COL11A1 acted as a downstream of Mist1 to promote the EMT in pancreatic cancer

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Research

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Abstract

Background Pancreatic cancer remains one of the deadliest cancers worldwide. The tumor microenvironment is closely related to the occurrence, growth, and metastasis of tumors. Collagen type XI alpha 1 chain (COL11A1), as a component of extracellular collagen, has been proven to be responsible for tumor development and drug resistance in various cancers. However, it's role in pancreatic cancer remains unknown.

Method The GEPIA (Gene Expression Profiling Interactive Analysis) web tool was used to clarify the differential expression of *COL11A1* and clinical prognosis in pancreatic cancer. Functional experiments were performed to assess the effect of COL11A1 on the state of pancreatic cells *in vitro*. Mouse xenograft models and pulmonary metastasis models were established to investigate the influence of COL11A1 *in vivo*. Chromatin immunoprecipitation (ChIP) assays and dual-luciferase assays were applied to assess the relationship between muscle, intestine and stomach expression 1 (Mist1) and COL11A1.

Results The upregulated expression of *COL11A1* in pancreatic cancer led to a worse prognosis and overall survival for patients with pancreatic cancer. Knockdown of *COL11A1* in pancreatic cancer cell lines inhibited their proliferation and invasion, while upregulating *COL11A1* increased those abilities. The ChIP and dual-luciferase assays clarified Mist1 could bind to the promoter of *COL11A1* as a transcription factor and repress its transcription. Meanwhile, we found that the N-terminal repressor region of Mist1 was capable of inhibiting *COL11A1* expression.

Conclusion We identified COL11A1 as a carcinogen in pancreatic cancer, and clarified a novel mechanism which Mist1 reverses the Epithelial-Mesenchymal Transition in pancreatic cancer by repressing *COL11A1* expression.

Background

Pancreatic ductal adenocarcinoma (PDAC) refers to one of the most malignant types of cancer, which ranks fourth among all cancer deaths in the USA (1). Numerous patients lost the opportunity for surgery because of early invasion of the main vessels and late clinical diagnosis (2). Pancreatic cancer has a poor prognosis, with a 5-year survival rate of about 7%, despite the application of surgical resection (3). Determining the molecular mechanism of pancreatic cancer metastasis might contribute to the development of effective therapies.

Collagen type XI alpha 1 chain (COL11A1) belongs to the collagen family and forms one of the two alpha chain of collagen type XI, acting as a major component of the interstitial extracellular matrix (4). COL11A1 was first identified in cartilage and several non-cartilaginous tissues, such as bone, vitreous humor, skin, heart, sternum, and arterial smooth muscle cells (5). Recently, studies have reported the effect of *COL11A1* upregulation on the malignancy of various tumors. Overexpression of *COL11A1* promoted lung metastasis and enhanced chemoresistance to cisplatin and paclitaxel in ovarian cancer (6–8). *COL11A1* knockdown in head and neck squamous cell cancer resulted in decreased migration and

invasion (9). Overexpression of *COL11A1* was observed in pancreatic cancer and was deemed to be a predictive prognostic biomarker (10). However, the regulation and role of COL11A1 in pancreatic cancer remains largely unknown. Previously, we found that overexpression of *MIST1*, encoding muscle, intestine and stomach expression 1, a member of the BHLH family, reversed the epithelial-mesenchyme transition (EMT) and inhibited the tumorigenicity of PDAC (11). In the present study, ChIP results show a correlation between Mist1 and COL11A1. We find that COL11A1 correlates with the progression and poor prognosis of PDAC and is the key molecule that mediates the role of Mist1 in the migration and invasion of pancreatic cancer cells.

Methods

Human pancreatic tissue samples

Pancreatic cancer and paracancerous tissue samples (n = 12) were obtained from patients at the Union Hospital of Huazhong University of Science and Technology (HUST). All tissues were stored immediately in liquid nitrogen after resection. The study was approved by the Ethics Committee at Tongji Medical College, HUST, China and written informed consent was obtained from patients before surgery as described before (11).

Cell Culture

Human pancreatic cancer cell lines PANC-1, BxPC-3, and SW1990 were obtained from the ATCC (Manassas, VA, USA). PANC-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), and BxPC-3 and SW1990 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% FBS. All Cells were cultured in 5% CO_2 at 37 ℃.

Western Blotting And Antibodies

Cells were washed with phosphate-buffered saline (PBS) three times and lysed in radioimmunoprecipitation assay (RIPA) buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and 1% Halt phosphatase inhibitor cocktail (Thermo Scientific) for 30 min. After quantification using a protein quantification kit (Sigma-Aldrich), the same amounts of protein were separated using 10% SDS-PAGE gels. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Pierce Biotechnology, Rockford, IL, USA) and blocked using 5% skim milk for 1 h, before being incubated overnight at 4 °C with specific primary antibodies, followed by incubation with secondary antibodies (BOSTER, Pleasanton, CA, USA) for 1 h at room temperature. The membranes were imaged using the Molecular Imager Chemi Doc XRS^+ imaging system (Bio-rad, Hercules, CA, USA) using ECL detection reagents (Thermo Fisher Scientific). The anti-Mist1 antibody (1:1000) was purchased from Cell Signaling Technology (Danvers, MA, USA; the anti-COL11A1 (1:1000) antibody was from Abcam (Cambridge, MA, USA. The anti-E-cad, N-cad, Vimentin, β-catenin, and Snail (1:1000) antibodies were from

Cell Signaling Technology. The anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:3000) antibody was purchased from Proteintech (Rosemont, IL, USA).

Quantitative Real-time Reverse Transcription PCR (QRT-PCR)

Total RNA was extracted using the TRIzol Reagent (Invitrogen, Waltham, MA, USA) and reverse transcribed to cDNA using a PrimeScript™ RT reagent Kit (Takara, Shiga, Japan). Next, the quantitative real-time reverse transcription PCR (qRT-PCR) step was performed using a SYBR Green PCR Kit (Takara). The primer sequences used for qRT-PCR are listed in Supplementary Table S1. The fold-enrichment was obtained from three independent replicate experiments and was normalized to the expression of *GAPDH*.

Chromatin Immunoprecipitation (CHIP) Assay And The Luciferase Reporter Assay

The ChIP assay was performed using a SimpleChIP® Plus Sonication Chromatin IP Kit (#56383, Cell Signaling Technology). According to the manufacturer's protocol, cells transfected with *MIST1* overexpression lentiviruses were cross-linked using 37% formaldehyde. DNA fragments were broken into 100–500 bp using ultrasound and separated by agarose gel electrophoresis. Then, the protein-DNA complexes were incubated vertically with 2 μg of anti-Mist1 antibodies or IgG overnight at 4 ℃. Then, protein G agarose beads were used to precipitate the cross-linked chromatin. Finally, the chromatin was isolated and purified for qPCR. The percent of the Input was calculated by:

$$5\% \times 2^{(CT5\%Input - CT\ Sample)},$$

Where CT is the cycle threshold.

Primer sequences for the *COL11A1* promoter are listed in Supplementary Table S1

For the luciferase reporter assay, PANC-1 cells were added to the wells of 24well plates, and co-transfected with luciferase reporter plasmids (Ribo biology, Guangzhou, China) and *MIST1* overexpression lentiviruses for 48 h. Then, the luciferase activities were measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA). Renilla luciferase activity was used as a control.

Overexpression And Knockdown

Lentiviruses for *MIST1* overexpression were purchased from Genechem (Shanghai China) and transfected into cells. Green fluorescent protein (GFP)-positive infected cells were selected using puromycin. The short hairpin RNA targeting *COL11A1* and the plasmid overexpressing *COL11A1* were procured from Sigma-Aldrich and Ribobiology (Guangzhou, China), respectively. Cells were transfected with the shRNA or plasmids using Lipofectamine 3000 (Invitrogen) and Opti-MEM media (Invitrogen). 24 h after transfection, the medium was replaced with fresh DMEM containing 10% FBS.

Cell Invasion And Migration Assay

For the Transwell assay, $1 \cdot 10^4$ cells were plated in 200 μL of PBSfree medium into upper chamber of a 24-well Corning Costar insert with 8-μm pores and 600 μL of DMEM with 30% FBS was added into the lower chamber. After incubation at 37 ℃ for 24 h, the cells that had moved into the lower chamber were fixed using 4% paraformaldehyde (PFA) and stained with crystal violet. The chambers were photographed under a under microscope and cells were counted in five random fields.

The wound healing assay was conducted in 6-well plates in which cells had grown to full confluence. Wounds (scratches) were made in the cell layers, which were photographed at 0, 24, 48, and 72 h after wounding to measure the wound.

Cell Proliferation

For MTT assay, 3000 cells in 200 μL of medium were plated into wells of a 96-well plate and grow for 5 days. On each incubated day, 50 μL of MTT solutions were added to seven independent replicate wells and incubated in the dark for 2 hours. Then 500 μL DMSO were added to dissolve the formazan crystals. The absorbances were measured at 570 nm using a microplate reader.

For the colony formation assay, 500 cells were seeded in 6-well plates. At 7 days after plating, the cells were washed with PBS three times, fixed in paraformaldehyde for 30 minutes, and then stained using crystal violet. The plates were photographed and the number of colony cells was counted.

Generation of PDAC xenografts model and pulmonary metastasis model in nude mice

Male athymic nude mice (4-weeks old) were purchased from Vitalriver, (Beijing, China). The xenografts models were established by subcutaneously inoculating $5 \cdot 10^6$ PANC-1 cells infected with sh-control or sh-COL11A1 lentiviruses diluted in 100 μL of PBS. Tumor volumes were calculated using the formula: tumor volume (mm3) = (Length x Width2)/2. All the mice were sacrificed at day 21 after injection. The pulmonary metastasis models were generated by intravenously injecting $5 \cdot 10^6$ cells diluted in 100 μL of PBS into the tail. Hematoxylin and eosin (H&E) staining was performed to detect the lung metastatic foci. The animal experiments were conducted under the guidelines approved by the local ethics committee (Tongji Medical College, HUST, China).

Statistical analysis

All data are expressed as the mean ± SD and were analyzed using GraphPad Prism 6 software (GraphPad Inc., La Jolla, CA, USA). Paired ttests and one-way analysis of variance (ANOVA) were used to asses statistical significance, which was set at $p < 0.05$. The clustering analysis was performed to group similar items together.

Discussion

The major finding of our study was that in patients with pancreatic cancer those who had relatively high *COL11A1* expression might have shorter survival, suggesting *COL11A1* as a candidate biomarker in

pancreatic cancer. Further investigation revealed that COL11A1 is the key molecular that mediates the role of Mist1 in migration and invasion in pancreatic cancer.

Increasing perspectives on cancer have revealed the important role of the ECM in regulating cell proliferation, migration, and invasion (12–14). COL11A1 is a minor fibrillar collagen whose importance on the progression, metastasis, and drug resistance in cancer has been highlighted in recent research. COL11A1 regulates Ets-1 binding to the *MMP3* promoter, thereby meditating the ovarian cell invasion through the expression of *MMP3* (8). Moreover, *COL11A1* upregulation has been shown to be associated with the TGF-β1, Wnt, and Hedgehog signaling pathways(8, 15, 16). A major feature of pancreatic cancer is the dense connective tissue around tumor cells, making them unresponsive to most chemotherapy drugs, which makes pancreatic cancer extremely difficult to treat (17, 18). It has been reported that *COL11A1* was upregulated in pancreatic cancer and was related to Wnt signaling pathway, which in turn led to pancreatic cancer invasion (15). In our report, *COL11A1* was upregulated in pancreatic cancer. Functional experiments confirmed that knockdown of *COL11A1* reduced pancreatic cancer cell migration, invasion, and proliferation *in vivo* and *in vitro*. Furthermore, overexpression of *COL11A1* upregulated the expression of mesenchymal markers, indicating that COL11A1 promotes the EMT process in pancreatic cancer. In addition, we found the *COL11A1* expression was associated with a poorer prognosis in patients with pancreatic cancer according to the TCGA database.

Silencing of the *MIST1* led to significant de-differentiation, greatly accelerating the progress of pancreatic intraepithelial neoplasia (PanIN), eventually giving rise to pancreatic ductal adenocarcinoma (19). Our previous study also showed that the overexpression of *MIST1* could reverse the EMT in pancreatic cancer cells (11). However, the study found that Mist1 could not act directly as a transcription factor for E-cadherin to alter its expression. Mist1 was first reported as an inhibitory transcription factor (20). On this basis, we conducted the ChIP-seq assay for Mist1 in pancreatic cancer cells. After identifying *COL11A1* as a downstream gene of *Mist1*, we performed ChIP and luciferase assays to determine that Mist1 conducted as a transcription factor for COL11A1 which regulated the expression of *COL11A1*, thereby affecting the development and metastasis of pancreatic cancer.

The BHLH family is one of the most widespread families of transcription factors (21, 22). They usually function as dimers with the longer helix binding to the -CANNTG- sequence (E-box) of the target DNA to regulate transcription (23). Binding of the BHLH dimer to the DNA E-box element generally resulted in the activation of the target genes (24). Lacking a classical active transcription domain, Mist1 instead contains a repressive N-terminal region capable of inhibiting transcription (20)(25, 26). We also came to the same conclusion using the UniProt web tool. The helix- loop- helix (HLH), the core structure of Mist1, is located in amino acids 75–125 and the 1–75 amino acid region acts as its Nterminal inhibitory domain, which agreed with the above reports (20) (25, 26). Therefore, we constructed the Mutant-Mist1 lacking amino acids 1–75. Functional experiments and luciferase reporter assays confirmed that Mist1 inhibited the expression of *COL11A1* through its N-terminus in pancreatic cancer cells.

Conclusions

We identified COL11A1 as a biomarker of pancreatic cancer, and revealed that overexpression of *COL11A1* in pancreatic cancer cells enhanced the cells' invasion and metastasis capacities. Moreover, Mist1 deficiency led to a loss of control of *COL11A1* expression, resulting in its upregulation, which promoted the development of pancreatic cancer.

Abbreviations

OS Overall survival

GEPIA Gene Expression Profiling Interactive Analysis

ChIP Chromatin immunoprecipitation

Mist1 muscle, intestine and stomach expression 1

PDAC Pancreatic ductal adenocarcinoma

TCGA The Cancer Genome Atlas

GTEx Genotype-Tissue Expression

EMT Epithelial-mesenchyme transition

PanIN pancreatic intraepithelial neoplasia

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee at Tongji Medical College, HUST, China.

Consent for publication

Not applicable.

Competing interests

No competing interests.

Funding

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Authors' contributions

YL and ZL performed the experiments; HD, YS, YL and DR collected the data; SG wrote the paper and analyzed the data. All authors read and approved the final manuscript.

Acknowledgments

Not applicable.

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Figures

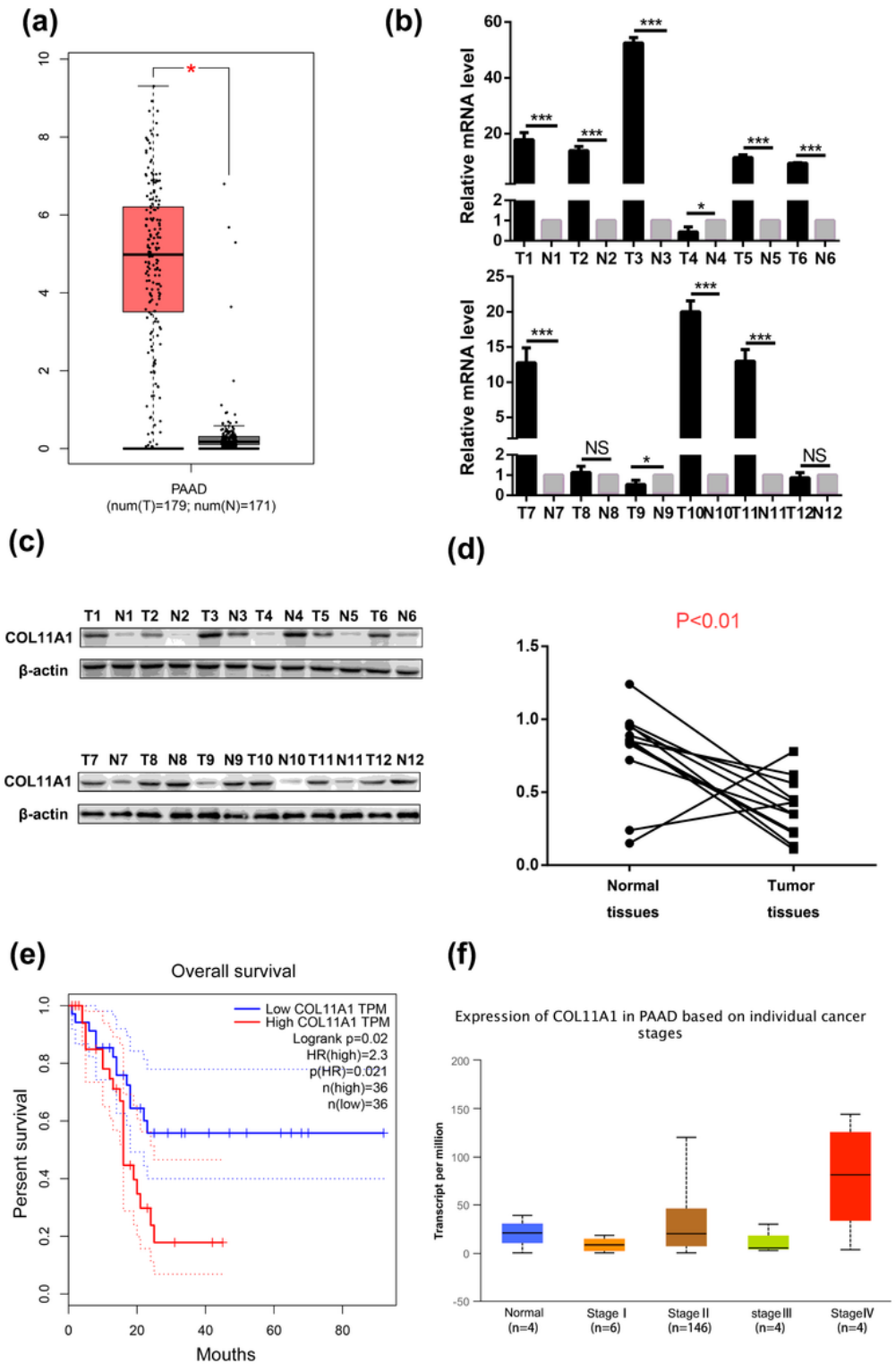

Figure 1

COL11A1 expression is elevated in PDAC and associated with poor prognosis. a. The expression of COL11A1 as shown by the GEPIA web tool. The box plots were analyzed using log2 (TPM + 1) for the log scale. *, P < 0.05. b. qRT-PCR of COL11A1 in 12 paired PDAC tissues (T) and normal tissues (N). c. Western blotting for the expression of COL11A1 in 12 paired PDAC tissues (T) and normal tissues (N). d. The proteins expression level quantified by Image J software. e. The overall survival of patients with

PDAC (P = 0.02, HR=2.3) was measured using the GEPIA web tool. f. The expression of COL11A1 based on cancer stage.

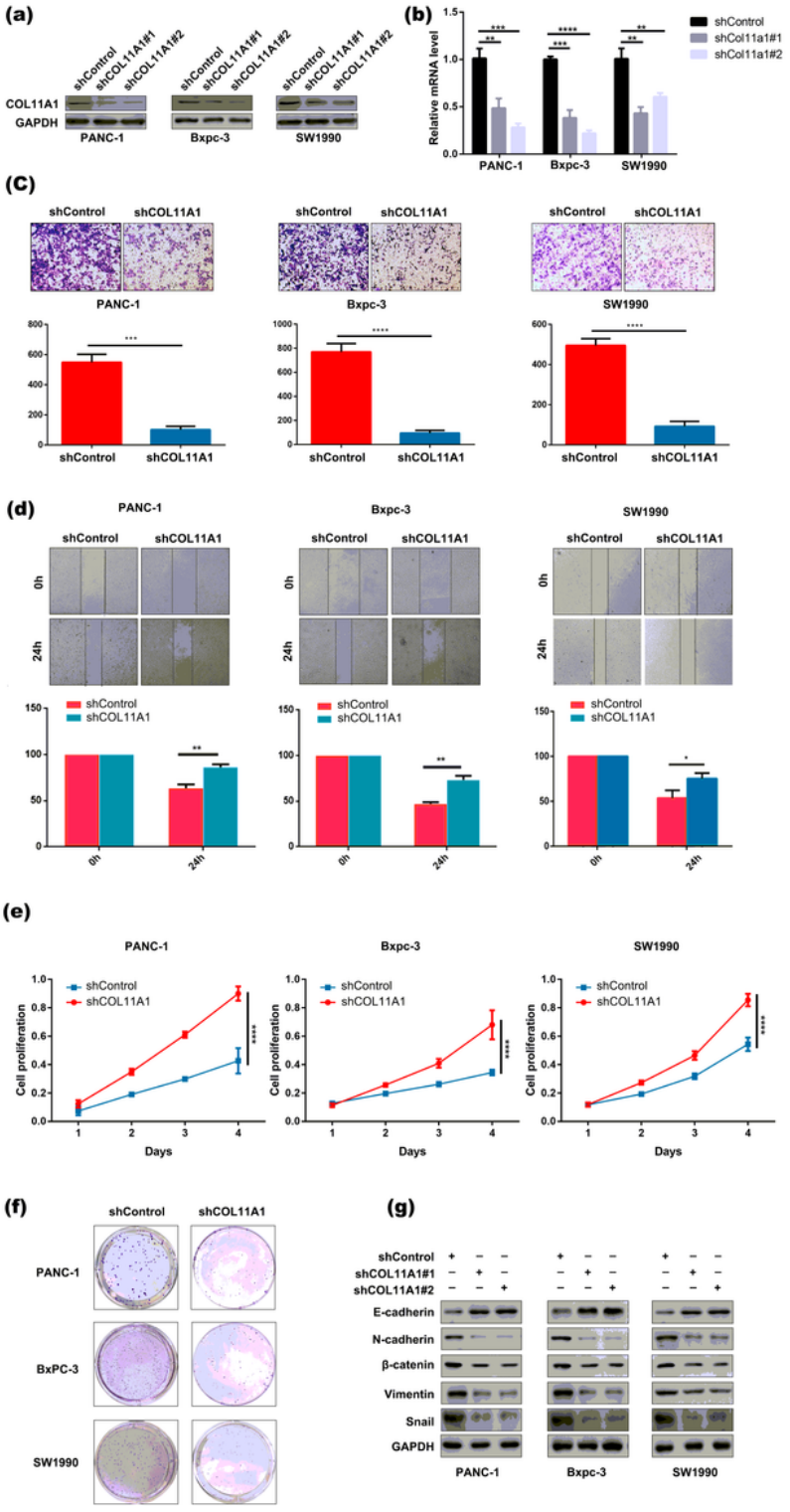

Figure 2

COL11A1 knockdown suppresses PDAC progression in vitro. a. b. Western blotting (a) and qRT-PCR (b) analysis of the expression of COL11A1 in PANC-1, Bxpc-3, and SW1990 cell lines transfected with shRNA-Control (shControl) and shRNA-COL11A1 (shCOL11A1). GAPDH acted as the internal reference. Data are

presented as the mean SD. Statistical analyses were performed using one way ANOVA and Tukey's multiple comparison tests; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Each experiment was performed separately three times. c-f. Pancreatic cancer cell lines (PANC-1, Bxpc-3, and SW1990) were transfected with shControl and shCOL11A1. Cell migration was evaluated using Transwell migration assays (c) and wound healing assays (d), and the relative migrated cell number and migration distance were analyzed statistically. Cell growth and viability were evaluated using the MTT assay (e). Plate cloning experiment to evaluate colony formation ability (f). g. Western blotting analysis of the proteins (E-cadherin, N-cadherin, β-catenin, Vimentin, and Snail) after 72 h of transfection of shCOL11A1. Data are shown as the mean SD. Each experiment was performed separately three times. Statistical analyses were performed using one-way ANOVA following Tukey's multiple comparison tests; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

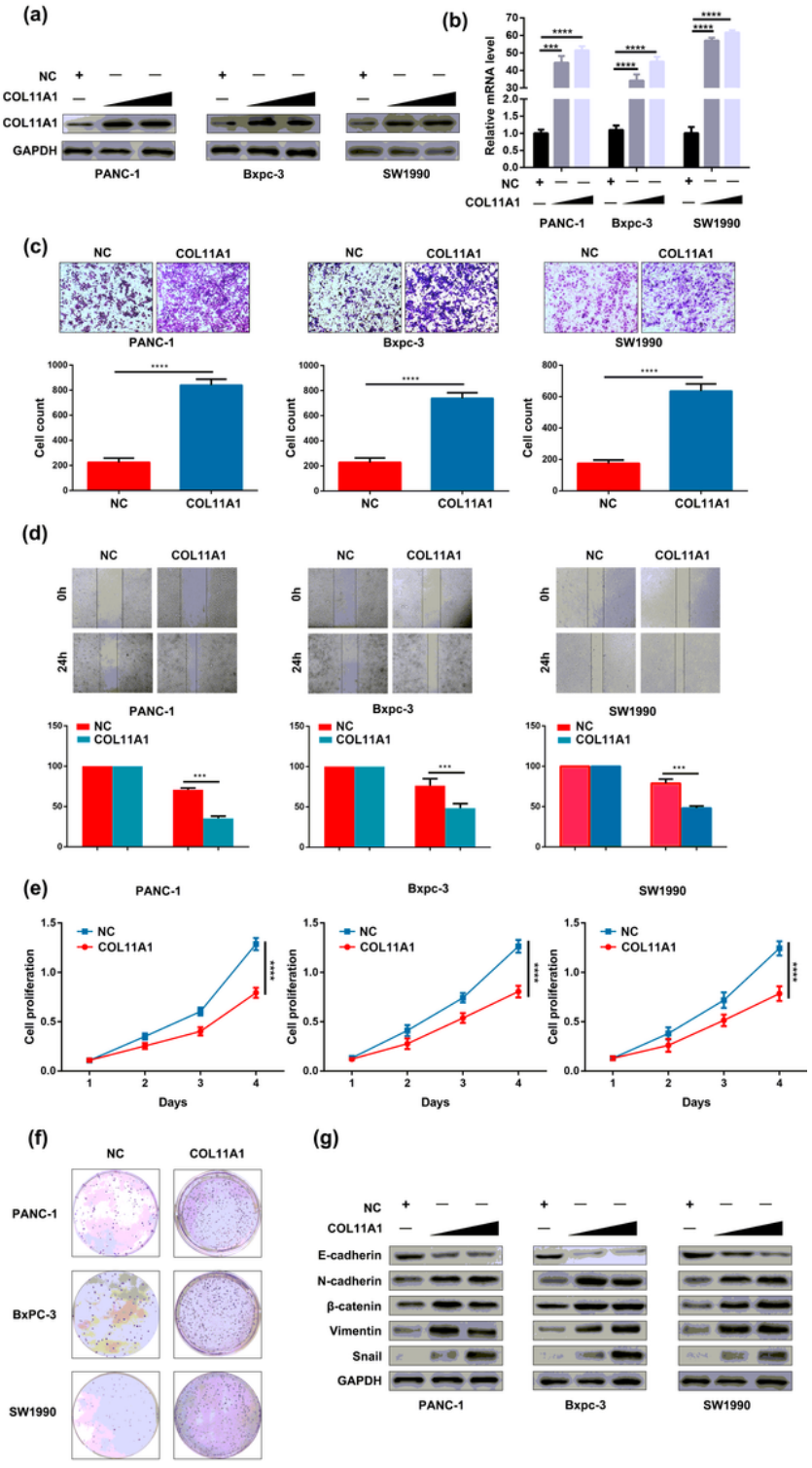

Figure 3

Overexpression of COL11A1 promotes tumor progression in vitro. a. b. Western blotting (a) and qRT-PCR (b) analysis for the expression of COL11A1 in PANC-1, Bxpc-3, and SW1990 cell lines transfected with NC or COL11A1 overexpression plasmids (COL11A1). GAPDH acted as the internal reference. Data are presented as the mean SD. Statistical analyses were performed using one-way ANOVA and Tukey's multiple comparison tests; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Each experiment was performed

separately three times. c-f. Pancreatic cancer cell lines (PANC-1, Bxpc-3 and SW1990) were transfected with NC and COL11A1 plasmid. Cell migration was evaluated using Transwell migration assays (c) and wound healing assays (d), and the relative migrated cell number and migration distance were analyzed statistically. Cell growth and viability were evaluated using the MTT assay (e). Plate cloning experiment evaluating the colony formation ability (f). g. Western blotting analysis of the proteins (E-cadherin, N-cadherin, β-catenin, Vimentin and Snail) involved in EMT after 72 h of transfection. Data are presented as the mean ± SD. Each experiment was performed separately three times. Statistical analyses were performed using one-way ANOVA following Tukey's multiple comparison tests; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

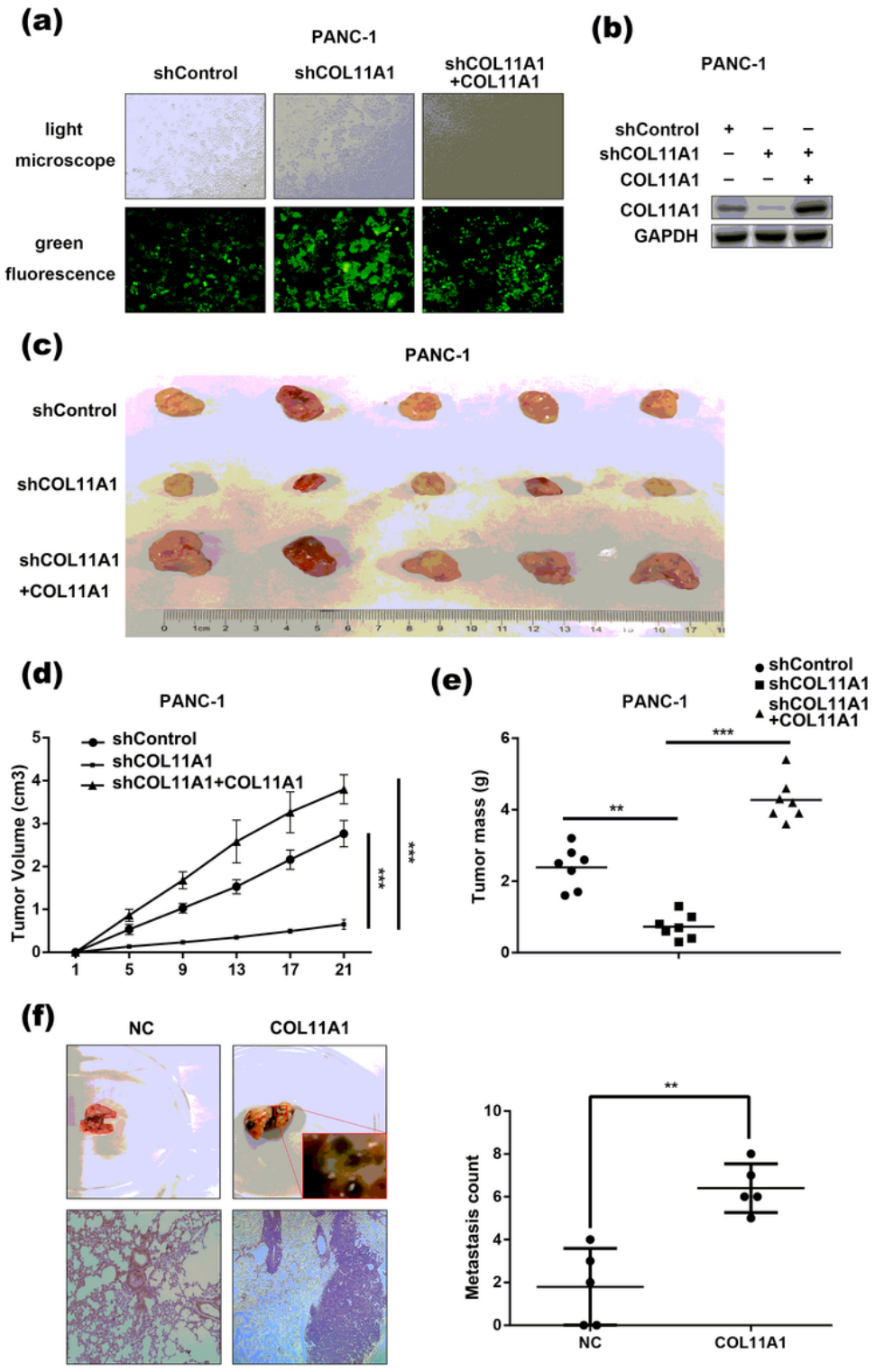

Figure 4

COL11A1 promotes PDAC progression in vivo. a. The green fluorescence of stable cell lines. b. Western blotting analysis verification of COL11A1 expression. c. Xenograft models were established by subcutaneously injecting shControl, shCOL11A1, or shCOL11A1 + COL11A1 cells. The mice were sacrificed after 6-weeks of growth and the tumors were excised. d and e. Tumor volume and weight measurement were shown as mean ± SD (n=6). shControl group was compared with shCOL11A1 group,

shCOL11A1 group was compared with shCOL11A1 + COL11A1 group. Statistical analyses were performed with two-way ANOVA followed by Sidak's multiple comparison's tests. **, P < 0.01, ***, P < 0.001. f. Pulmonary metastasis models were established by tail intravenous injection of LV-NC cells or the LV-COL11A1-overexpression construct and allowed to grow for 6 weeks. The mice were sacrificed and pulmonary metastases were then exposed. H&E staining of lung tissues indicating the pulmonary metastasis tumors. Statistical analysis of the metastasis foci was calculated using T-test. Means ± standard deviation from five independent experiments. **, P < 0.01.

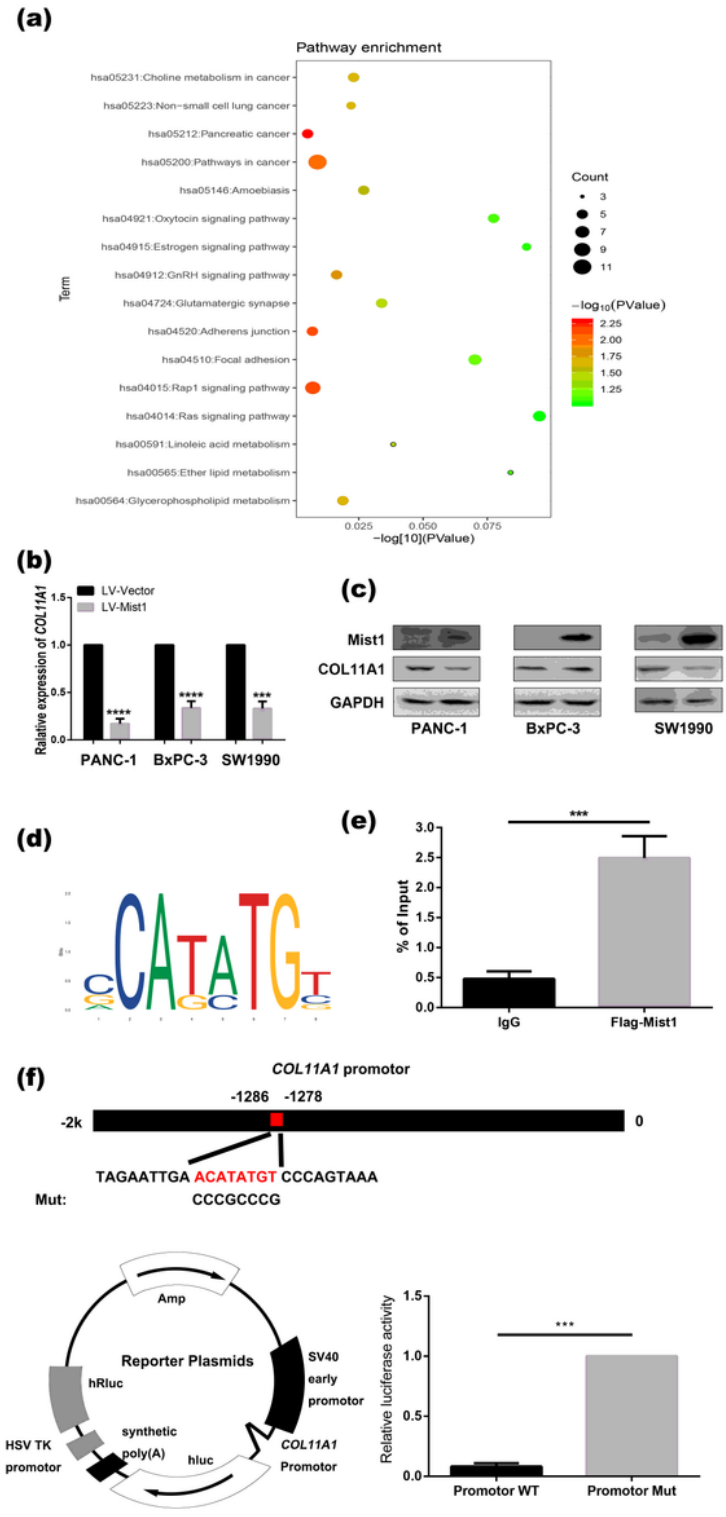

Figure 5

Mist1 reduced COL11A1 expression by acting as its transcription factor. a. Clustering analysis made based on the ChIP-seq results. b and c. COL11A1 downregulation under conditions of Mist1 overexpression. qRT-PCR (b) and western blotting (c) for COL11A1 were performed in Mist1 overexpression cells. d. The transcription binding site motif of Mist1 provided by the JASPAR database. e. The ChIP assay was conducted to analyze the local enrichment of Mist1 across the COL11A1 promote region in PANC-1 cells. f. Luciferase assay in PANC-1 cells co-transfected with COL11A1 luciferase reporter plasmids and Mist1 overexpression plasmid. Values represent the mean ± SD of three independent experiments. The luciferase activities were measured using the Dual-Glo luciferase assay system. ***, $P < 0.001$.

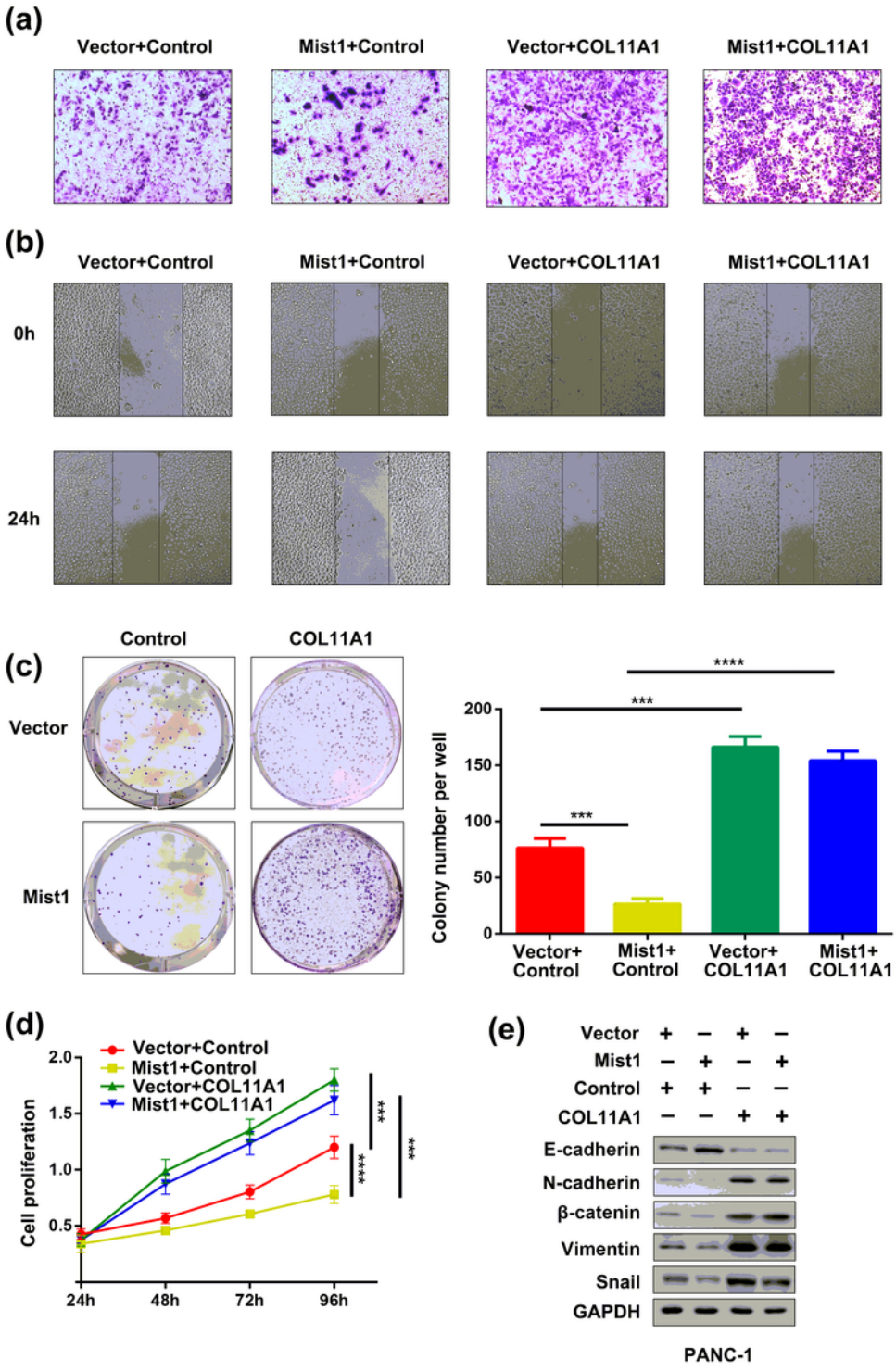

Figure 6

Mist1 repress tumor progression by downregulating COL11A1 expression. PANC-1 cells were transfection with shControl, shCOL11A1, Vector, or Mist1. a and b. Cell migration evaluated using transwell migration assay (a) and wound healing assay (b). c and d. Cell growth and viability evaluated using plate cloning experiment (c) and MTT assay (d). The relative migrated cell number and migration distance were

analyzed statistically. e. Western blotting analysis of the proteins (E-cadherin, N-cadherin, β-catenin, Vimentin and Snail) after 72 h of transfection.

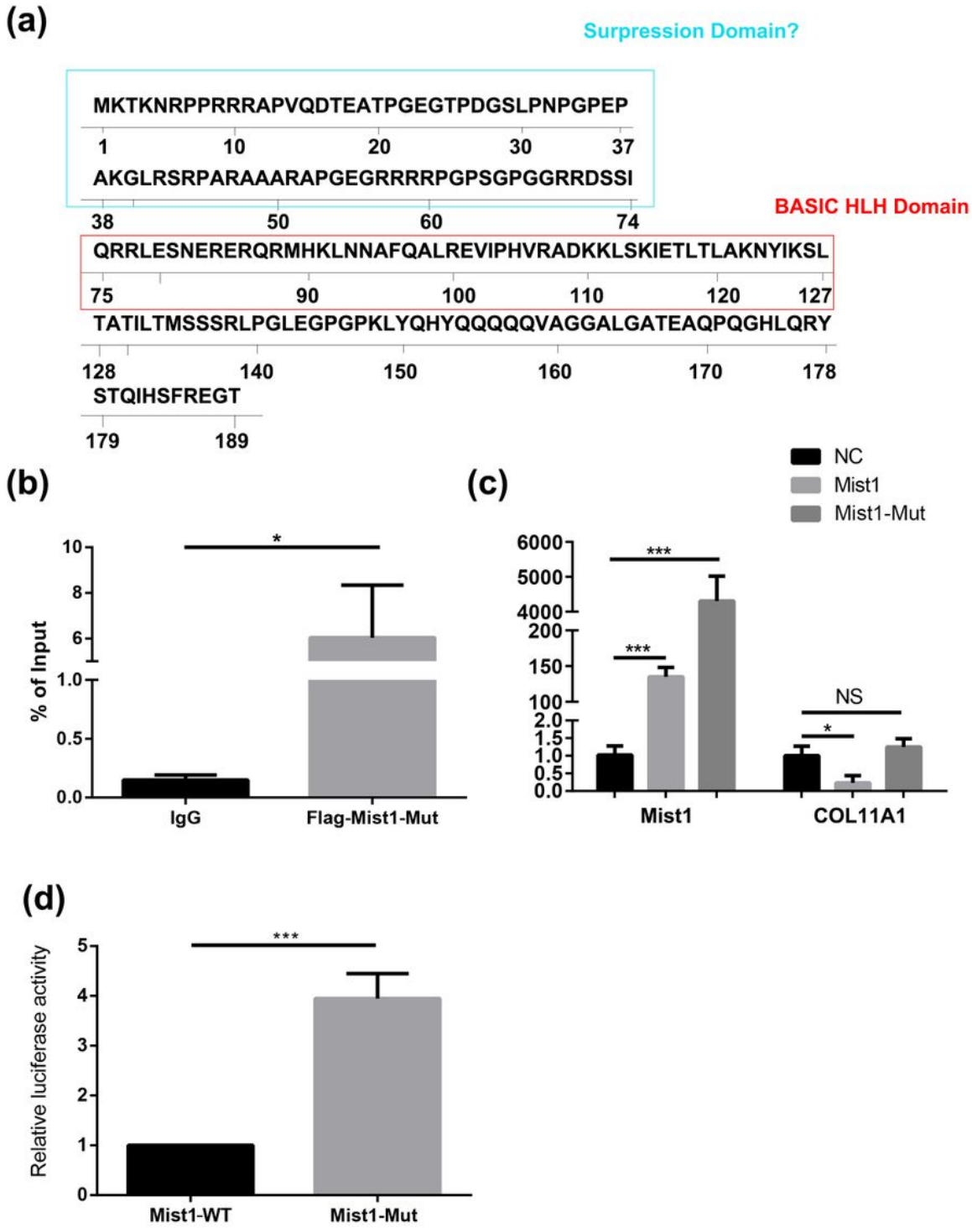

Figure 7

The N-terminal domain of Mist1 is important for downregulating COL11A1 expression. a. The amino acid sequence of Mist1. The sequence in the red box is a predicted inhibitory domain (rank 1 to 72), while the sequence in the blue box is the classic BHLH domain, according to the UniProt web tool. b. The ChIP

assay was conducted to analyze the local enrichment of FLAG-tagged Mist1-Mut across the COL11A1 promote region in FLAG-tagged Mist1-Mut transfected PANC-1 cells compared with empty vector transfected cells. *, P < 0.05. c. qRT-PCR of Mist1-Mut. Mist1-Mut lost the inhibitory effect on the expression of COL11A1 compared with that of wild-type Mist1. NS, not significant; *, P < 0.05; ***, P < 0.001 d. Luciferase assay in PANC-1 cells co-transfected with COL11A1 luciferase reporter plasmids and Mist1 overexpression plasmid or Mist1-Mut overexpression plasmid. Values represent the mean ± SD of three independent experiments. The luciferase activities were measured using the Dual-Glo luciferase assay system. ***, P<0.001.

Supplementary Files

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