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Original Article

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LINC00891 regulated by miR-128-3p/GATA2 axis impedes lung cancer cell proliferation, invasion and EMT by inhibiting RhoA pathway

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Abstract

Long non-coding RNA (lncRNA) LINC00891 knockdown is associated with poor prognosis of lung adenocarcinoma, but the underlying mechanism remains to be further explored. Here, we found that LINC00891 expression is downregulated in lung cancer tissues and cell lines compared with that in adjacent normal tissues and normal lung epithelial cells. LINC00891 overexpression impedes cell proliferation, invasion, migration and epithelial-to-mesenchymal transition (EMT) process in lung cancer cells. Mechanistic research showed that GATA2 directly binds to LINC00891 promoter and transcriptionally regulates LINC00891 expression. Meanwhile, GATA2 was identified as a target of miR-128-3p, and it is negatively regulated by miR-128-3p. Moreover, overexpression of GATA2 suppresses lung cancer cell proliferation, invasion, migration, and EMT process. Furthermore, LINC00891 restrains the RhoA pathway activity, and treatment with CCG-1423 (a specific RhoA pathway inhibitor) antagonizes the promoting effect of LINC00891 knockdown on cell malignant behaviors. Additionally, silencing of LINC00891 promotes xenograft tumor growth, which can be reversed by administration with CCG-1423. In summary, LINC00891 regulated by the miR-128-3p/GATA2 axis restrains lung cancer cell malignant progression and hinders xenograft tumor growth by suppressing the RhoA pathway.

Key words LINC00891, miR-128-3p/GATA2 axis, RhoA pathway, lung cancer

Introduction

Lung cancer is the second most commonly diagnosed cancer in 2020 and the leading cause of cancer deaths, accounting for about 10% of cancers diagnosed and 5% of deaths [1]. Non-small cell lung cancer (NSCLC) accounts for the majority of diagnosed lung cancer cases with unacceptable high mortality rate [2,3]. The main cause of high mortality of lung cancer is the difficulty in early diagnosis, and the 5-year survival in populations with lung cancer varies from 4% to 17% [4]. Therefore, finding the biological indicators and effective targets for early diagnosis of lung cancer is of great significance for the early diagnosis, treatment and prognosis.

Long non-coding RNAs (lncRNAs) are a class of RNAs with a length greater than 200 nt, which are located in the nucleus or cytoplasm and not involved in protein coding [5]. A previous study indicated that the expression of lncRNAs is imbalanced in various tumors and shows a certain tissue specificity [6]. LncRNAs can promote the proliferation, invasion and metastasis of tumor cells, and play important roles in the occurrence and development of tumors [7,8]. It was reported that lncRNA LINC00891 is specifically down-regulated in endometrial cancer [9]. Moreover, low expression of LINC00891 indicates a poor prognosis for patients with lung adenocarcinoma (LUAD) [10]. However, the further regulation mechanism that LINC00891 involved in lung cancer is still obscure.

GATA2 is a member of the GATA family of zinc finger proteins that act as transcriptional activators or repressors of multiple genes to regulate various cellular processes during development

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and carcinogenesis [11]. GATA2 is considered as the main regulator of hematopoietic stem cells and progenitor cells, and its role in hematopoietic system development and malignant tumors has been fully established [12]. Moreover, GATA2 is identified as one of the genes for epigenetic silencing in non-small cell lung cancer (NSCLC) cells [13]. A previous study showed that the expression of GATA2 is markedly reduced in lung cancer [13], while its specific role in lung cancer remains to be further explored.

miRNAs are small, non-coding RNA molecules of approximately 19–25 nucleotides in length [14]. It has been reported that miRNAs involved in the proliferation, migration, invasion and drug resistance of lung cancer cells [15–17]. It was reported that miR-128-3p can act as a key regulator of the malignant phenotype of lung cancer cells, and it promotes epithelial plasticity through the regulation of mesenchymal gene expression [18].

RhoA is a core member of the Rho GTPase family and a Rho GTP-binding protein of the Ras super-family [19]. RhoA plays important roles in many key cellular activities, including morphological changes, chemotaxis, and motility [19]. Liu *et al.* [20] demonstrated that Wnt5a promotes gastric cancer cell migration through RhoA activation. Moreover, a Rho kinase inhibitor fasudil can suppress cell proliferation, invasion, adhesion and migration, and induce apoptosis in lung cancer cells. In addition, RhoA overexpression suppresses apoptosis and promotes proliferation of lung cancer cells [21], revealing its important role in lung cancer progression.

In this study, we found that LINC00891 is downregulated in lung cancer tissues and cell lines, and LINC00891 overexpression suppresses lung cancer cell malignant progression. Mechanistic research showed that LINC00891 is regulated by miR-128-3p/GATA2 axis, and the tumor-suppressing effect of LINC00891 might be achieved by regulating the RhoA pathway.

Materials and Methods

Tissue sample collection

The paired tumorous and adjacent non-tumorous human lung tissues (more than 2 cm from the edge of the tumor) were obtained from 35 patients admitted to the First Affiliated Hospital of Xi'an Jiaotong University from 2019 to 2020. Inclusion criteria: (1) patients who were diagnosed as non-small cell lung cancer (NSCLC) at stage I and stage II by histopathological evaluation and reviewed by experienced pathologists; (2) patients and their family members understood the entire experimental protocol and signed informed consent. Exclusion criteria: (1) patients with diseases other than NSCLC; (2) patients who received preoperative chemotherapy, radiotherapy, targeted therapy, or other anti-cancer therapy prior to admission. This study was approved by the Ethics Committee of Xi'an Jiaotong University, and adhered to the Ethical Guidelines of the Declaration of Helsinki. All patients signed informed consent forms before surgery.

Cell culture

BEAS-2B, A549 (EGFR wild type/KRAS mutant type), H1975 (EGFR mutant type/KRAS wild type), H1650 (EGFR mutant type/KRAS wild type), H460 (EGFR wild type/KRAS mutant type), and HEK293T cell lines were purchased from the American Type Culture Collection (Manassas, USA). Cells were cultured in DMEM medium (Gibco, Rockville, USA) supplemented with 10% fetal bovine serum (Gibco) and 100 U/mL penicillin under an atmosphere containing 5% CO_2 at 37°C.

Cell transfection

siRNAs against LINC00891, GATA2 and negative control (NC) siRNA were purchased from GenePharma (Shanghai, China). miR-128-3p mimic, miR-128-3p inhibitor, NC mimic and NC inhibitor were synthesized by Invitrogen (Carlsbad, USA). Lentiviral-mediated LINC00891 short hairpin RNA (shRNA) and NC shRNA vectors were obtained from Genechem (Shanghai, China). Full-length cDNA of LINC00891 and full-length DNA coding sequence of GATA2 were inserted into the pcDNA3.1 empty vector. All transfections were performed by using Lipofectamine® 3000 (Thermo, Waltham, USA) according to the manufacturer's instructions. The sequences used in this study are as follows: LINC00891 siRNA: 5′-AGUGGAUGUUUGUGGUCUAAC-3′; GATA2 siRNA: 5′-UUCUUGGA CUUGUUGGACAUCUUCC-3′; NC siRNA: 5′-AAUUCUCCGAACGU GUCACGU-3′; LINC00891 shRNA: 5′-GAAGAATCATGGAAGCTTA-3′; miR-128-3p mimic: 5′-UCACAGUGAACCGGUCUCUUU-3′; NC mimic: 5′-UUCUCCGAACGUGUCACGU-3′; miR-128-3p inhibitor: 5′-AAAGAGACCGGUUCACUGUGA-3′; NC inhibitor: 5′- GGCUCAUCG AUAGUCAUUA-3′.

RT-qPCR

Total RNA in cells and tissue samples was extracted by using TRIZOL reagent (Thermo). Superscript III reverse transcriptase (Thermo) was used to synthesize cDNA templates. RT-qPCR was carried out using a SYBR ExScript qPCR kit (Takara, Dalian, China) under the following conditions: pre-denaturation at 95°C for 2 min, degeneration at 94°C for 15 s, annealing at 55°C for 25 s, and extension at 72°C for 15 s with 35 cycles. *GAPDH* was used as the internal reference for GATA2 and LINC00891, and *U6* was used as the internal reference for miR-128-3p. The relative expression of the candidate genes was calculated according to the $2^{-\Delta\Delta CT}$ method. Primer sequences are as follows: *U6* forward 5′-CTCGCTTCGGC AGCACA-3′, and reverse 5′-AACGCTTCACGAATTTGCGT-3′; *GAPDH* forward 5′-AAGAAGGTGGCCTGCGCAGGC-3′, and reverse 5′-TCCACCACCCGGTTGTTGCGC-3′; *miR-128-3p* forward 5′-GCC GGCGCCCGAGCTCTGGCTC-3′, and reverse 5′-TCACAGTGAACC GGTCTCTTT-3′; *LINC00891* forward 5′-AAGGCACCTGACATCA CCTG-3′, and reverse 5′-GGGTCATGAGACACCTGTGG-3′;

Western blot analysis

Total protein from cells and tissue samples was extracted by using RIPA lysis buffer (Beyotime Biotechnology, Nantong, China). The protein concentrations were determined by using the Pierce BCA Protein Assay Kit (Thermo). The samples (30 μg for each sample) were separated by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. After being blocked with 5% skimmed milk, the membranes were incubated at 4°C overnight with primary antibodies including: anti-E-cadherin (ab231303, 1:1000; Abcam, Cambridge, UK), anti-Vimentin (ab137321, 1:1500; Abcam), anti-Snail (ab31787, 1:1000; Abcam), anti-Slug (ab27568, 1:1000; Abcam), anti-GATA2 (ab109241, 1:1000; Abcam), anti-β-actin (ab8224, 1:2000; Abcam). Next, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Enhanced chemiluminescence was used to visualize protein bands. Lab Works 4.5 was used to quantitatively analyze the integrated

optical density.

RhoA activation assay

RhoA activity was assessed using RhoA Activation Assay Kit (Merck Millipore, Billerica, USA) according to the manufacturer's instructions. RhoA activity is increased due to its binding with GTP, while the hydrolysis of GTP to GDP can make it inactivated. In brief, cells were washed with PBS and lysed with lysis buffer. Samples were incubated at 4°C together with 100 μL of Rhotekin agarose beads for 1 h to precipitate the GTP-bound RhoA. The agarose beads were washed and resuspended in 25 μL of 2 × Laemmli reducing sample buffer and boiled for 5 min. The activated RhoA was detected by western blot analysis. The relative RhoA activity was expressed by the relative protein expression level of RhoA-GTP.

Luciferase reporter gene assay

Full-length DNA coding sequence of GATA2 was inserted into pGL3-basic vector (Promega, Madison, USA), and then three vectors containing different inserts cloned from LINC00891 were constructed downstream GATA2. HEK293T cells were transfected with pGL3-GATA2-LINC00891 report vectors. The wild-type and mutant miR-128-3p putative targets on GATA2 3′UTR were cloned into pGL3-basic vector. HEK293T cells were transfected with miR-128-3p mimic and wild-type or mutant GATA2 report vectors. Luciferase activity was measured by using a Dual-Luciferase reporter system (Promega, Madison, USA) according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using ChIP-IT Expression Chromatin Immunoprecipitation Kit (Active motif, Carlsbad, USA). It mainly included the following steps: (1) formaldehyde fixation; (2) separation and ultrasonic chromatin; (3) immunoprecipitation; and (4) elution and chromatin cross-linking. Twenty percent chromatin that was not incubated with the antibody was used as the input. Finally, semi-quantitative PCR analysis was performed.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8) assay was performed to detect cell proliferation. Cells were seeded into 96-well plates. A total of 10 μL of CCK-8 solution (Dojindo, Tokyo, Japan) was added into each well and incubated for 2 h at 37°C with 5% CO_2. The absorbance was measured at 450 nm with an automatic microplate reader (Bio-Rad, Hercules, USA).

Transwell assay

Cell invasion was detected using 24-well Transwell chambers with 8-μm-pore size membrane (Corning Incorporated, Corning, USA). Cells (2×10^5) were suspended in 500 μL of serum-free RPMI 1640 medium, and the cell suspension was transferred to the upper chamber, which was pre-coated with Matrigel for 30 min. The lower chamber was filled with RPMI-1640 medium containing 10% FBS. After 24 h, cells on the lower surface of the membrane were stained with 0.5% crystal violet (Sigma, St. Louis, USA) at 25°C for 20 min, and counted under a microscope. For the migration assay, the transwell membranes were not coated with Matrigel, and the other steps were the same as those of the invasion assay.

Tumor xenograft assay

Nude mice (8 weeks old) were obtained from SLAC Laboratory Animal Center (Shanghai, China) and fed in specific-pathogen-free (SPF) condition. A total of 2×10^6 A549 cells infected with Lv-sh-NC ($n = 6$) or Lv-sh-LINC00891 ($n = 12$) were subcutaneously injected into nude mice. Next, six mice were randomly selected from the Lv-sh-LINC00891 treatment group and intraperitoneally injected with CCG-1428 (0.15 mg/kg) once a day until day 28. When the tumor volume reached about 100 mm^3 (day 7), the tumor size was measured with calipers every 7 days, and the following formula was used to calculate tumor volumes: $v = 0.5 \times (\text{length} \times \text{width}^2)$. After 28 days, the tumors were excised and evaluated. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals established by United States National Institutes of Health (Bethesda, USA).

Statistical analysis

Data were presented as the mean ± standard deviation (SD) and were processed using SPSS 22.0. Comparisons between the two groups were assessed by using Student's *t*-test. ANOVA was used to evaluate the significance of the differences in mean values within and among multiple groups. $P < 0.05$ was considered to be statistically significant.

Results

LINC00891 is downregulated in lung cancer tissues and cell lines

The expression of LINC00891 in 35 pairs of lung cancer tissues and adjacent normal tissues was determined by RT-qPCR. The results showed that LINC00891 expression was decreased in lung cancer tissues compared with that in paracancerous normal tissues (Figure 1A). Similarly, lower LINC00891 expression was observed in lung cancer cell lines (A549, H1975, H1650 and H460) than in normal lung epithelial cells (BEAS-2B) (Figure 1B).

LINC00891 inhibits cell proliferation, invasion, migration, and EMT in lung cancer cells

To explore the role of LINC00891 in lung cancer progerssion, KRAS-mutated lung cancer cell lines A549 and H460 were transfected with LINC00891 overexpression vector (pcDNA-LINC00891), LINC00891 siRNA or respective negative controls. The expression of LINC00891 was observably upregulated after transfection with pcDNA-LINC00891 and significantly reduced after transfection with LINC00891 siRNA (Figure 2A). The results of CCK-8 and transwell assays showed that LINC00891 overexpression decreased the proliferative, invasive and migratory capacities of A549 and H460 cells, whereas LINC00891 knockdown showed an opposite effect

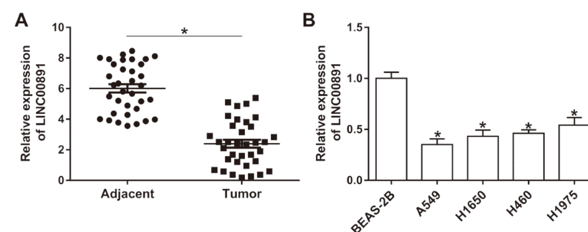

Figure 1. LINC00891 is down-regulated in lung cancer cells and tissues (A) LINC00891 expression in lung cancer tissues and paracancerous normal tissues was detected by RT-qPCR ($n = 35$ in each group). (B) LINC00891 expression in lung cancer cell lines (A549, H1975, SPCA1 and H226) and normal lung epithelial cells (BEAS-2B) was measured by RT-qPCR. $n = 5$ in each group. $^*P < 0.05$, $^{**}P < 0.01$.

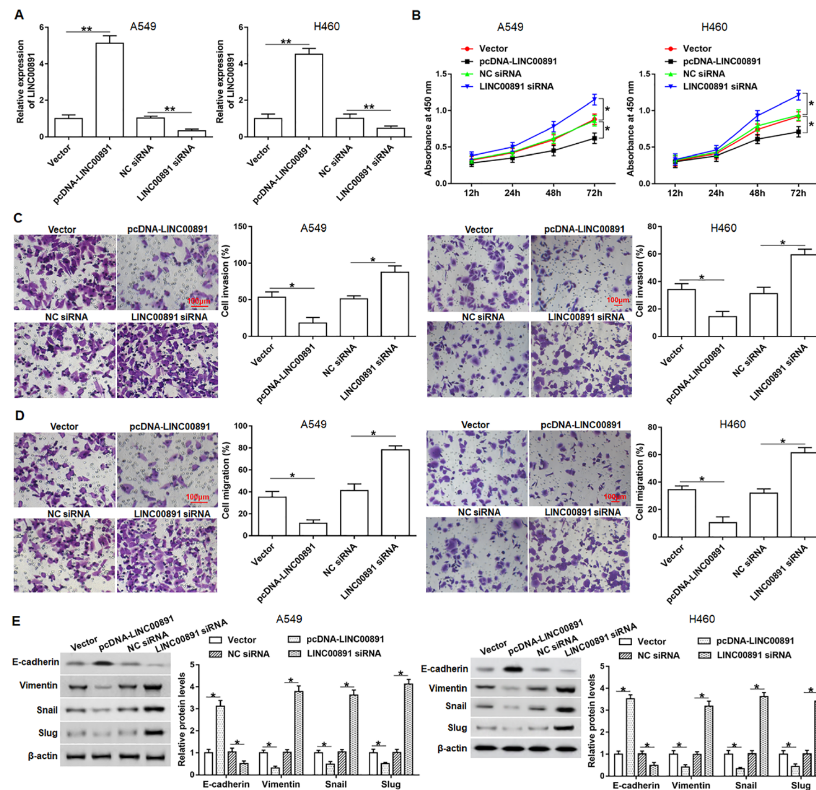

Figure 2. LINC00891 suppresses cell proliferation, invasion, migration, and EMT in A549 and H460 cells A549 and H460 cells were transfected with LINC00891 overexpression vector (pcDNA-LINC00891), LINC00891 siRNA or respective negative controls. (A) LINC00891 expression was detected by RT-qPCR. (B) Cell proliferation was detected by CCK-8 assay. (C, D) Cell invasion and migration were analyzed by Transwell assay. (E) Western blot analysis was used to determine the protein levels of E-cadherin, Vimentin, Snail and Slug. $n=5$ in each group, $^{*}P<0.05$, $^{**}P<0.01$.

(Figure 2B–D). It was also found that the expression of E-cadherin, an epithelial marker, was increased significantly, while the expressions of Vimentin, Snail and Slug which are interstitial markers were observably decreased after LINC00891 overexpression (Figure 2E). Meanwhile, LINC00891 knockdown downregulated E-cadherin expression and upregulated the expressions of Vimentin, Snail and Slug (Figure 2E). In addition, we explored the effect of LINC00891 on the behaviors of an EGFR-mutated lung cancer cell line, H1975. The results showed that LINC00891 expression was observably upregulated after transfection with pcDNA-LINC00891 and decreased after transfection with LINC00891 siRNA (Figure 3A). LINC00891 overexpression suppressed cell proliferation, invasion and migration, as well as the expressions of Vimentin, Snail and Slug, but increased the expression of E-cadherin (Figure 3B–E). While LINC00891 knockdown facilitated cell proliferation, invasion and migration, as well as the EMT process (Figure 3B–E). These data indicated that LINC00891 suppressed the malignant behaviors of lung cancer cells by inhibiting cell proliferation, migration, invasion and EMT process.

GATA2 directly binds to the LINC00891 promoter

In order to investigate the upstream regulatory factors of LINC00891, we analyzed the promoter region of LINC00891 by using ChIPBase database, and found a potential binding site between LINC00891 and GATA2. The potential binding region locates at 115 bp upstream of the transcription initiation site of LINC00891 (Figure 4A). Next, we constructed three luciferase reporter vectors, which included GATA2 sequence together with different truncated sequences of LINC00891 promoter. Both luciferase activities of the reporters containing only the binding region and full-length sequence of the LINC00891 promoter were notably increased compared with the control (Figure 4B). Next, ChIP assay was performed to confirm the binding relationship between LINC00891 and GATA2. As shown in Figure 4C, LINC00891 promoter was markedly enriched in the anti-GATA2 antibody-precipitated complex compared to the IgG group. Next, to study the regulatory effect of GATA2 on LINC00891 expression, cells were transfected with pcDNA-GATA2, GATA2 siRNA or respective controls. Western blot analysis results showed that the protein level of GATA2 was markedly upregulated after pcDNA-GATA2 transfection and reduced after GATA2 siRNA treatment (Figure 4D). Moreover, overexpression of GATA2 prominently up-regulated the expression of LINC00891, and knockdown of GATA2 reduced LINC00891 expression in A549 and H460 cells (Figure 4E). These data indicated that GATA2 directly bound to the LINC00891 promoter and promoted LINC00891 transcription.

GATA2 suppresses cell proliferation, invasion, migration, and EMT in A549 and H460 cells

Next, we explored the effect of GATA2 on lung cancer cell functions. The expression of GATA2 was detected in tissues and cell lines of lung cancer. The results showed that GATA2 expression was decreased in lung cancer tissues and cell lines compared with that in paracancerous normal tissues and normal lung epithelial cells (Figure 5A,B). Next, pcDNA-GATA2, GATA2 siRNA and respective negative controls were transfected into A549 and H460 cells, and

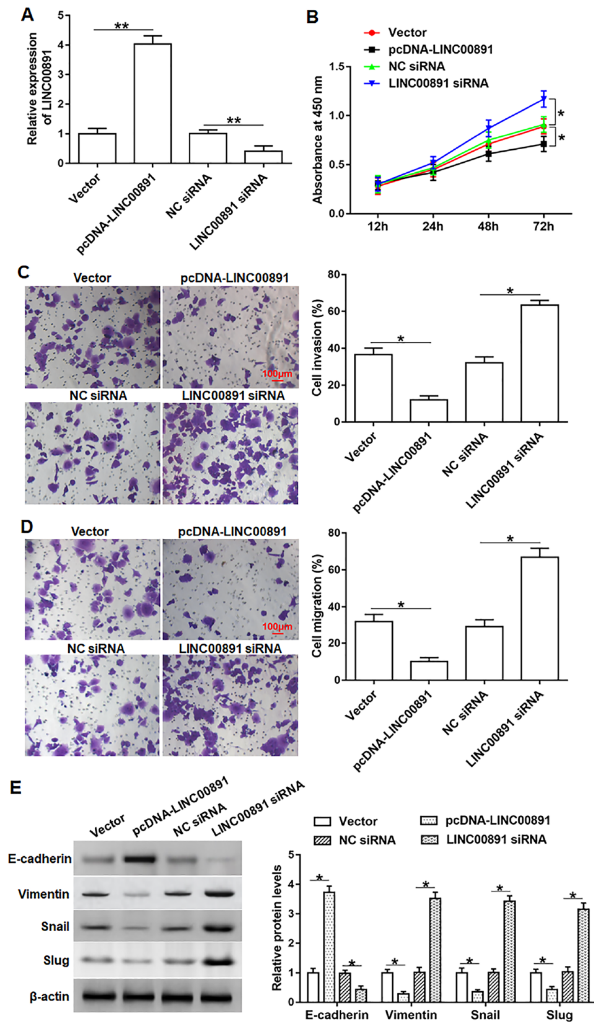

Figure 3. LINC00891 inhibits cell proliferation, invasion, migration, and EMT in H1975 cells H1975 cells were transfected with pcDNA-LINC00891, LINC00891 siRNA or respective negative controls (A) LINC00891 expression was measured by RT-qPCR. (B) Cell proliferation was assessed by CCK-8 assay. (C,D) Cell invasion and migration were determined by Transwell assay. (E) Western blot analysis was used to analyze the protein levels of E-cadherin, Vimentin, Snail and Slug. $n=5$ in each group, $*P<0.05$, $**P<0.01$.

the cell proliferation, migration, invasion and EMT marker gene expression were detected. The results showed that overexpression of GATA2 decreased cell proliferation, invasion and migration, whereas knockdown of GATA2 showed an opposite effect (Figure 5C–E). Furthermore, highly expressed GATA2 led to an obvious decrease in Vimentin, Snail and Slug expression, and a significant increase in E-cadherin expression, while GATA2 knockdown played an opposite role (Figure 5F). These data indicated that overexpression of GATA2 could hinder the malignant progression of lung cancer cells by inhibiting cell proliferation, migration, invasion and EMT process.

LINC00891 is regulated by the miR-128-3p/GATA2 axis in A549 and H460 cells

It was reported that miR-128-3p is an important regulator of the malignant phenotype of lung cancer cells and promotes the EMT process [18]. We used TargetScan to predict miRNAs that might regulate GATA2 expression, and found a potential binding site between miR-128-3p and GATA2 (Figure 6A). Subsequent results of dual luciferase reporter gene assay demonstrated that transfection with miR-128-3p mimic significantly decreased the luciferase activity of GATA2 wild-type reporter, without altering the luciferase activity of GATA2 mutant reporter (Figure 6B). Next, miR-128-3p was overexpressed or silenced in cells to examine the regulatory effect of miR-128-3p on GATA2 expression. As shown in Figure 6C, the protein level of GATA2 was markedly reduced after transfection with miR-128-3p mimic and up-regulated after transfection with miR-128-3p inhibitor (Figure 6D). Furthermore, LINC00891 expression was reduced after miR-128-3p overexpression, which was reversed by the high expression of GATA2 (Figure 6E). These data indicated that GATA2 was a target gene of miR-128-3p, and LINC00891 was regulated by the GATA2/miR-128-3p axis.

LINC00891 regulates A549 and H460 cell functions by suppressing the RhoA pathway

It was reported that LINC00891 is associated with negative regulation of small GTPase-mediated signal transduction [10]. To investigate the effect of LINC00891 on RhoA pathway, cells were transfected with pcDNA-LINC00891, LINC00891 siRNA or respective negative controls to evaluate RhoA activity. The results showed that the relative RhoA activity in A549 and H460 cells was weakened after LINC00891 overexpression but enhanced after LINC00891 knockdown (Figure 7A). To further explore whether LINC00891 modulates cell behaviors by regulating RhoA activity, a specific RhoA pathway inhibitor (CCG-1423) was used to suppress RhoA activity. As expected, RhoA activity was observably decreased after treatment with CCG-1423 (Figure 7B). Next, A549 and H460 cells were treated with LINC00891 siRNA alone or together with CCG-1423. The results showed that CCG-1423 reversed the increase of cell proliferation, invasion, migration, and the expressions of Vimentin, Snail and Slug, and the decrease of E-cadherin expression caused by LINC00891 knockdown (Figure 7C–F). These data indicated that knockdown of LINC00891 facilitated the malignant behaviors of lung cancer cells by increasing the RhoA pathway activity.

LINC00891 inhibits *in vivo* tumor growth

To investigate the role of LINC00891 in tumor growth, we generated a xenograft mouse model by subcutaneous injection of A549 cells transfected with NC shRNA or LINC00891 shRNA. It was found that silencing of LINC00891 prominently increased the tumor volume and weight in mice within 28 days (Figure 8A–C). Compared with the LINC00891 knockdown group, CCG-1423 administration significantly inhibited tumor growth (Figure 8A–C). Moreover, silencing of LINC00891 reduced LINC00891 expression and upregulated RhoA activity in tumor tissues, and the injection of CCG-1423 only decreased the RhoA activity without altering LINC00891 expression (Figure 8D,E). These data indicated that knockdown of LINC00891 promoted lung cancer xenograft tumor growth by increasing the RhoA pathway activity.

Discussion

In this study, we found that ectopic expression of LINC00891 hindered lung cancer cell proliferation, migration, invasion and the EMT progress, and impeded the growth of xenograft tumor in mice. Mechanistic studies showed that LINC00891 was transcriptionally

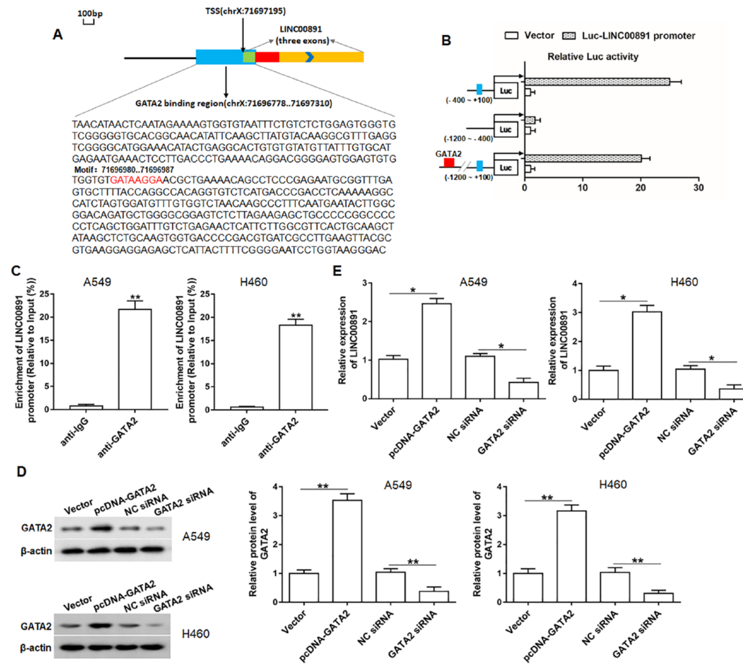

Figure 4. GATA2 directly binds to LINC00891 promoter and regulates LINC00891 expression (A) Graphical representations of the detailed information of GATA2-binding region. GATA2-binding region was presented in blue and the three exons of LINC00891 were presented in green, red and orange. The mazarine arrow indicates transcription direction. The binding motif was shown in red bases. (B) Relative luciferase activities were detected by dual-luciferase reporter gene assay. These reporters consisted of different sequences of LINC00891 promoter regions, which were located upstream of the luciferase reporters and downstream of *GATA2*. (C) Anti-GATA2 and anti-IgG were used to perform ChIP assay, and the enrichment of LINC00891 promoter was detected by semi-quantitative PCR. (D, E) A549 and H460 cells were transfected with GATA2 overexpression vector, GATA2 siRNA or respective negative controls, and the expressions of GATA2 and LINC00891 were measured by western blot analysis and RT-qPCR respectively. $n=5$ in each group, $*P<0.05$, $**P<0.01$.

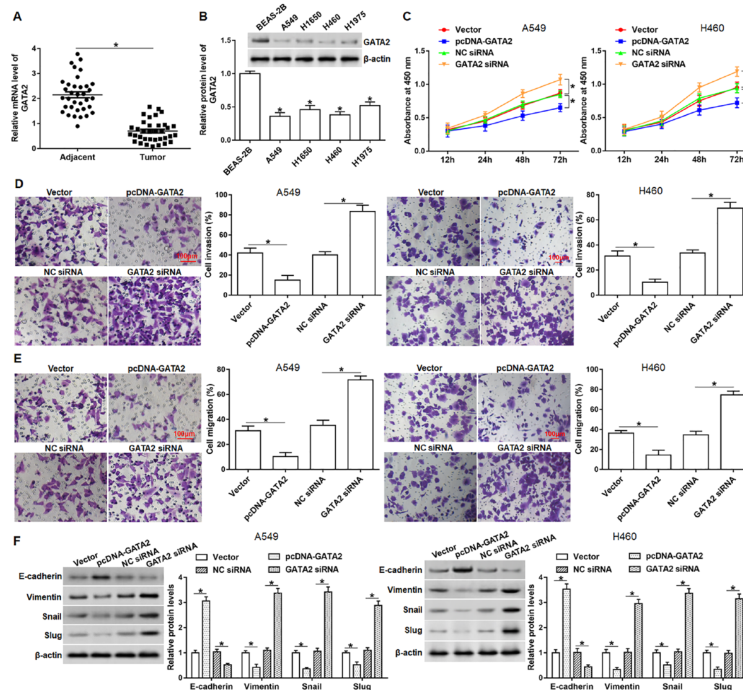

Figure 5. GATA2 inhibits cell functions of A549 and H460 cells (A) GATA2 expression in lung cancer tissues ($n=35$) and adjacent normal tissues ($n=35$) was detected by RT-PCR. (B) The protein level of GATA2 in lung cancer cell lines (A549, H1975, SPCA1 and H226) and normal lung epithelial cells (BEAS-2B) was measured by western blot analysis. A549 and H460 cells were transfected with GATA2 overexpression vector, GATA2 siRNA or respective negative controls. Cell proliferation (C), invasion (D), and migration (E) were analyzed. (F) Western blot analysis was performed to measure the protein levels of E-cadherin, Vimentin, Snail and Slug. $n=5$ in each group, $*P<0.05$, $**P<0.01$.

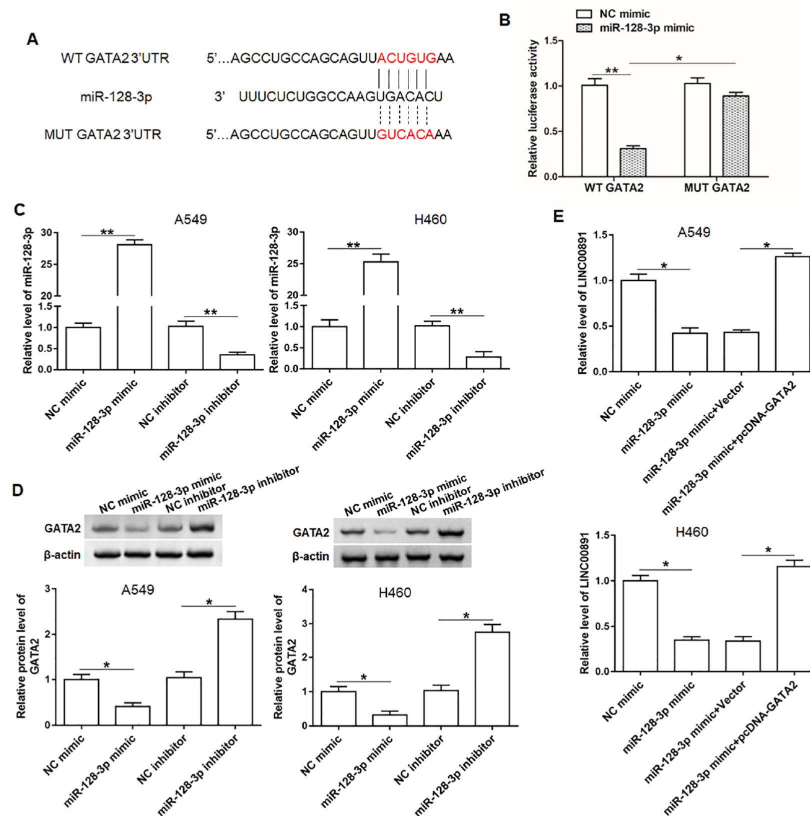

Figure 6. LINC00891 is regulated by miR-128-3p/GATA2 axis in A549 and H460 cells (A) The potential binding site between GATA2 and miR-128-3p. (B) miR-128-3p mimic and GATA2 wild type or mutant type reporters were transfected into HEK293T cells, and dual-luciferase reporter gene assay was performed to determine the relative luciferase activity. A549 and H460 cells were transfected with miR-128-3p mimic, miR-128-3p inhibitor or respective controls, (C) miR-128-3p expression was detected by RT-qPCR. (D) The protein level of GATA2 was measured by western blot analysis. (E) miR-128-3p mimic was transfected into A549 and H460 cells alone or together with pcDNA-GATA2, and LINC00891 expression was evaluated by RT-qPCR. $n=5$ in each group, $^{*}P<0.05$, $^{**}P<0.01$.

regulated by GATA2, a target gene of miR-128-3p, and played a tumor-suppressing role in lung cancer by suppressing the RhoA pathway activity (Figure 9).

lncRNAs play important roles in the occurrence and development of lung cancer. LncRNAs that are closely related to lung cancer mainly include metastasis associated in lung adenocarcinoma transcript 1 (MALAT-1), HOX transcript antisense intergenic RNA (HOTAIR), maternally expressed gene 3 (MEG3), etc. MALAT-1 can induce tumor growth and metastasis and is associated with poor prognosis in patients with NSCLC [22]. HOTAIR promotes the invasion and metastasis of lung cancer cells, which is of great significance for the prognosis evaluation of lung cancer individuals [23]. MEG3 restrains A549 cell proliferation, induces apoptosis, and prevents tumorigenesis by regulating p53 [24]. A previous study showed that LINC00891 is down-regulated in LUAD tumor tissues and associated with the prognosis of LUAD [10]. Moreover, LINC00891 is involved in negative regulation of small GTPase-mediated signal transduction [10]. Our results demonstrated that LINC00891 expression was lower in lung cancer tissues than in adjacent normal tissues. It was reported that the two most common mutated oncogenes in lung cancer are EGFR and KRAS, which are active research topics in targeted therapy [25]. So we measured the expression of LINC00891 in two KRAS mutant (A549 and H460) and two EGFR mutant (H1650 and H1975) lung cancer cell lines, and found that LINC00891 expression was downregulated in the four cells compared with that in normal lung epithelial cells. Patients with surgically treated EGFR-mutated NSCLC show better disease-free survival (DFS) and overall survival (OS) compared with KRAS, which show poor DFS and OS [26]. The complexity of KRAS biochemistry hinders the development of direct KRAS inhibitors, making KRAS mutations more difficult to target than EGFR mutations [26]. Therefore, we used A549 and H460 cells to focus on the role of LINC00891 in KRAS mutant NSCLC. Our data revealed that LINC00891 overexpression increased cell proliferation, invasion and migration, as well as the EMT process in A549 and H460 cells. Furthermore, in order to verify whether the important role of LINC00891 depends on KRAS mutation in lung cancer cells, we examined the effect of LINC00891 on the behaviors of EGFR mutant lung cancer cells. The results showed that highly expressed LINC00891 also suppressed the malignant progression of H1975 cells. In addition, LINC00891 knockdown promoted xenograft tumor growth *in vivo*, suggesting the anti-cancer effect of LINC00891 in lung cancer.

The expression of miR-128 is dysregulated in several types of human cancers, indicating its important role in tumorigenesis [27]. In NSCLC, miR-128-3p is a molecule that promotes tumor development, especially in facilitating tumor chemoresistance and metastasis [28]. However, a previous study reported that miR-128 increases apoptosis in lung cancer cells by directly targeting NIMA-related kinase 2, and exerts a tumor-suppressing effect [29]. The

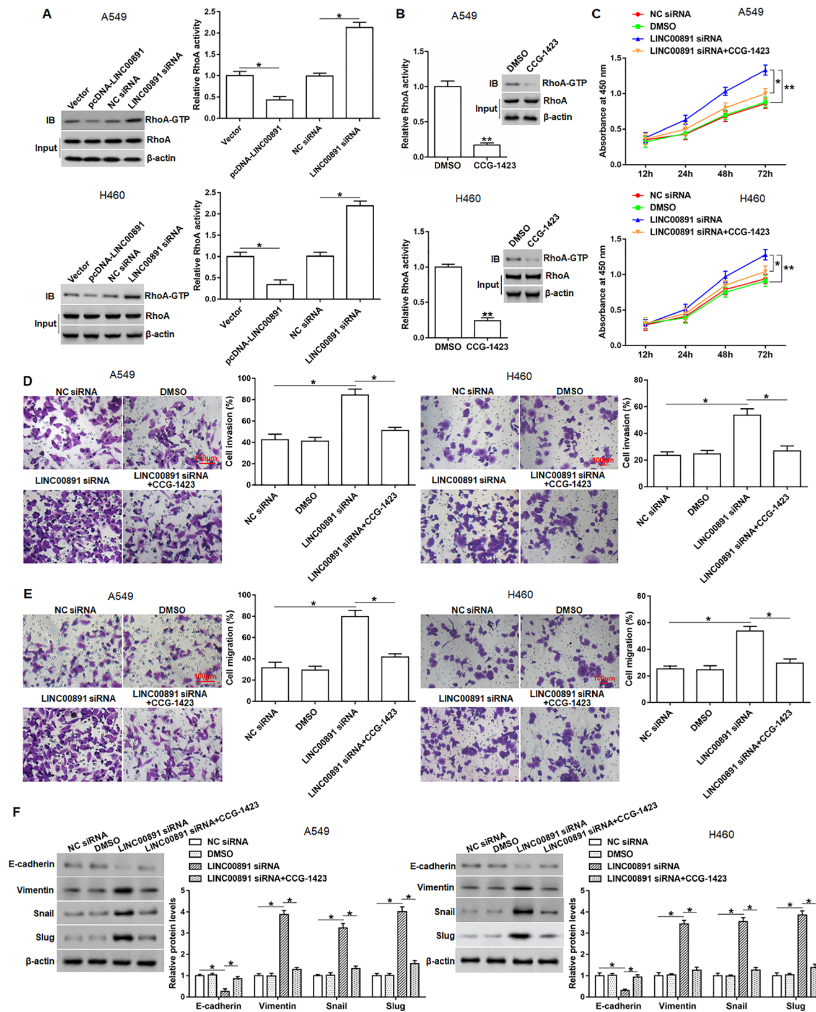

Figure 7. LINC00891 regulates A549 and H460 cell functions via the RhoA pathway (A) A549 and H460 cells were transfected with LINC00891 overexpression vector, LINC00891 siRNA or respective negative controls, and the activated RhoA was determined using RhoA Activation Assay Kit. (B) Cells were treated with 15 μM CCG-1423 for 24 h, and the activated RhoA was assessed using RhoA Activation Assay Kit. Cells were treated with LINC00891 siRNA alone or together with 15 μM CCG-1423, and cell proliferation (C), invasion (D), and migration (E) were analyzed. (F) Western blot analysis was used to measure the protein levels of E-cadherin, Vimentin, Snail and Slug. $n=5$ in each group, *$P<0.05$, **$P<0.01$.

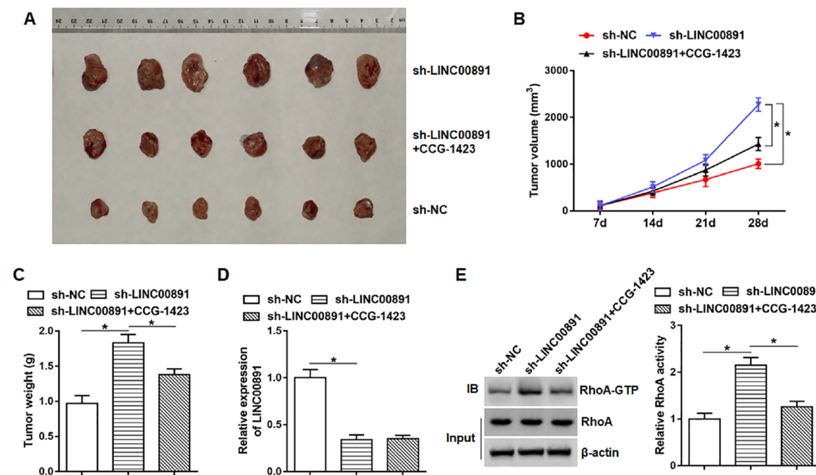

Figure 8. LINC00891 inhibits *in vivo* tumor growth An xenograft mouse model was generated by subcutaneous injection of A549 cells (2×10^6) treated with NC shRNA ($n=6$) or LINC00891 shRNA ($n=6$). Next, mice were injected with CCG-1428 (0.15 mg/kg) once a day until day 28 ($n=6$). (A) Representative images of tumors. (B) Tumor volume was measured every 7 days. (C) Tumor weight was detected on day 28. LINC00891 expression (D) and RhoA activity (E) in tumor tissues were assessed on day 28. *$P<0.05$.

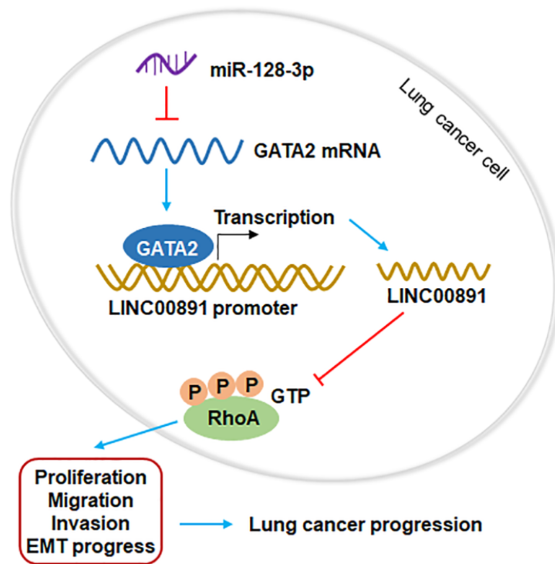

Figure 9. A schematic diagram for the role of LINC00891 in lung cancer progression As a target gene of miR-128-3p, GATA2 promotes the transcription of LINC00891 by binding to the promoter region of LINC00891. Highly expressed LINC00891 hinders cell proliferation, migration and invasion, as well as the EMT progress of lung cancer cells by suppressing the activity of the RhoA pathway, thus impeding the progression of lung cancer.

function of miR-128-3p is controversial in the development of lung cancer. There is evidence that the function of miR-128-3p ranges from cancer-promoting to tumor suppression, which explains its two-way role in lung cancer [18]. Our results indicated that GATA2 is a target gene of miR-128-3p, and is negatively regulated by miR-128-3p. Given the important role of miR-128-3p in lung cancer, we speculate that GATA2 may also play an important role in lung cancer progression.

Previous studies indicated that changes in GATA2 expression are related to clinical outcomes in patients with multiple solid cancers such as breast, prostate, colorectal, renal, and hepatocellular carcinoma [30–35]. And GATA2 expression is decreased in clear cell renal cell carcinoma and hepatocellular carcinoma, and its low expression is associated with an increased risk of disease recurrence [30,31]. RNAi-mediated knockdown of GATA2 significantly enhances the proliferation, migration and invasion of HepG2 cells (human liver cancer cells) [30]. Moreover, it was reported that knockdown of GATA2 significantly inhibited the growth of xenograft tumors induced by lung cancer cells with RAS pathway mutation [13]. Our results revealed that GATA2 was downregulated in both lung cancer tissues and cell lines compared with that in adjacent normal tissues and normal lung epithelial cells. Overexpression of GATA2 suppressed cell proliferation, invasion and migration, as well as the EMT process in KRAS-mutant lung cancer cells. As a transcription factor, GATA2 can regulate the transcriptional activation or repression of many genes. We analyzed the promoter region of LINC00891 using ChIPbase database and found that there is a potential GATA2-binding site 115 bp upstream of the transcription start site of LINC00891. We further demonstrated that GATA2 directly binds to the LINC00891 promoter and transcriptionally regulates LINC00891 expression.

Activation of the Rho/Rho kinase pathway is important for cell migration, intercellular adhesion, cell proliferation and apoptosis, invasion and mitosis [36]. In NSCLC, inhibition of the Rho/Rho-kinase pathway impedes tumor migration and invasion [37]. Knockdown of RhoA reduces the proliferation, adhesion, migration and invasion of SPCA1 lung cancer cells [38]. We found that RhoA pathway inactivation reversed the promoting effect of LINC00891 silencing on cell proliferation, migration and invasion. Moreover, CCG-1423 treatment impeded LINC00891 knockdown-induced increase in mouse xenograft tumor growth. RhoA activity is closely related to the EMT progress of tumor cells. Down-regulation of lncRNA NORAD inhibits the expression levels of mesenchymal markers N-cadherin, Vimentin, and ZEB1, and increases the expression of epithelial marker E-cadherin in pancreatic cancer cells, which is reversed by treatment with the RhoA inhibitor CCG-1423, indicating that NORAD regulates EMT process by modulating RhoA activity [39]. RhoA knockout decreased cyclin D1, MMP-9 and Vimentin, and upregulated E-cadherin expression in lung adenocarcinoma cells [40]. We found that high expression of LINC00891 inhibited RhoA activity. In view of the regulatory effect of RhoA on the progression of EMT, we speculate that LINC00891 may also be involved in the regulation of EMT in lung cancer cells. As expected, silencing of LINC00891 reduced E-cadherin expression and increased Vimentin, Snail and Slug expressions, which was rescued by CCG-1423.

In summary, we demonstrated that silencing of LINC00891 facilitates the malignant progression of lung cancer cells *in vitro*, and promotes xenograft tumor growth *in vivo*. Mechanistic studies indicated that LINC00891 is regulated by miR-128-3p/GATA2 axis and acts as a cancer suppressor by restraining the RhoA pathway. Our research may provide a promising molecular target for the treatment of lung cancer.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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