which modulates cell mobility. Several EMT transcription factors such as Snail, Zeb1, FoxC2, and Twist, which are used for mesenchymal morphologic alterations, mediate E-cadherin inhibition and enhance N-cadherin expression [30-33]. In the present study, we found that CASZ1 positively regulated EMT in lung cancer cells and that both cell morphological and molecular characteristics were affected by CASZ1. The regulation of EMT proteins by CASZ1 in ovarian cancer cells favors the mesenchymal type [14]. However, further detailed information about the mechanism used by CASZ1 in regulating EMT has not yet been revealed. Here, we further found that the major enriched downregulated genes in CASZ1 knockout cells were adherens junctions and ECM components via a Gene Ontology (GO) analysis. The mechanical properties and composition of the ECM and the ECM-receptor interaction network are key drivers in regulating EMT [34]. Integrin-mediated cell adhesion to the ECM also activates down-

stream signaling pathways and triggers EMT [35]. Further studies exploring how CASZ1 regulates ECM composition will be helpful for understanding the complex interactions involved in tumor metastasis.

Herein, we demonstrated a strong relationship between ITGAV overexpression and poor prognosis in lung adenocarcinoma patients and showed that CASZ1 promotes cell migration and invasion by driving ITGAV expression. Integrins are heterodimeric cell surface receptors that govern the positioning and activity of proteolytic enzymes during ECM remodeling, which enhance cancer cell invasion and migration [15, 20]. Integrin expression patterns change throughout tumor growth and metastasis, promoting cell invasion through matrix metalloproteinase 9 (MMP9) activation [36]. Furthermore, integrins promote cancer cell invasion and metastasis by interacting with other cell types, such as endothelial cells and fibroblasts, in the tumor microenvironment [16]. Integrins alpha V, particularly alpha V beta 6 and alpha V beta 8, activate TGF-β and thereby promote the expression of mesenchymal matrix proteins and their cognate integrin receptors [37]. Integrin alpha V beta 6 expression is strongly connected to the expression of other EMT transcription factors, such as ZEB1 and ZEB2 [4]. Additionally, integrins alpha V knockdown inhibited proliferation, invasion, and colony formation in breast and colorectal cancer and laryngeal and hypopharyngeal carcinoma [17, 38]. Furthermore, ITGAV overexpression is a poor prognostic indicator for various malignancies, including stomach, liver, and pancreatic cancer [18, 20, 27].

In conclusion, in this study we confirmed that CASZ1 acts as an oncogene in LUADs and associated with cancer metastasis and poor overall survival rate. CASZ1 suppresses cell migration, invasion, and cancer metastasis by directly regulating ITGAV expression. This study opens up the potential new therapies for LUAD patients by targeting the ITGAV or CASZ1.

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Disclosure of conflict of interest

None.

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