The positive RNASEH1-AS1/has-miR-218-5p/NET1 feedback loop mediated by POU2F1 contributes to the development and progression of human lung squamous carcinoma

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Abstract

Background

At the molecular level, competing endogenous RNAs (ceRNAs) regulate other RNA transcripts by competing for shared microRNAs (miRNA). Notably, miRNAs negatively regulate gene expression at the levels of mRNA stability and translation suppression.

Methods

We measured theexpression of miR-218-5p and RNASEH1-AS1 in clinical lung squamous cell carcinoma tissues using qRT-PCR. In an attempt to explore the roles of miR-218-5p and RNASEH1-AS1 in determining the malignant phenotype of NCI-H520 cells, colony formation and MTT assays were performed to measure cell viability and proliferation, and transwell invasion and wound healing assays were performed to examine cell migration and invasion. A ChIP assay was conducted to confirm the direct binding of POU2F1 to the RNASEH1-AS1 promoter.

Results

In this investigation, the expression of the lncRNA RNASEH1-AS1 is upregulated in human lung cancer tissues, and it functions as a miRNA sponge for hsa-miR-218-5p in human lung squamous carcinoma cells. The lncRNA RNASEH1-AS1 facilitates the growth and motility of lung squamous carcinoma cells, while miR-218-5p exerts the opposite effects. NET1 and POU2F1 are validated as direct and functional targets of miR-218-5p. The downregulation of miR-218-5p releases the suppression of NET1 and POU2F1. POU2F1 binds directly to the lncRNA-RNASEH1-AS1 promoter and functions as transcription factor to enhance the promoter activity of RNASEH1-AS1.

Conclusions

Overall, the positive RNASEH1-AS1/hsa-miR-218-5p/NET1/POU2F1 feedback loop can help us understand the regulatory mechanism underlying the genesis and progression of human lung squamouscarcinoma, possibly providing new biomarkers for its diagnosis and treatment.

Background

Lung cancer is the most regular and fatal cancer worldwide[1]and is recognized as a cluster of distinct diseases with high levels of genetic, cellular and molecular heterogeneity[2-4]. In general, 80–85% of human lungcancers are non-small cell lung cancer (NSCLC), of which lung adenocarcinoma (LUAD) and lung squamous carcinoma (LUSC) are the mainsubtypes. Moreover, the genetic and epigenetic alterations differ substantially between LUSC and LUAD. The exploitation ofdrugstargeting particular gene mutations hasdramatically improved the treatment forpatients with advanced LUAD. However, only asmall number of LUSC specimenscontains driver gene mutations, leadingto anextremely low five-year survival ratebecause of compromised efficacy ofplatinum-based chemotherapy inpatients with LUSC[5]. Thus,

studies aimingto further address the molecular mechanisms underlying thepathogenesis of LUSC for more effective therapeutic options are crucial.

Recent investigations have unexpectedly discovered a large group consisting of 70 transcripts of non-protein-coding RNAs (ncRNAs) in mammalian cells [6-9].In contrast, small ncRNAs, such as miRNAs, siRNAs and piRNAs, longncRNAs (lncRNAs) are a new class of mRNA-like transcripts with sizelonger than 200 nucleotides[10]. Mature miRNAs regulate the expression of most protein-coding genes by binding to the 3'-UTR of target genes, thereby leading to the degradation of target mRNA and suppression of protein translation [11, 12]. In contrast, lncRNAs function as molecular decoys for miRNAs in the cytoplasm and cell nucleus [13, 14]. The dysregulation of lncRNAs has been reported in many types of cancers, along with its importance in key cancer signalling networks and malignantbehaviours[15], such as in prostate cancer [16], breast cancer [17] and liver cancer [18, 19]. Further studiesexamining the lncRNAs and miRNAs that are dysregulated in LUSCare still urgently needed. LncRNAs were expressed at high levels in lung cancer in our early stage screen. However, the role of RNASEH1-AS1 in LUSC progression and the possible target miRNAs have not been extensively investigated in previous reports. We conducted the in vitro cell experiment and in vivo xenograft assay to explore the effect of RNASEH1-AS1 on LUSC. Notably, miR-218 functions as a lncRNA target and influences the malignant behaviours of many cancer cells, such as hepatocellular carcinoma [20], pancreatic cancer[21] and breast cancer [22]. The expression of miR-218-5p was suppressed in clinical LUSC tissues.

POU2F1(POU class 2 homeobox 1) is also known as OCT1, OTF1 or oct-1B. The POU2F1 transcription factor was among the first identified members of the POU transcription factor family [23]. It is a ubiquitous transcription factor that regulates the transcription of genes involved in inflammation and the cell cycle by binding to cis-acting octamer elements[24]. In our study, we investigated the effect of miR-218-5p on POU2F1 expression and the relationship between POU2F1 and RNASEH1-AS1 expression in LUSC cells. The NET1 (neuroepithelial cell transforming 1) gene is part of the family of Rho guanine nucleotide exchange factors. The protein encoded by this gene interacts with RhoA within the cell nucleus and may play a role in repairing DNA damage after exposure to ionizing radiation.NET1 is reported to be overexpressed in many human cancers, including non-small cell lung cancer [25-27]. We further examined its expression and its upstream regulatory network in LUSC.

Methods

Bioinformatics predictions and screening.

The long non-coding RNAs that areexpressed at high levels in lung cancer were screened using StarBase V3.0 (http://starbase.sysu.edu.cn/).The miRNAsthat bind to the lncRNAs and the possible downstream targets of miR-218-5p were predicted by the most frequently used algorithms TargetScan, miRDB and PicTar, and we noticed the overlappingmiRNAs and targets identified by the three algorithms. The putative promoter of miR-218-5p was predictedusing Promoter Scan (http://www-bimas.cit.nih.gov/molbio/proscan/).

Cell line and human LUSC tissue

NCI-H520 cells were cultured with the RPMI-1640 (Gibco, Grand Island, NY, USA) containing10%foetal bovine serumand 1,000 U/mL P/S. The human LUSC cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.Cells were transfectedusing Lipofectamine 2000 Reagent (Invitrogen, USA) according to the manufacturer's instructions. The NCI-H520 cells and three pairs of human lung squamous cell carcinoma tissues were provided by the Department of Cancer Institute, North China University of Science and TechnologyAffiliated People's Hospital. The number, gender, classification and age of the patients with LUSC are shown in Table 1. Informed consent was obtained from all subjects or their direct relatives.The cell and tissue studies weresubmitted to and approved by both the Ethics Committee of North China University of Science and Technology and the Ethics Committee of Hebei Medical University.

qRT-PCR.

Total RNA was extracted from lung cancer tissues and the cell line with the mirVana miRNA Isolation Kit (Ambion, USA) according to the manufacturer's instructions. Onemicrogram of RNA was reverse transcribed into cDNAswith Moloney murine leukaemia virus reverse transcriptase (Takara, Japan). qRT-PCR was conducted with a SYBR® Taq™ kit (Takara Bio, Japan) and the iQ5 Real Time PCR Detection System. The level of hsa-miR-218-5p in the transcripts was normalized to U6 as the internal control. The levels of the RNASEH1-AS1, NET1 and POU2F1 mRNAs were normalized to β-actin as the internal control. The quantity of the negative control group was defined as 1, and the relative fold change of the experimental group was calculated. All the RT and qPCR primer sequences are listed in the Table 2.

Plasmid and miRNA mimics.

The miR-218-5p overexpression mimics and ASO-miR-218-5pplasmids were commercially synthesized. The sequencesare listed in Table 2.The sh-RNASEH1-AS1 and sh-POU2F1 pSilencer vectors were generated by annealing the sense and antisense strands of the hairpin RNA followed by insertion onto the pSilencer2.1-U6 neo vector between the BamHI and HindIII sites.

The wild type and mutant 3'-UTRs of the NET1 or POU2F1 genesharbouring the predicted miR-218-5p binding site were inserted into the 3' end of the reporter gene of the pmirGLO vector. Similarly, the putative promoter area and miR-218-5p binding site in lnc-RNASEH1-AS1 were amplified by PCR and inserted into the 3' end of the reporter gene of the pGL3-basic-luciferase vector.

All the insertions mentioned above were verified by DNA sequencing. All primers used in this study are listed in Table 2.

Dual-luciferase reporter assay.

NCI-H520 cells were cultivated in 48-well plates at a density of 6×10^4 cells per well. The miR-218-5p transfection reagents were prepared at a final concentration of 20µM and incubated for 4h. After 24 h, pmirGLO-targetgene-3'UTR/mut transfection regents were added into cultured cells at a final concentration of 0.5µg. After transfection for 4 h, the transfection mixture was replaced with 300 µL of fresh complete 1640 medium. After cotransfection for 48 hours, the luciferase activity was measured usingthe Dual-Luciferase Reporter System (Thermo, USA) according to the manufacturer's instructions.

Cell viability and proliferation assays.

In the MTT assay, NCI-H520 (1000 per well) cells were plated into 96-well plates. At 24, 48, and 72h after transfection, MTT was added to every well, and the plates were incubated for 4h. The absorbance was measured at a wavelength of 570 nm to evaluate cell viability.

In the colony formation assay, transfected NCI-H520 cells (200 cells per well) were trypsinized, plated into 12-well plates and cultured for 7 to14 days at 37 °C. The colonies were stained with a solution composed of 0.2% crystal violet and 20% methanol. Colonies with greater than 50 cells were counted and analysed. The colony formation rate was calculated using the following formula: colony formation rate = (number of colonies/number of seeded cells) × 100%. All cell experiments were conducted at least three times.

Transwell invasion and wound healing assays.

In the transwell invasion assay, NCI-H520 cells (1×10^5 per well) were seeded in the upper chamber of every insert (Millipore, USA) containing 50 µl of Matrigel (Millipore, USA). Eight hundred microliters of DMEM supplemented with 20%FBS (JIBCO, USA) were added to the lower chambers. After 72h, cells that had attached to the lower surface were stained f with crystal violet or 15 min. Afterwards, we captured images and counted the cells.

In the wound-healing assay, NCI-H520 cells were cultured in 12-well plates. When cell confluence reached 70-80%, scratches were generated with a 50 µl pipette tip, and non-adherent cells were removed by three washes with PBS. Wounded cells were cultured in medium lacking FBS for 0, 24, and 48 h. Images of three randomly selected fields of view were captured in each well.

Western blotting.

NCI-H520 cells were collected and lysed with lysis buffer (100 mM Tris-HCl, 2% SDS, 1 mM mercaptoethanol and 25%glycerol). Cell extracts were heated in loading buffer and the same amounts of cell extracts were separated on a 10% SDS-PAGE gels. After electrophoretic transfer to PVDF membranes (Millipore, USA), the protein bands were probed with its corresponding primary antibodies (anti-NET1, anti-POU2F1, Saier Biotechnology, China)overnight at 4 °C. The secondary antibody (anti-GAPDH, Saier Biotechnology, Tianjin, China) was added and incubated with the membrane at room temperature for 1.5 hour. The PVDF membranes were washed with PBS four times and the immunoreactive target bands

were visualized using the chemiluminescence imaging system (Huqiu Image Instruments, Suzhou, China). Band intensities were quantified using LabWorks image analysis software (UVP, USA).

Animal model.

Twenty BALB/c-nu mice (female) aged 5 to 6 weekswere purchased from the Institute of Experimental Animals, Chinese Academy of Medical Sciences (Beijing).They were randomly divided into two groups.In total, 2×10^7 NCI-H520 cells were transfected with siR-RNASEH1-AS1 or siR-NC and suspended in 100 μl of serum-free RPMI1640 for every nude mouse. The cell suspension was injected directly into the left side of the back of the mice. The tumour volume was measured every three days after injection. Four weeks later, anaesthetized mice were sacrificed by cervical dislocation, and their tumourmasseswere harvested. **No premature deaths were documented.** The tumour weight was measured and the average tumour weight was calculated. The tumour tissues were stored at -80°Cor used to perform haematoxylin-eosin (HE) and immunohistochemical staining.All studies were performed under the American Association for the Accreditation of Laboratory Animal Care guidelines and adhered to national and international standards. All animal experiments were approved by the Ethics Committee of Hebei Medical University.

CHIP assay.

The binding of POU2F1 to the lnc-RNASEH1-AS1 promoter was confirmed by ChIP assay according to the instructions of Chromatin Immunoprecipitation Kit (Millipore, USA). NCI-H520 cells were used in the cross-linking step after reaching 80-90% confluence. Isolated chromatin was sonicated to shear the DNA. Next, immunoprecipitation and elution of cross-linked protein/DNA were performed according to the manufacturers' protocol.Three to five micrograms of anti-RNA polymerase or anti-Rabbit IgG were used as the positive and negative control groups, respectively.Three to five microgramsof anti-POU2F1was used in the experimental group. Cross-links of protein/DNA complexes were reversed to free DNA, and spin columns were used for DNA purification. Primers flanking the predicted POU2F1 binding site in the lnc-RNASEH1-AS1 promoter were used for PCR.The primers used in this studyare listed in Table 2.The LabWorks image acquisition and analysis system (UVP, USA) was used to capture images and quantify the intensities of target signals after gel electrophoresis. The quantity of the input group or anti-POU2F1-ChIP group was 3481or 8041, respectively. The quantity of the input group was defined as 1, and the fold change (2.30) in the anti-POU2F1-ChIP group was calculated.

Statistical evaluation.

The data were analysed using GraphPad Prism 6 Software (GraphPad Software, USA) with the two-tailed Student's t test. The results are presented as the means±S.D. of three separate experiments. Unpaired Student's t-test was used to compare the two groups. A p value less than 0.05 was regarded as a statistically significant difference (* p⬚0.05, ** p⬚0.01, and ***p⬚0.001).

Results

The lncRNA RNASEH1-AS1 is expressedat high levels and functions as a miRNA sponge for miR-218-5p in human lung cancer.

We used StarBase V3.0 to identify lncRNAs that are dysregulated in human lung cancer tissues. The screening results revealed high RNASEH1-AS1 expression in 526 cancer samples compared to 59 normal samples (Fig.1A). We alsodetected theexpression level of the RNASEH1-AS1 mRNA in three pairs of clinical lung squamous cell carcinoma tissues, and RNASEH1-AS1 was expressedat higher levels in LUSC tissues than in theadjacent normal tissues (Fig. 1B). Base-pairing complementation showed that lnc-RNASEH1-AS1 contains a putative binding sitewithobvious complementarity to the seed region of miR-218-5p (Fig. 1C). When wild typelncRNA RNASEH1-AS1 was cotransfected with miR-218-5p mimics or ASO-218-5p, the luciferase activity of NCI-H520 cells was obviously reduced or increased, respectively, compared withtheir corresponding controls (Fig. 1C). However, both overexpression and inhibition of miR-218-5p did not exert an apparent effect on the luciferase intensity of cells transfected with the lnc-RNASEH1-AS1mutant (Fig. 3C).

The level of the RNASEH1-AS1 mRNA was measured using qRT-PCR to assess the effect of miR-218-5p on endogenous lncRNA RNASEH1-AS1 expression.The expression of RNASEH1-AS1 was reduced upon miR-218-5p overexpression (Fig. 1D). Meanwhile, a small interfering RNA (siRNA) for RNASEH1-AS1 was synthesized, and the effect of pSilencer/shRNA-RNASEH1-AS1 was verified using qRT-PCR (Fig. 1E). Knockdown of RNASEH1-AS1 resulted in 2.9-fold increases in the endogenous miR-218-5p level in NCI-H520 cells (Fig. 1F).In contrast to the lncRNA RNASEH1-AS1, miR-218-5p was expressed at lower levels in clinical lung squamous cell carcinoma samples than in adjacent normal tissue samples(Fig. 1G).

miR-218-5p suppresses the proliferation and motility of LUSC cells in vitro, while lncRNA-RNASEH1-AS1 exerts the opposite effects.

We transfected the miR-218-5p overexpression construct and ASO mimic into NCI-H520 cells and performed some functional experiments to explore the effect of miR-218-5p on the malignant phenotype of LUSC cells. In the MTT assay, miR-218-5p obviously reduced the OD value of NCI-H520 cells at 48 and 72 hours after transfection, while its ASO mimicsincreased cell viability at 24, 48 and 72 hours(Fig. 2A). As shown in the colony formation assay, miR-218-5p apparently decreased the colony formation rate of NCI-H520 cells, while its ASO mimics increased the colony formation rate compared to its corresponding control (Fig. 2C). In other words, miR-218-5p inhibited both the viability and the growth of LUSC cells in vitro.

We also conducted transwell invasion and wound-healing assaysusing NCI-H520 cells to investigate the function of miR-218-5p in cell motility. The data revealed that miR-218-5p reduced the number of invasive cells (Fig. 2E) and enlarged the distance of cell gaps at 48 hours (Fig. 2G) compared with the control group, while its ASO mimic exerted the opposite effects (Fig. 2E and 2G). Our study focused on lnc-RNASEH1-AS1 at the same time. Theviability (Fig. 2B), growth (Fig. 2D), migration (Fig. 2G) and invasion (Fig. 2F) of NCI-H520 cells was suppressed after lnc-RNASEH1-AS1 interference. The miR-218-5p ASO

mimics counteracted the inhibitory effect of sh-RNASEH1-AS1 on the growth of LUSC cells (Fig. 2D), but did not result in significance differencesin cell viability, migration and invasion (Fig. 2B, 2F and 2G).

NET1 and POU2F1 were identified as direct targets of miR-218-5p.

We screened the potential downstream targets of miR-218-5p using the TargetScan, miRDB and PicTar databases to determine the mechanism by which miR-218-5p regulates the oncogenesis of lung squamous carcinoma cells (Fig. 3A). Among the overlapping target genes, NET1 and POU2F1 were selected for further investigation.

The 3' UTRs of both NET1 and POU2F1 contain putative sites that are conserved among many species and displayapparent complementarity with the seed region of miR-218-5p, according to the base-pairing rules (Fig. 3B and 3C). The 3'UTRs of NET1 or POU2F1 was co-transfectedwith miR-218-5p into NCI-H520 cells.The transfection of miR-218-5p reduced the fluorescence intensity, while the transfection ofits ASO mimicsincreased the fluorescence intensity in the dual-luciferase reporter assay (Fig. 3B and 3C). In contrast, neither the overexpression nor knockdown of miR-218-5p exerted an obviouseffect on the fluorescence intensity of NCI-H520 cells transfected with constructs containing the 3'UTR of NET1 or POU2F1with the mutated miR-218-5p binding sequence (Fig. 3B and 3C). These results revealed that miR-218-5p directly binds to the 3'-UTR of NET1 and POU2F1.According to the qRT-PCR results, miR-218-5p downregulatedthe expression of the endogenous NET1 and POU2F1 mRNAs in NCI-H520 cells (Fig. 3D). Western blot analysis revealed that miR-218-5papparently reduced the levelsofthe endogenous NET1 and POU2F1 proteins (Fig. 3E and 3F), indicating that miR-218-5p negatively regulates NET1 and POU2F1 expression by binding to their 3'-UTRs.

Wesubsequently examined the expression of the NET1 mRNA in three pairs of clinical lung squamous cell carcinoma samples. As expected, NET1 was expressed at higherlevels in LUSC tissues than in corresponding normal tissues (Fig. 3G), in contrast to miR-218-5p and similar to lnc-RNASEH1-AS1.

Downregulation of lncRNA-RNASEH1-AS1 inhibitsthe growth of tumours formed by LUSC cells in vivo.

We conducted a tumourigenicity assayin nude mice to assess the effect of the lncRNA RNASEH1-AS1 in vivo. Twenty mice were randomly divided into two groups of ten mice per group. NCI-H520 cells transfected with siR-RNASEH1-AS1 or siR-NC were subcutaneously injected into the left back of the nude mice. Figure 4A shows images of the two groups of nude mice at 4 weeks after injection, and figure 4B shows images of the tumour masses after the animals were sacrificed. The tumoursgrew slower in nude micefrom the lnc-RNASEH1-AS1 interference group than the control group (Fig. 4C), and the average tumour volume of the lnc-RNASEH1-AS1 interference group was smaller thanthe control group (Fig.4D). The weight of the tumour mass was lighter than the control group (Fig. 4E).Figure 4F shows the loci oftumours derived from transfected cellsdetected using haematoxylin-eosin (HE)staining and NET1 or POU2F1expression detected using immunohistochemical staining. NET1 and POU2F1 expression were significantly reduced after RNASEH1-AS1 interference, as evidenced by the immunohistochemical staining (Fig. 4F).The expression of miR-218-5p was apparently increased and the expression of the

targets of miR-218-5p were decreased in xenograft tumours after RNASEH1-AS1 interference, as evidenced by the qRT-PCR results (Fig. 4G). Moreover, the protein levels of its targets were also decreased,as determined using westernblotting (Fig. 4H).Based on these data, the downregulation of the lncRNA RNASEH1-AS1 promoted the upregulation of miR-218-5p and downregulation of its target genes and suppressed the growth of lung squamous carcinomacells in vivo.

POU2F1 binds directly to the lncRNA-RNASEH1-AS1 promoter and stimulates its promoter activity.

We constructed a POU2F1 interfering plasmid and validated its efficiency using qRT-PCR to verify whether POU2F1 is a potential transcription factor for RNASEH1-AS1 (Fig. 5A). As shown in figure 5A and 5B, knockdown of POU2F1 effectively reduced the expression and promoter luciferase activity of RNASEH1-AS1. A ChIP assay was conducted with an anti-POU2F1 antibody using NCI-H520 cell lysates, followed by PCR with primers designed toamplify the RNASEH1-AS1 promotor. **The intensity of target signal was higher than the input (Fig. 5C and 5D).** The band indicated the direct interact and positive binding of POU2F1 to the RNASEH1-AS1 promoter. Figure 5E shows a schematic of the regulatory mechanism of the lncRNARNASEH1-AS1/hsa-miR-218-5p/NET1/POU2F1 axis in human lung squamous carcinoma cells.

Discussion

Recently, researchers have discovered that the interaction of the miRNA seed sequence with mRNA is not unidirectional, and the pool of pseudogenes, mRNAs, long non-coding RNAs (lncRNAs), circular RNAs (circRNAs) compete for the same pool of miRNAs[28, 29]. These competitive endogenous RNAs (ceRNAs) serve as molecular sponges for miRNAs by interacting with their miRNA binding sites, consequently de-repressing all targets of the corresponding miRNA family. An increasing number of researchers have paid close attention to lncRNAs as competing endogenous RNAs for miRNAs during tumourigenesis and progression in recent years. Numerous lncRNAs are abnormally expressed or mutated in many types of cancers [30]. HULC was upregulated in both tumours and plasma from patients with hepatocellular carcinoma and was a possible biomarker for HCC [31].Abnormal X chromosome inactivation caused by aberrantly expressed XIST promotes carcinogenesis in leukaemia[32]. PCA3 is over-expressed in ninety-five percent of prostate cancer clinical samples and is a marker with high specificity in the urine of patients with benign and malignant prostate cancer [33, 34]. In ourstudy, we firstdiscovered that the lncRNARNASEH1-AS1 was expressed at high levels in 526 lung cancer samples compared to 59 normal tissue samples by screening StarBase and was expressed at higher in LUSC tissues than in the adjacent normal tissues. Moreover, the viability, proliferation, invasion and migration of NCI-H520 cells were reduced after RNASEH1-AS1 interference, suggesting the oncogenic character of RNASEH1-AS1 in LUSC. We observed the same oncogenic effect of RNASEH1-AS1 on LUSC in the in vivo xenograft animal model and in vitro cell experiment.

Our study also discovered that RNASEH1-AS1 functions as a ceRNA by directly interacting with the seed region of miR-218-5p in LUSC samples. The expression of miR-218-5p in LUSC cells was suppressed due to the upregulation of RNASEH1-AS1. In contrast to RNASEH1-AS1, miR-218-5p inhibited LUSC cell

growth and motility and functioned as a tumour suppressor gene in lung squamous carcinoma. According to a recent study by Yu et al[35],circRNA-104718 functions as a ceRNA and promotes HCC progression by regulating the microRNA-218-5p-TXNDC5 signalling pathway. Li et al[36]found that oncogenic KSHV-encoded interferon regulatory factor upregulates HMGB2 and CMPK1 to promote cell invasion by disrupting the lncRNA-OIP5-AS1-miR-218-5p network. Ye et al[37] also discovered that the E2F1-mediated MNX1-AS1-miR-218-5p-SEC61A1 feedback loop is involved in the progression of colon adenocarcinoma. Our results expand the recognition of miR-218-5p and its competing endogenous RNA as playing a role in the development and progression of LUSC.

Half of all genes in the genome are estimated to be targets of miRNAs, spanning a large regulatory mechanismat the post-transcriptional level [11]. We identified both NET1 and POU2F1 as direct targets of miR-218-5p. Importantly, miR-218-5p negatively regulated NET1 and POU2F1 expression by directly binding to their 3'-UTRsin LUSC cells. The levels of the NET1 and POU2F1 mRNAs and proteins were decreased in xenograft tumour tissues after RNASEH1-AS1 interference. In other words, downregulation of the lncRNA RNASEH1-AS1 led to the upregulation of miR-218-5p, thereby resulting in the downregulation of its target genes. POU2F1 is also regulated by miR-665 and miR-9-5p in human osteosarcoma[38, 39], and by miR-449 in liver cancer [40]. Our study provides the first evidence that identified POU2F1 and NET1 as the downstream molecules of miR-218-5p and RNASEH1-AS1 in lung squamous cell carcinoma. The RhoA activating protein NET1 makes fundamental contributions to mammary gland tumorigenesis and metastasis [41]. NET1 expression was strongly associated with the patients' pathological characteristics, including clinical stage, lymph node metastasis, distant metastasis and differentiation degree and had a significant role in the tumorigenesis of human non small cell lung cancer [27]. However, the influence of NET1 on the malignant phenotype of lung squamous carcinoma cells have not been discovered in any reports before and needs to be studied in the future.

More importantly, our data revealed that POU2F1 interference attenuates the activity of the RNASEH1-AS1 promoter in LUSC cells. The direct binding of POU2F1 to the RNASEH1-AS1 promoter was validated by the results of the chromatin immunoprecipitation assay. POU2F1 not only represents a downstream target gene of miR-218-5p but also the upstream transcription factor for RNASEH1-AS1.

Conclusion

Taken together, RNASEH1-AS1 functions as an oncogene by serving as a molecular sponge for miR-218-5p. The downregulation of miR-218-5p releases the suppression of NET1 and POU2F1. POU2F1 binds directly to the RNASEH1-AS1promoter to induce its expression, thereby forming a closedlncRNA-miRNA-GENE-TF regulatory loop in LUSC. These findings expand our understanding of the positive feedback loop of RNASEH1-AS1/hsa-miR-218-5p/NET1/POU2F1 and the mechanism underlying the genesis and progression of human lung squamous carcinoma, possibly providing new biomarkers for its diagnosis and treatment.

Abbreviations

ceRNAs: Competing endogenous RNAs

miRNA: MicroRNA

LncRNA: Long non-coding RNA

CircRNA: Circular RNA

NSCLC: Non-small cell lung cancer

LUSC: Lung squamous cell carcinoma

LUAD: Lung adenocarcinoma

RNASEH1-AS1: RNASEH1 antisense RNA 1

POU2F1: POU class 2 homeobox 1

NET1: Neuroepithelial cell transforming 1

Declarations

Ethical approval and consent to participate

All studies were performed according to the American Association for the Accreditation of Laboratory Animal Care guidelines and adhered to national and international standards. Prior to obtaining thetissue specimens, informed consent was obtained from all subjects or their direct relatives.All cell and tissue studies were submitted to and approved by both the Ethics Committee of North China University of Science and Technology and the Ethics Committee of Hebei Medical University. All animal experiments were submitted to and approved by the Ethics Committee of Hebei Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Competing interests

All authors have no conflicts of interest to declare.

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Authors' contributions

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Tables

Table 1. Clinical Tissue Samples Used in This Study.

Number	Gender	Classification	Age	Date
1	Male	LUSC	63	2019.2.26
2	Male	LUSC	50	2019.3.6
3	Male	LUSC	62	2019.4.29

Table 2. The PCR Primers Used in This Study.

Primer Name	Primer Sequence (5′ →3′)
hsa-miR-218-5p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACACATGGTTAG
hsa-miR-218-5p-Fwd	TGCGGTTGTGCTTGATC
U6 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATGGAAC
U6 Forward	TGCGGGTGCTCGCTTCGGCAGC
Reverse	CCAGTGCAGGGTCCGAGGT
RNASEH1-AS1-qPCR-S	GAGAAGCACCCGCACCTGG
RNASEH1-AS1-qPCR-AS	GCCTCTAATCCCAACACT
NET1-qPCR-S	GAAAGGTGAATCCGAGTG
NET1-qPCR-AS	GTGCCGTTCGTTCCGTGT
POU2F1-qPCR-S	GTAACCGCCGCCAGAAAG
POU2F1-qPCR-AS	TGGAGGCTGAGGCAGAAGG
B-actin-qPCR-S	CGTGACATTAAGGAGAAGCTG
B-actin-qPCR-AS	CTAGAAGCATTTGCGGTGGAC
RNASEH1-AS1-Chip-S	TCTGAGGGAGACTGATTC
RNASEH1-AS1-Chip-AS	ACAGGTGTAGAGTCTGACGTGC
RNASEH1-AS1-target-Top	AAACTAGGAATTCATGAATTAGAATAAGCACAAAGGAGGGCGAGAGGCT
RNASEH1-AS1-target-Bot	CTAGAGCCTCTCGCCCTCCTTTGTGCTTATTCTAATTCATGAATTCCTAGTTT
RNASEH1-AS1-target-mut-Top	AAACTAGGAATTCATGAATTAGAATCGTACTGCAGGAGGGCGAGAGGCT

RNASEH1-AS1-target-mut-Bot	CTAGAGCCTCTCGCCCTCCTGCAGTACGATTCTAATTCATGAATTCCTAGTTT
NET1-3'UTR-Top	AAACTAGGAATGATACTATTAAAAAAAAAAAAAAGCACACACATAATCACCCTGCT
NET1-3'UTR-Bot	CTAGAGCAGGGTGATTATGTGTGTGCTTTTTTTTTTTTAATAGTATCATTCCTAGTTT
NET1-3'UTR-mut-Top	AAACTAGGAATGATACTATTAAAAAAAAAAACGTACTGCACATAATCACCCTGCT
NET1-3'UTR-mut-Bot	CTAGAGCAGGGTGATTATGTGCAGTACGTTTTTTTTTTTAATAGTATCATTCCTAGTTT
POU2F1-3'UTR-Top	AAACTAGGTTGGGGGAAAAAAAAGCACAACTATACCTCTTTAATGTTATTTTCCT
POU2F1-3'UTR-Bot	CTAGAGGAAAATAACATTAAAGAGGTATAGTTGTGCTTTTTTTTCCCCCAACCTAGTTT
POU2F1-3'UTR-mut-Top	AAACTAGGTTGGGGGAAAAAACGTACTGCCTATACCTCTTTAATGTTATTTTCCT
POU2F1-3'UTR-mut-Bot	CTAGAGGAAAATAACATTAAAGAGGTATAGGCAGTACGTTTTTTCCCCCAACCTAGTTT
sh-POU2F1-Top	GATCCCCAGTCAACACCAAAGCGAATCTCGAGATTCGCTTTGGTGTTGACTGGTTTTTGA
sh-POU2F1-Bot	AGCTTCAAAAACCAGTCAACACCAAAGCGAATCTCGAGATTCGCTTTGGTGTTGACTGGG
Sh1-RNASEH1-AS1-top	GATCCGAGAAGCACCCGCACCTGGAGCTCGAGCTCCAGGTGCGGGTGCTTCTCTTTTTGA
Sh1-RNASEH1-AS1-bot	AGCTTCAAAAAGAGAAGCACCCGCACCTGGAGCTCGAGCTCCAGGTGCGGGTGCTTCTCG

Figures
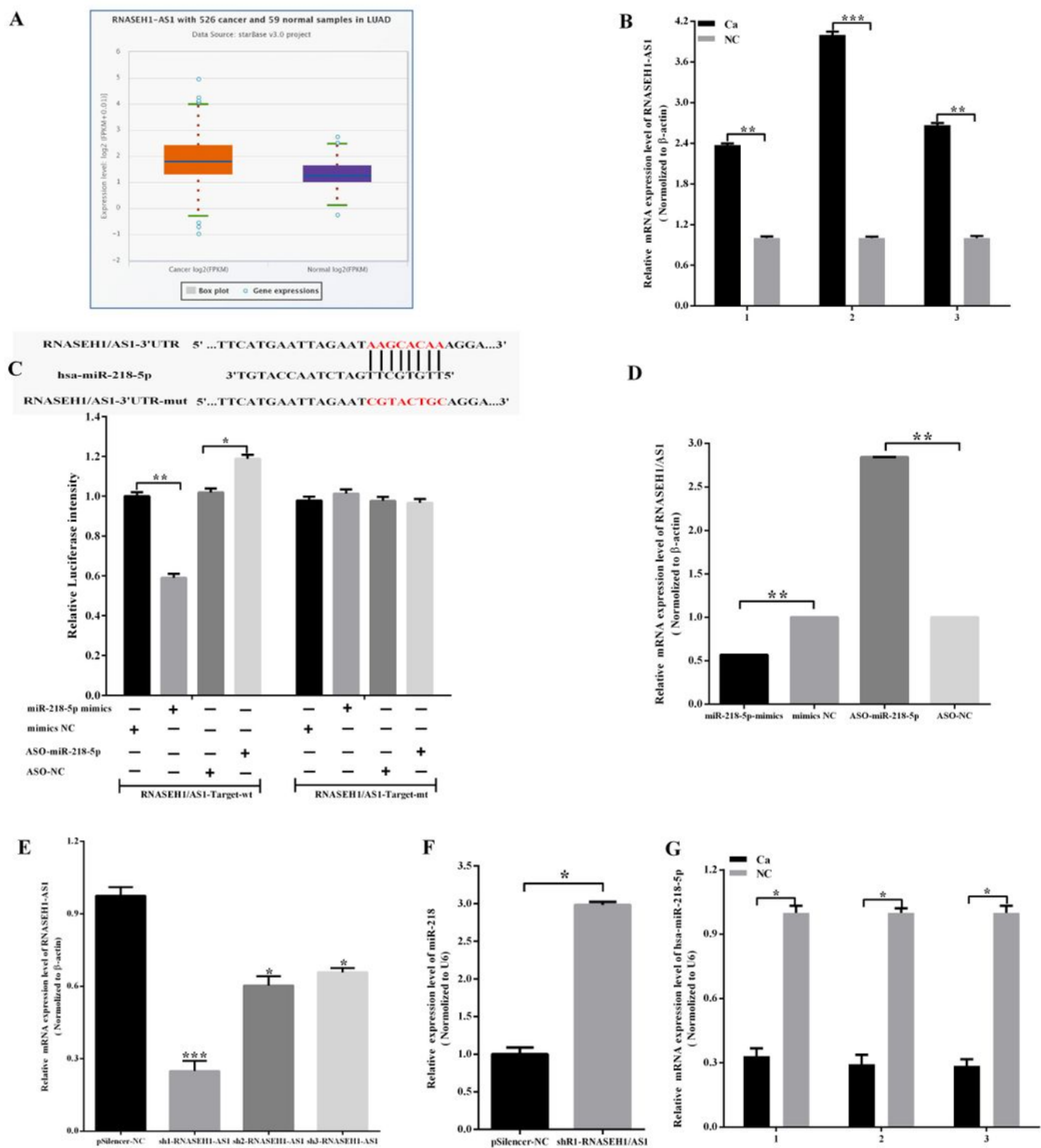
Figure 1

The lncRNA RNASEH1-AS1 is ceRNA for miR-218-5p. (A) RNASEH1-AS1 expression in cancer and normal tissue samples determined by screening StarBase V3.0. RNASEH1-AS1 was obviously upregulated in cancer samples compared to normal tissue samples (p◻0.05). (B) RNASEH1-AS1 expression in three pairs of clinical tissue samples analysed using qRT-PCR. The relative foldchange in RNASEH1-AS1 expression was calculated using β-actin as the internal control. The Y axis presents the means±S.D. of three separate

experiments. * p⎕0.05, ** p⎕0.01, and ***p⎕0.001. (C)The direct interaction between RNASEH1-AS1 and miR-218-5p was confirmed using a luciferase report assay. (D) The effect of miR-218-5p on the level of the endogenous lncRNA RNASEH1-AS1 determined using qRT-PCR. (E) The interfering effect of sh-RNASEH1-AS1 plasmid. (F) The effect of the lncRNA RNASEH1-AS1 on the endogenous miR-218-5p level determined using qRT-PCR. (G) Expression of miR-218-5p in three pairs of clinical tissue samples measured using qRT-PCR.
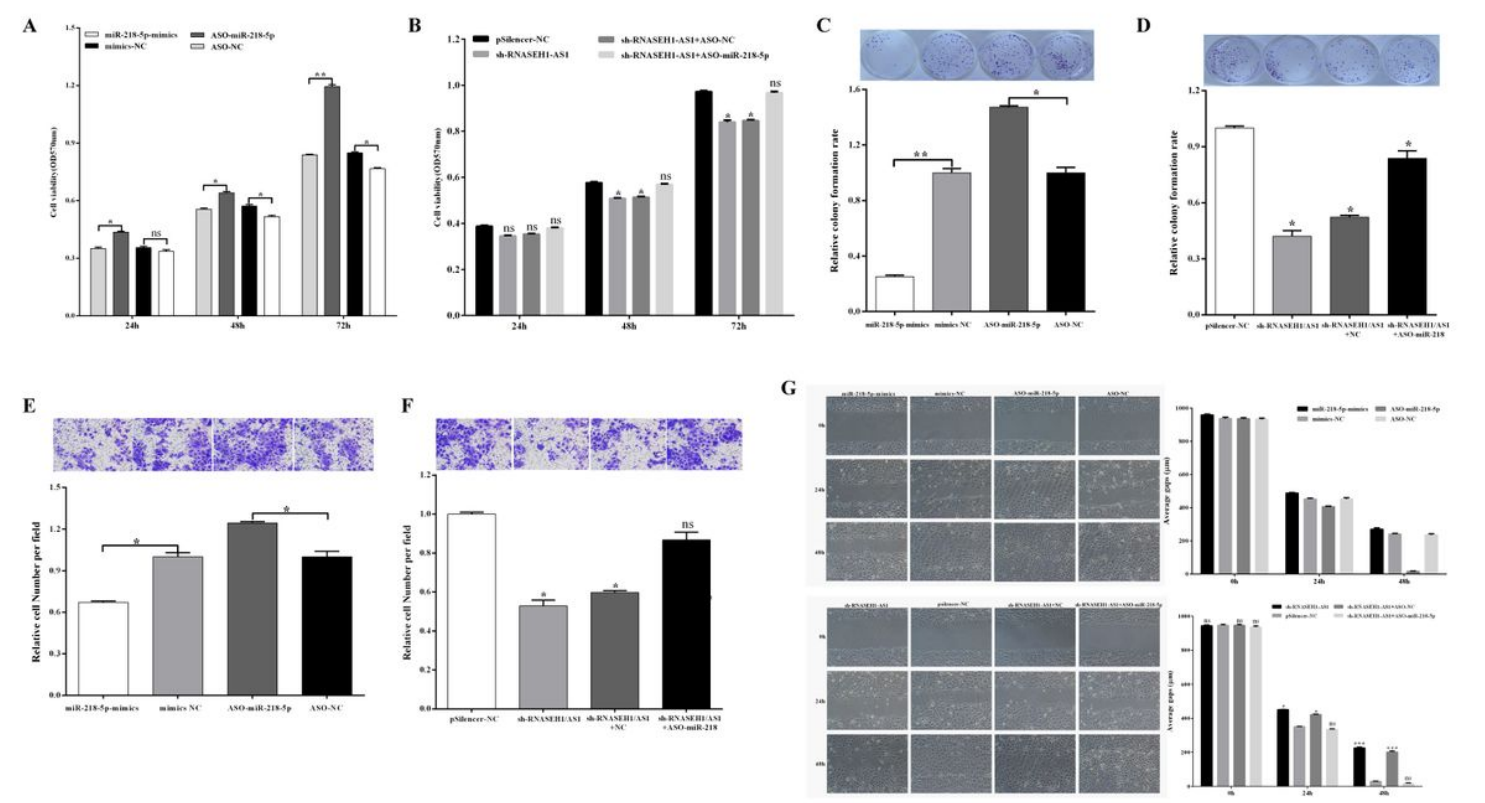
Figure 2

The functions of miR-218-5p and RNASEH1-AS1 in LUSC cells. (A and B) Results of the MTT assay performed in NCI-H520 cells. (C and D) Results of the colony formation assay performed in NCI-H520 cells. (E and F) Results of the transwell invasion assay conducted with NCI-H520 cells. (G) Results of the wound-healing assay performed using NCI-H520 cells.
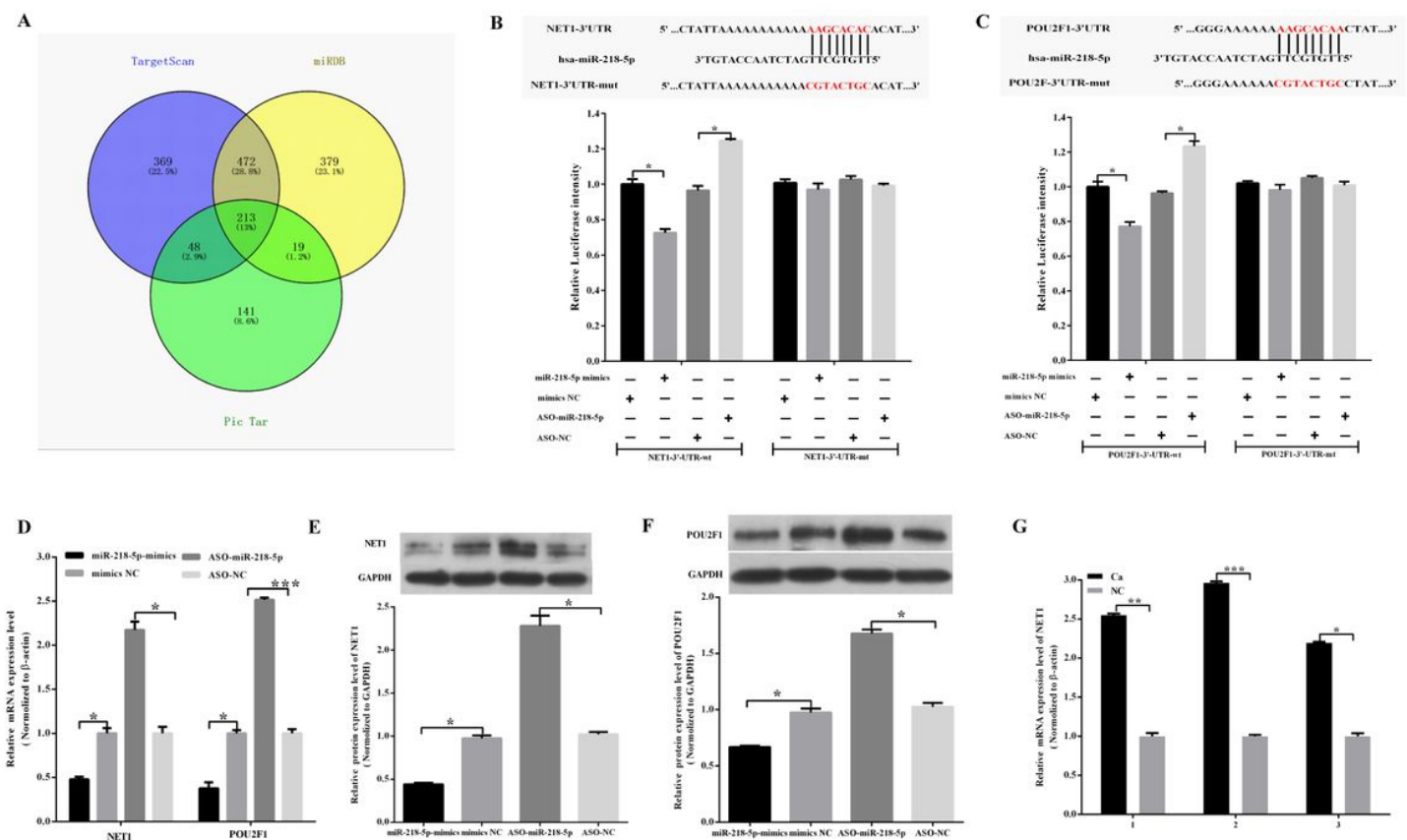
Figure 3

miR-218-5p directly targets NET1 and POU2F1. (A) Targets predicted by Target Scan, miRDB and PicTar. (B) The direct interaction between miR-218-5p and NET1 was confirmed by conducting a luciferase reporter assay. (C) The direct interaction between miR-218-5p and POU2F1 was confirmed by performing a luciferase reporter assay. (D) The effect of miR-218-5p on the expression of the endogenous NET1 and POU2F1 mRNAswas determined using qRT-PCR. (E and F) The effect of miR-218-5p on levels of the endogenous NET1 and POU2F1 proteinswere determined using western blotting. The full-length blots are presented in Supplementary Figure S1 and Supplementary Figure S2. (G) NET1 expression in three pairs of clinical tissue samples measured using qRT-PCR.
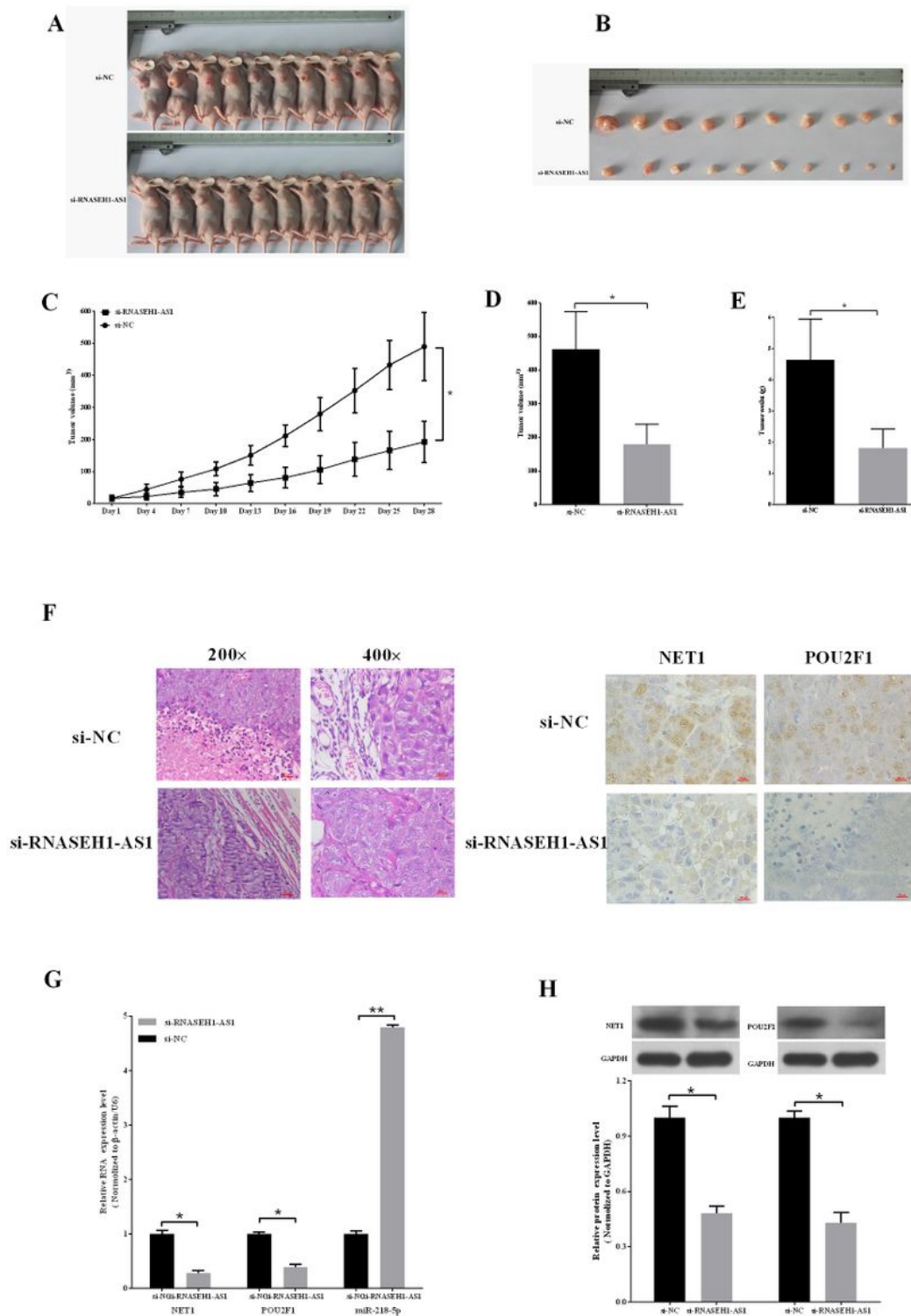
Figure 4

The tumour-promoting effect of RNASEH1-AS1 in the in vivo tumourigenicity assay. (A and B) The tumour mass generated from transfected NCI-H520 cells. (C) The tumour volume was measured every three days. (D and E) The average tumour volume and tumour weight were measured. (F) H-E staining reveals the interface of the metastatic tumour and the adjacent liver tissue. Bar, 200 μm. Immunohistochemical staining showing NET1 and POU2F1 expression in xenograft tumours. (G) The NET1, POU2F1 and miR-

218-5p mRNA levels in xenograft tumoursdetected using qRT-PCR. (H) western blots showing levels of the NET1 and POU2F1 proteinsin xenograft The full-length blots are presented in Supplementary Figure S3.
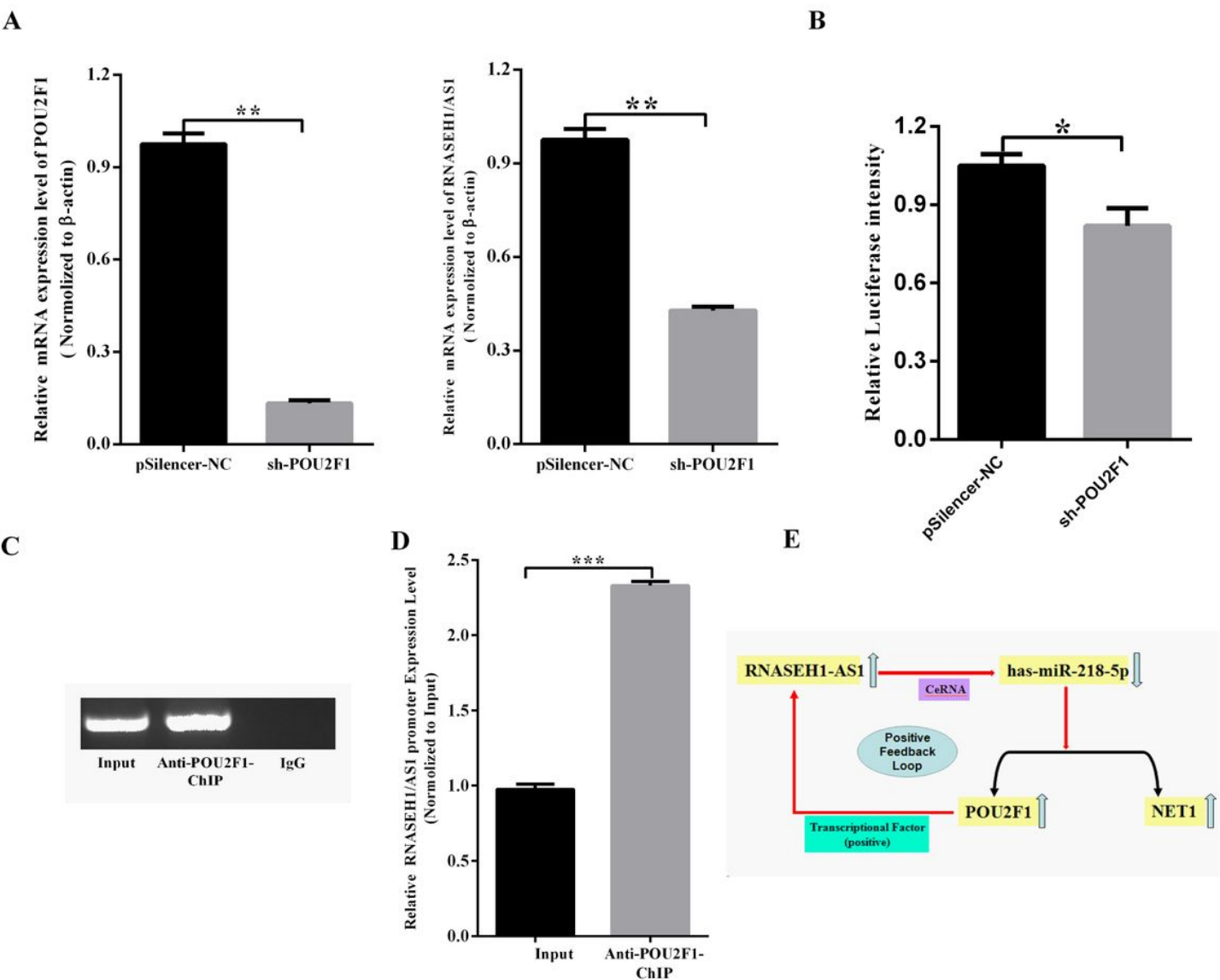
Figure 5

POU2F1is a transcription factor for RNASEH1-AS1. (A) The interfering effect of the sh-POU2F1 plasmid. Decreased level of RNASEH1-AS1 after POU2F1 knockdown. (B) Decreased activity of the RNASEH1-AS1 promoter after knockdown of POU2F1. (C and D) The positive relationship between POU2F1 and RNASEH1-AS1 expression in the CHIP assay. The quantity of the input group was defined as 1, and the fold change (2.30) of the anti-POU2F1-ChIP group was calculated. The full-length blots are presented in Supplementary Figure S4. (E) Summary of the results from this study.