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# GATA-6 transcriptionally inhibits Shh to repress cell proliferation and migration in lung squamous cell carcinoma

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ABSTRACT

GATA-6 is a transcription factor that participates in cell lineage differentiation and organogenesis in many tissue types. The abnormal expression of GATA-6 is associated with the development of diverse cancers. GATA-6 acts as an oncogene or tumor suppressor based on tumor origin. Here, we investigated the effects of GATA-6 on lung squamous cell carcinoma (LSCC). We found that GATA-6 was significantly reduced in LSCC tissues compared with the paired normal tissues. The overexpression of GATA-6 inhibited LSCC cell proliferation and migration. Importantly, a luciferase reporter assay and chromatin immunoprecipitation assay demonstrated that GATA-6 negatively regulated the expression of sonic hedgehog (Shh) by directly binding to its promoter region. Furthermore, N-Shh stimulation rescued the inhibition of LSCC cell proliferation and migration upon GATA-6 overexpression. Thus, GATA-6 inhibited the proliferation and migration of LSCC cells by transcriptionally inhibiting the expression of Shh, indicating that targeting GATA-6 may be a potential approach for LSCC therapy.

## 1. Introduction

Lung cancer remains the leading cause of cancer-related mortality around the world (Siegel et al., 2018). According to the histological characteristics, lung cancer cases are classified as nonsmall-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). NSCLC mainly includes lung adenocarcinoma (LAC), lung squamous cell carcinoma (LSCC) and large cell carcinoma (LCLC) (Justilien et al., 2014). LSCC comprises approximately 30% of NSCLC cases and is strongly associated with smoking (Ettinger et al., 2015). Treatment options for NSCLC chemotherapy are subtype-dependent. Agents targeting *EGFR* mutations and *ALK* gene rearrangement are used to treat LAC. However, these targeted agents are nearly ineffective against LSCC, and chemotherapy remains the standard treatment for LSCC. Although oncogenic alterations, such as alterations in *FGFR1*, *DDR2* and components of the PI3K/mTOR pathway, have been previously described in LSCC (Drilon et al., 2012), the development of effective target therapies is urgent.

GATA-6 is a member of the GATA transcription factor family, which contains six members, namely, GATA-1, -2, -3, -4, -5, and -6. GATAs contain evolutionarily conserved C2-type zinc finger DNA binding domains that bind to the WGATAR motif (Molkentin, 2000). GATA-1, -2, and -3 are mainly expressed in the hematopoietic system, whereas GATA-4, -5, and -6 are expressed in many organs, including the gut, liver and lung (Molkentin, 2000). GATA-6 is associated with tumorigenesis and regulates target genes that participate in various biological processes, such as cell differentiation, cell survival, cell proliferation and signal transduction. In pancreatic ductal adenocarcinoma and colon cancer, GATA-6 acts as a tumor promoter. GATA-6 copy number is negatively correlated with the overall survival of pancreatic ductal adenocarcinoma patients (Zhong et al., 2011). The forced overexpression of GATA-6 promotes the proliferation and colony formation of colon cancer cells (Belaguli et al., 2010). In contrast, GATA-6 is lost in ovarian cancer, gastric cancer, astrocytoma, and hepatocellular carcinoma, and it acts as a tumor suppressor. The loss of GATA-6 expression is closely associated with the neoplastic transformation of the ovarian surface epithelium (Capo-chichi et al., 2009; Shen et al., 2019). The of GATA-6 results in the enhanced proliferation and transformation of astrocytes, and knocking down GATA-6 expression in human malignant astrocytoma cells reduces tumorigenic growth (Kamnasaran et al., 2007). Gastric cancer patients with high levels of GATA-6 methylation tend to exhibit shorter overall survival (Wu et al., 2016). The low expression of GATA-6 is detected in hepatocellular carcinoma tissues, and the silencing of GATA-6 promotes cell migration and the

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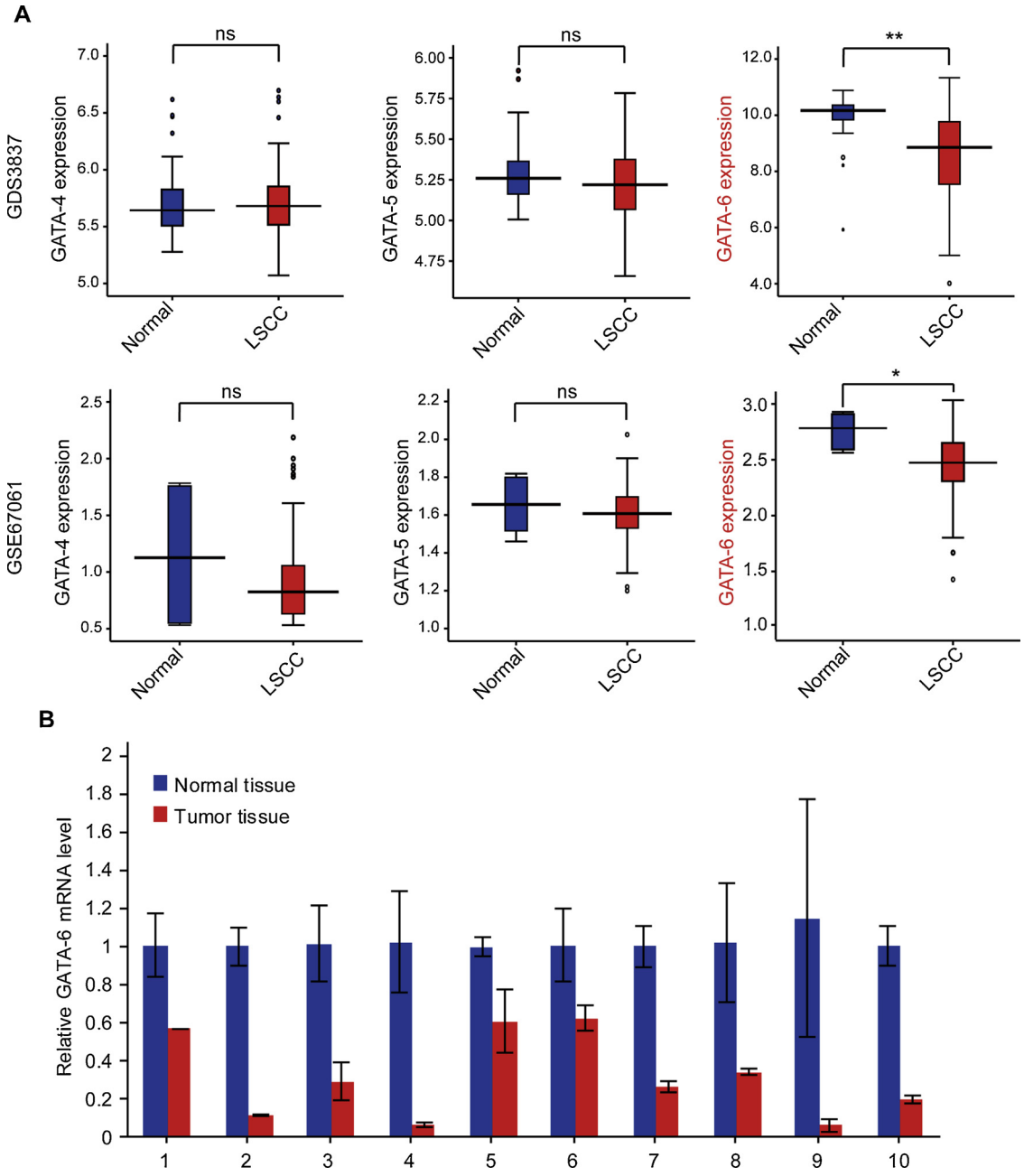


**Fig. 1. GATA-6 is downregulated in LSCC.** (A) GATA-4, -5, and -6 mRNA levels were analyzed in LSCC tissues and compared with those in normal tissues from the GDS3837 and GSE67061 datasets from the GEO database. *$p < 0.05$, **$p < 0.01$, ns, no significance. (B) Expression of GATA-6 was repressed in 10 freshly dissociated LSCC tumor tissues compared with that in paired adjacent lung tissues, as examined by real-time PCR.

invasion of hepatocellular carcinoma cells (Tan et al., 2019). GATA-6 has been implicated in the transcriptional regulation of genes within the respiratory epithelium of the lung, which are important for lung development and branching morphogenesis (Liao et al., 2018; Molkentin, 2000). GATA-6 has been reported to inhibit the metastasis of LAC (Cheung et al., 2013). However, the effects of GATA-6 in LSCC remain to be revealed.

The Hedgehog (Hh) signaling pathway is involved in body patterning and the regulation of adult stem cells. The abnormal activation of Hh signaling is associated with cancers including medulloblastoma, basal cell carcinoma, liver cancer, gastric cancer and lung cancer (Oro et al., 1997; Shao et al., 2017; Szczepny et al., 2017; Teglund and Toftgård, 2010). Sonic hedgehog (Shh) is essential for proper embryonic development and is the most widely characterized Hh ligand (Ingham and McMahon, 2001). The *Shh* gene encodes a 45-kDa precursor protein, which is autocatalytically cleaved to generate activated Shh, a 19-kDa N-terminal peptide fragment (N-Shh) (Pepinsky et al., 2000). The activated Shh binds to the PTCH (Patched) receptor and activates Hh signaling. It has been reported that Hh signaling is hyperactivated in LSCC and that its components are overexpressed in specimens from patients with LSCC (Gialmanidis et al., 2009; Shi et al., 2011). The inhibition of the Hh signaling transcription factor Gli2 significantly reduces the proliferation and induce the apoptosis of LSCC cells (Huang et al., 2014). Whether GATA-6 regulates Shh expression in LSCC needs to be elucidated.

In this work, we found that GATA-6 was drastically reduced in LSCC tissues compared with normal tissues. The upregulation of GATA-6 inhibited LSCC cell proliferation and migration. Furthermore, it was demonstrated that GATA-6 negatively regulated the expression of Shh by directly binding with its promoter region, and N-Shh stimulation

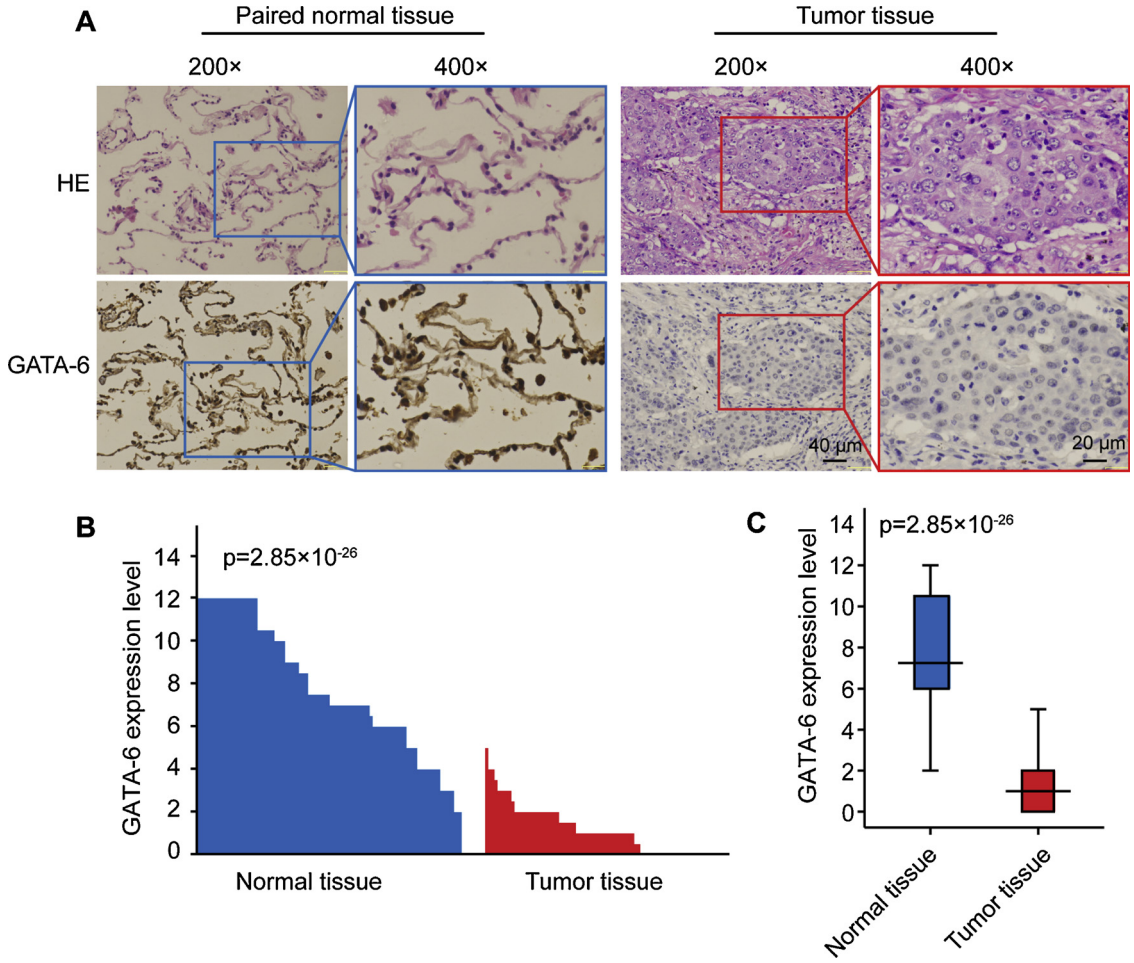


**Fig. 2. Expression of GATA-6 is repressed in LSCC.** (A) The expression of GATA-6 was repressed in LSCC tumor tissues compared with that in paired normal tissues, as examined by IHC. Representative photos and magnified local images reflecting detailed information are shown on the right. (B–C) The GATA-6 expression level detected by IHC assay was scored. Plots of GATA-6 scores in each LSCC tissue and normal tissues (B) and box plots of scores of GATA-6 expression (C) are shown. Statistical significance was analyzed by using the Mann-Whitney U test, n = 78.

reversed the GATA-6 overexpression-induced inhibition of LSCC cell proliferation and migration. Taken together, these results suggest that GATA-6 plays an important role in the progression of LSCC, and targeted agents for the upregulation of GATA-6 expression may be effective for clinical LSCC treatment.

## 2. Materials and methods

### 2.1. Reagents, antibodies and constructs

Protease inhibitor cocktail was obtained from Sigma-Aldrich (St. Louis, MO). Lipofectamine 2000 transfection reagent was purchased from Invitrogen (Carlsbad, CA). An anti-Shh antibody (SC365112) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), an anti-GATA-6 antibody (5851S) was obtained from Cell Signaling Technology (Danvers, MA), and an anti-Flag antibody (F3165) was purchased from Sigma-Aldrich.

GV358-GATA-6-Flag was purchased from Genechem (Shanghai), and pcDNA3.1-GATA-6-Flag was generated by PCR-cloning human GATA-6 into pcDNA3.1 using the Hind III and Xho I sites. The Shh promoter reporter plasmid (pGL4.2-Shh-pro) was generated by inserting the Shh promoter region (-2000 to +500 bp) into the pGL4.2 vector using the Kpn I and Xho I sites. All constructs were verified by DNA sequencing.

### 2.2. Cell culture

The human embryonic kidney 293T cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The human LSCC SK-MES-1 cell line was obtained from the cell bank of the Chinese Academy of Sciences. These cells were cultured as recommended by the suppliers in a humidified incubator with 5% $CO_2$ at 37 ˚C.

### 2.3. Cell transfection and lentivirus infection

The transient transfection of cells was performed with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Stably overexpressing and knockdown cells were generated using a lentiviral expression system. The Lenti-GATA-6 construct (pReceiver-Lv201) for GATA-6 overexpression was generated, packed, and purified by GeneCopoeia (Rockville, MD). Lentivirus infection was performed according to the protocol provided by the manufacturer.

### 2.4. Real-time PCR assay

Total RNA was extracted from the cells using TRIzol Reagent (Life Technologies, and 1 μg of total RNA was used to generate cDNA via reverse transcription using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara, RR047A). Quantitative real-time PCR was performed

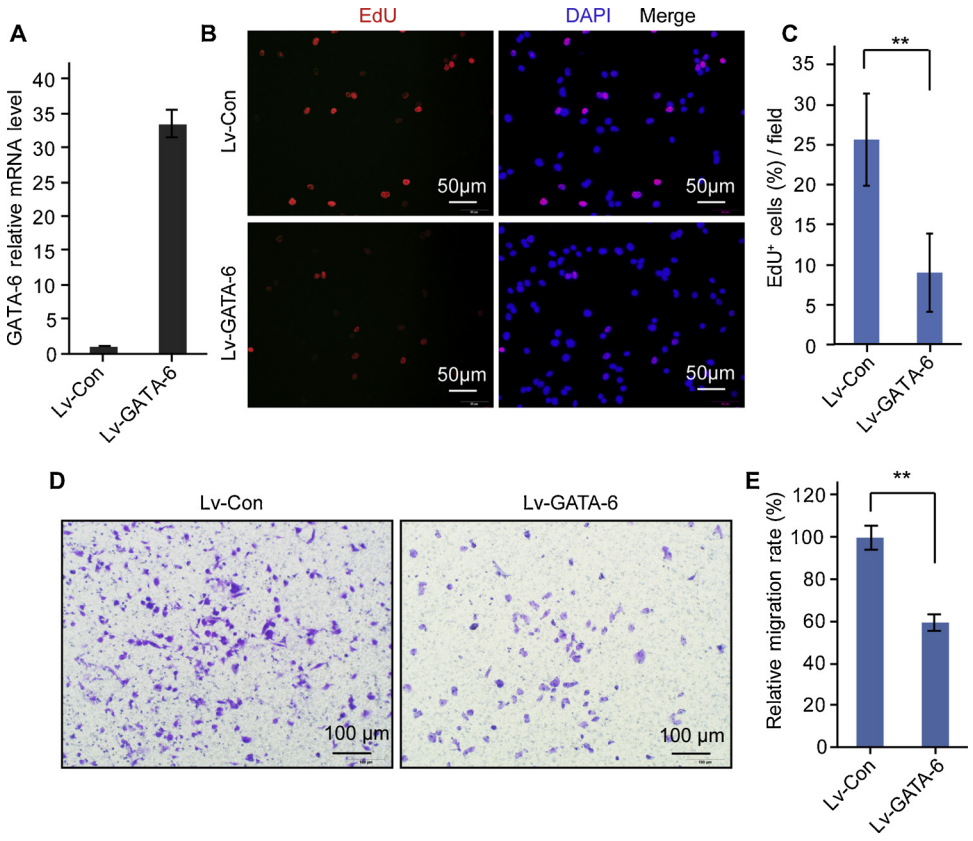


**Fig. 3. GATA-6 overexpression inhibits cell proliferation and migration in LSCC.** (A) GATA-6 was stably overexpressed in SK-MES-1 cells. SK-MES-1 cells were infected with a control lentivirus (Lv-Con) and a GATA-6 overexpression lentivirus (Lv-GATA-6), and the GATA-6 expression level was determined by real-time PCR assay. (B–C) The overexpression of GATA-6 inhibits the proliferation of LSCC cells. The quantification of the percentage of EdU-positive cells was carried out. The data is presented as the mean ± SD, n = 3, $^{**}p < 0.01$. (D–E) The upregulation of GATA-6 expression reduces the migration of LSCC cells. Representative images of cells stained with crystal violet are shown. The migration ratio was quantified. The data is presented as the mean ± SD, n = 3, $^{**}p < 0.01$.

using TB Green Premix Ex Taq II (Takara, RR820A) with an ABI StepOnePlus Real-Time PCR System (Applied Biosystems Inc.). The following primers were used: *GATA-6*-forward, 5′-CCAGGAAACGAAA ACCTAAGAAC-3′ and *GATA-6*-reverse, 5′-TGAGGCTGTAGGTTGTG TTG-3′; *Shh*-forward, 5′-CTACGAGTCCAAGGCACATATC-3′ and *Shh*-reverse, 5′-CAGGTCCTTCACCAGCTTG-3′; and *GAPDH*-forward, 5′-ACATCGCTCAGACACCATG-3′ and *GAPDH*-reverse, 5′-TGTAGTTGA GGTCAATGAAGGG-3′. The data represent the mean ± SD from at least three independent biological experiments.

### 2.5. Western blot analysis

The cells were lysed with RIPA buffer containing protease inhibitor cocktail, and the protein concentration was measured with a Pierce BCA protein assay kit (Thermo Scientific). Cell lysates containing 50 μg of total protein were separated on 10% SDS–PAGE gels, and the proteins were transferred to nitrocellulose (NC) membranes. Then, the NC membranes were blocked with 5% nonfat dry milk for 1 h at room temperature and incubated with primary antibodies overnight at 4 °C followed by incubation with peroxidase-conjugated secondary antibodies. Finally, the protein bands were exposed to Kodak film. The film was scanned with an EPSON 1680 scanner, and the intensity of the blot bands was quantified using ImageJ software.

### 2.6. Immunohistochemistry

Immunohistochemical (IHC) staining was performed as described previously (Shao et al., 2017). To distinguish cancer tissues from adjacent normal epithelium, consecutive sections were stained by H&E.

### 2.7. Cell proliferation assay

Cell proliferation was determined using the EdU incorporation assay, which was carried out using the Cell-Light EdU imaging detecting kit according to the manufacturer's protocol (Ruibo Biotechnology, Guangzhou). EdU is a thymidine analog that is incorporated into the DNA of dividing cells.

### 2.8. Cell migration assay

A Transwell assay without precoated Matrigel was also used to evaluate cell migration ability. Briefly, $3 \times 10^4$ cells in 100 μl of medium containing 1% FBS were seeded into the upper chambers. The bottom wells of the system were filled with 600 μl of growth medium supplemented with 10% FBS. After being cultured in an incubator for 36 h, the cells on the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. The cell number was detected under an optical microscope. Each experiment was repeated at least three times.

### 2.9. Human tissue specimens

Human LSCC tumor tissue and paired normal tissue were obtained by surgical intervention from patients that did not undergo prior radiotherapy or chemotherapy at The First Affiliated Hospital of Nanchang University from June 2011 to March 2014. The study protocol was approved by the Institutional Review Board of The First Affiliated Hospital of Nanchang University. All patients gave informed consent.

### 2.10. Luciferase assay

293T or SK-MES-1 cells were seeded into a 24-well plate. After the cells were cultured overnight, they were cotransfected with the pGL4.2-Shh-pro reporter plasmid and the pRL-TK plasmid with pcDNA3.1-GATA-6-Flag or pcDNA3.1 vector. Luciferase assays were performed 30 h after transfection using the Dual Luciferase Reporter Assay System according to the supplier's protocol (Promega, WI).

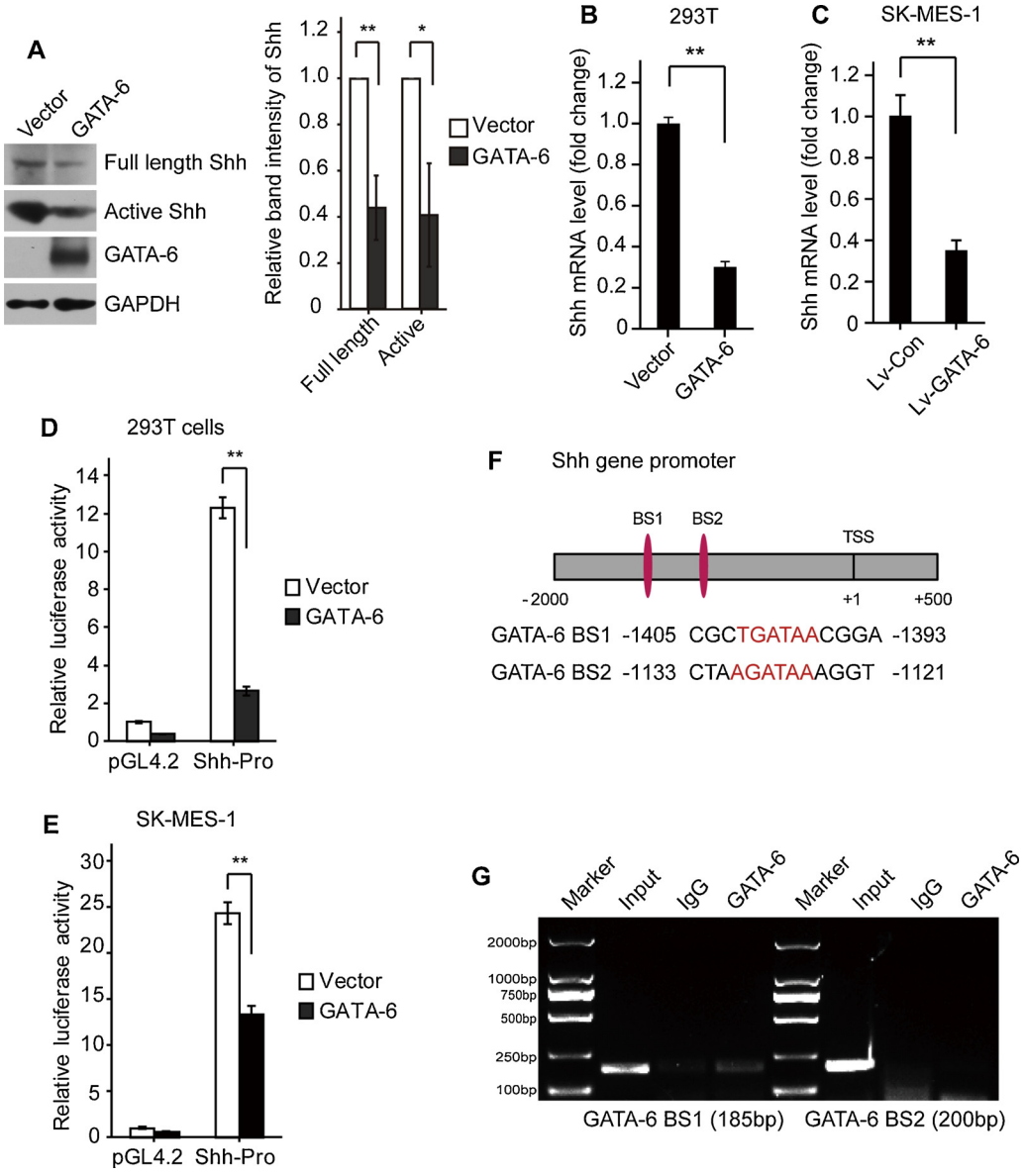


**Fig. 4. GATA-6 transcriptionally represses Shh expression.** (A–B) GATA-6 overexpression results in the downregulation of Shh expression. 293T cells were transfected with a GFP-GATA-6 or control vector, and 48 h later, real-time PCR (A) and western blot (B) were used to determine Shh expression. The data is presented as the mean ± SD, n = 3, $^{*}p < 0.05$, $^{**}p < 0.01$. (C) The upregulation of GATA-6 inhibits Shh expression in LSCC cells. SK-MES-1 cells stably overexpressing GATA-6 and control cells were analyzed for Shh expression by real-time PCR. The data is presented as the mean ± SD, n = 3, $^{**}p < 0.01$. (D–E) GATA-6 inhibits Shh promoter activity. 293T (D) and SK-MES-1 (E) cells were cotransfected with a Shh promoter luciferase construct (Shh-Pro) or pGL4.2 and pcDNA3.1-GATA-6 or pcDNA3.1. Thirty hours later, promoter activity was measured. The data is presented as the mean ± SD, n = 3, $^{**}p < 0.01$. (F) Schematic diagrams of the Shh promoter regions indicating the putative transcription factor binding sites. (G) GATA-6 interacts with the Shh promoter sequence BS 1. Chromatin DNA was isolated from 293T cells, and ChIP assays were performed with control (IgG) and anti-GATA-6 antibodies. Specific primers for each putative binding element were used for the PCR assay.

### 2.11. Chromatin immunoprecipitation (ChIP) assay

The cells were cross-linked with 1% (v/v) formaldehyde for 10 min at room temperature with gentle shaking and 0.125 M glycine was added to terminate the reaction. The cells were lysed with lysis buffer on ice. After sonication, the chromatin DNA in the cell lysate was sheared into 250–800 bp fragments. Then, an anti-GATA-6 antibody and protein G-agarose were added to enrich the DNA fragments that bound to GATA-6 through immunoprecipitation. The precipitated DNA was analyzed by semi-quantitative PCR to assess the Shh promoter regions containing putative GATA-6 binding sites after decrosslinking.

### 2.12. Statistical analysis

All experiments were performed at least three times. Student's *t*-test was employed to assess the difference between two groups. If $p < 0.05$, the differences were considered significant. All analyses were performed using SPSS v. 13.0 software (SPSS Inc., Chicago, IL).

## 3. Results

### 3.1. GATA-6 was downregulated in LSCC tissues

By analyzing the datasets from the GEO database, we found that the expression of GATA-6 was repressed in LSCC tissues compared with normal tissues, while GATA-4 and GATA-5 expression showed no significant difference between the LSCC tissues and normal tissues (Fig. 1A). We employed a real-time PCR assay to test the GATA-6 mRNA level in 10 paired LSCC tumor tissues and LSCC normal tissues and found that the GATA-6 mRNA level was sharply reduced in the LSCC tumor tissues compared with the paired normal tissues (Fig. 1B).

To further determine the protein expression level of GATA-6 in LSCC, we recruited 78 eligible LSCC cases and analyzed the expression of Shh by IHC assay. The results demonstrated that the protein level of GATA-6 was significantly decreased in LSCC tissues compared to paired normal tissues, and the immunoreactivity for GATA-6 in several LSCC tumor tissues was negative (Fig. 2A). We used an immunoreactive score of 0–12 (the intensity score multiplied by the extent score) to estimate the expression of GATA-6, and the intensity of nucleic staining of each specimen (no staining = 0; weak staining = 1, moderate staining = 2, strong staining = 3) and the extent of cell staining (0% = 0,

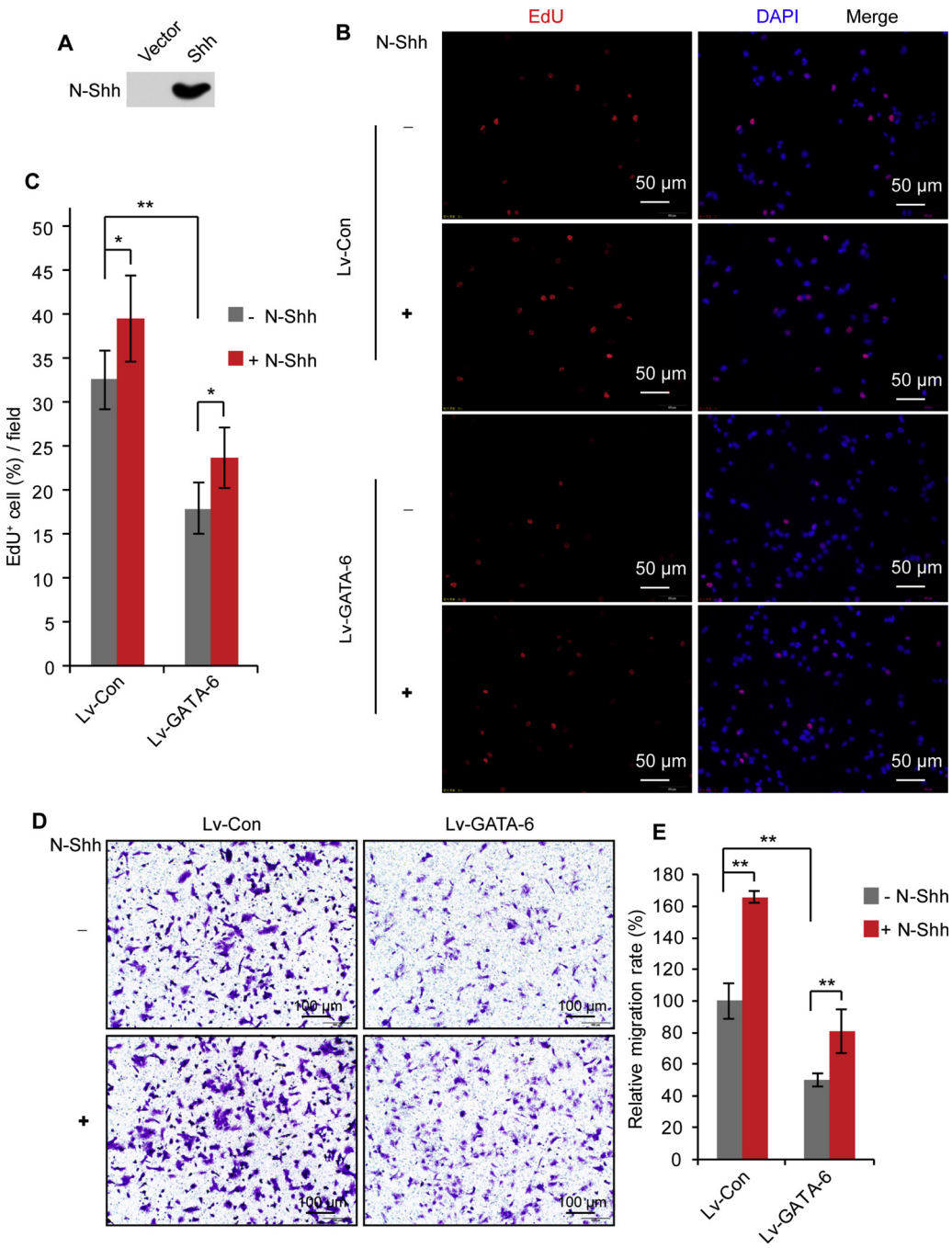


**Fig. 5. GATA-6 inhibits the proliferation and migration of LSCC cells by repressing Shh expression.** (A) N-Shh stimulation medium with active Shh was evaluated by western blot assay. (B–C) N-Shh stimulation rescued the inhibitory effect of GATA-6 overexpression on LSCC cell proliferation. SK-MES-1 cells stably overexpressing GATA-6 or control cells were treated with N-Shh stimulation medium or control medium and subjected to an EdU incorporation assay. The percentage of EdU-positive cells was quantified. The data is presented as the mean ± SD, n = 3, $*p < 0.05$, $**p < 0.01$. (D–E) The inhibitory effect of GATA-6 overexpression on cell migration was attenuated by N-Shh stimulation. The data is presented as the mean ± SD, n = 3, $**p < 0.01$.

1–24% = 1, 25–49% = 2, 50–74% = 3, 75–100% = 4) were scored. As shown in Fig. 2B and C, the GATA-6 score of LSCC tissues was much lower than that of normal tissues, and the GATA-6 score of approximately 50% of LSCC tissues was 0, which is consistent with the results of the IHC assay. These results indicate that the expression of GATA-6 is significantly repressed in LSCC tissues, implying that GATA-6 may contribute to the progression of GATA-6.

### 3.2. Upregulation of GATA-6 inhibited cell proliferation and migration in LSCC

We tested the functional role of GATA-6 in the oncogenic processes of LSCC and measured the effect of GATA-6 on LSCC cell proliferation and migration. GATA-6 was stably overexpressed in LSCC SK-MES-1 cells (Fig. 3A). As shown in Fig. 3B and C, the EdU incorporation assay showed that the overexpression of GATA-6 drastically inhibited cell proliferation. Moreover, the Transwell assay revealed that GATA-6 overexpression significantly inhibited cell migration (Fig. 3D and E). These results indicate that GATA-6 plays a very important role in the proliferation and migration of LSCC cells.

### 3.3. GATA-6 directly inhibited Shh transcription in LSCC

GATA-6 has been reported to regulate Shh in the limb buds (Kozhemyakina et al., 2014), raising the prospect that GATA-6 regulates Shh expression in LSCC. To investigate this possibility, first, GATA-6 was ectopically expressed in 293T cells, and we found that the forced expression of GATA-6 resulted in reduced protein and mRNA levels of Shh (Fig. 4A and B). In the LSCC SK-MES-1 cell line, GATA-6 overexpression also resulted in the downregulation of Shh expression (Fig. 4C). To further investigate the mechanism underlying the role of GATA-6 in regulating Shh expression, we sought to evaluate whether

GATA-6 regulates Shh promoter activity. We cotransfected the pGL4.2-Shh-pro construct with pcDNA3.1-GATA-6-Flag or the control vector, and the results demonstrated that GATA-6 inhibited the luciferase activity driven by the Shh promoter (Fig. 4D and E). Potential GATA-6 binding elements within the Shh promoter from the −2000 to +500 bp region were predicted by MatInspector software (Genomatix). Two putative GATA-6-binding elements (referred to as BS 1 and BS 2) with matrix similarity is greater than 0.95 were selected and further validated by chromatin immunoprecipitation (CHIP) (Fig. 4F). As shown by the CHIP assay results, GATA-6 bound to the promoter of Shh through BS 1 (Fig. 4G). Overall, we concluded that Shh is a transcriptionally inhibited target gene of GATA-6 in LSCC.

### 3.4. GATA-6 inhibited cell proliferation and migration by downregulating Shh in LSCC

Our previous publication reported that Shh can promote the proliferation and migration of cancer cells (Shao et al., 2017) and that GATA-6 transcriptionally represses the expression of Shh in LSCC cells. We hypothesized that GATA-6 inhibits cell proliferation and migration by downregulating Shh in LSCC. To examine this possibility, we tested whether Shh stimulation rescues the decrease in LSCC proliferation and migration upon GATA-6 overexpression. SK-MES-1 cells transfected with a GATA-6 overexpressing or control lentivirus were treated with N-Shh stimulation medium. The N-Shh stimulation medium contained secreted and active 20-kDa Shh, which was produced according to previously described methods (Fig. 5A) (Shao et al., 2017). We found that N-Shh stimulation rescued GATA-6 overexpression-induced inhibition of cell proliferation (Fig. 5B and C). Furthermore, the GATA-6 upregulation-induced repression of cell migration was rescued by N-Shh stimulation (Fig. 5D and E).

## 4. Discussion

It has been reported that GATA-6 is aberrantly expressed in diverse cancers, and it can be a tumor promoter or a tumor suppressor based on the tumor origin. GATA-6 is a tumor promoter in pancreatic ductal adenocarcinoma and colon cancer (Belaguli et al., 2010; Zhong et al., 2011). In contrast, in ovarian cancer, astrocytoma, gastric cancer, hepatocellular carcinoma and LAC, GATA-6 acts as a tumor suppressor (Capo-chichi et al., 2009; Cheung et al., 2013; Kamnasaran et al., 2007; Liu et al., 2019; Tan et al., 2019). We found that GATA-6 acted as a tumor repressor in LSCC. GATA-6 was downregulated in LSCC tissue compared with paired normal lung tissue (Figs. 1 and 2). The forced expression of GATA-6 repressed the proliferation and migration of LSCC cells (Fig. 3), indicating that regulating GATA-6 levels can target the growth and migration ability of LSCC cells. The expression of GATA-6 can be regulated in several ways. MicroRNAs (miRNAs) have been proven to regulate GATA-6 expression, miR-455 inversely regulates GATA-6 expression in colorectal cancer and miR-196b can downregulate GATA-6 levels in lung cancer (Li et al., 2018; Yunqi et al., 2019). On the other hand, it has been reported that epigenetic modification is associated with GATA-6 expression. The GATA-6 promoter region is hypermethylated in gastric cancer (Wu et al., 2016). GATA-6 gene silencing has been found to be correlated with the hypoacetylation of histones H3 and H4 and the loss of histone H3-K4 trimethylation at its promoter in ovarian cancer cell line (Caslini et al., 2006). Hepatocellular carcinoma cells treated with the demethylation agent 5′-aza-2′-deoxycytidine express higher levels of GATA-6 (Tan et al., 2019). The histone deacetylase inhibitor trichostatin A restores the expression of GATA-6 in ovarian cancer cells (Caslini et al., 2006). In lung cancer, the GATA-4 and GATA-5 promoters are methylated in primary lung cancer samples, but the promoter of GATA-6 is unmethylated (Guo et al., 2004). Whether DNA methylation and the histone epigenetic modification of the GATA-6 promoter region regulate the expression of GATA-6 in LSCC remain to be determined.

Canonical Hh signaling is initiated when activated Shh binds to the 12-pass transmembrane receptor PTCH, causing the inhibitory effect of PTCH on smoothened (Smo) to be lost. Then, Smo in turn actives the final component of Hh signaling, Gli transcription factors, leading to the transcriptional activation of target genes (Briscoe and Therond, 2013; Humke et al., 2010). The target genes of Hh signaling have important roles in various cellular processes; for example, *Cyclin D1* has a role in cell cycle regulation, *Myc* is involved in cell proliferation, *Bcl2* plays a role in cell apoptosis, *Snail* and *MMP9* are involved in epithelial-mesenchymal transition and *Nanog* and *Sox2* have roles in cell self-renewal (Scarpa and Scarpa, 2016). Thus, the precise regulation of Shh expression is critical for controlling the activation of Hh signaling, and several mechanisms have been reported to control Shh expression. Fibroblast growth factor (FGF) signaling is known to promote the expression of Shh in the posterior limb buds (Zhang et al., 2009). However, ETV4 and ETV5, which are FGF-activated transcription factors, inhibit Shh expression in the anterior limb buds (Zhang et al., 2009). Neural stem cells in which the transcription factor Sox2 is deleted do not express Shh, and CHIP has demonstrated that Shh is a Sox2 target (Favaro et al., 2009). The overexpression of chick Pcl2 (Polycomb-like 2) represses Shh expression in Hensen's node, and chick Pcl2 has been found to directly repress Shh promoter activity (Wang et al., 2004). The transcription factor GATA-6 inhibits Hh signaling by repressing the transcription of Shh to regulate pancreatic endoderm specification during patterning of the gut tube (Xuan and Sussel, 2016). GATA-6 can repress Shh expression in limb buds via interacting with an enhancer sequence that lies 1 Mb upstream of the Shh coding region (Kozhemyakina et al., 2014). However, whether GATA-6 regulates Shh expression in LSCC is unclear. In our study, we found that the overexpression of GATA-6 results in the downregulation of Shh and that GATA-6 can directly interact with the promoter sequence (−1405 to −1393 bp) of the Shh gene to negatively regulate Shh expression in LSCC cells (Fig. 4).

The dysregulation of Hh signaling has been implicated in various key biological processes in cancers, including cell proliferation, cell survival, and EMT. Thus, the inhibition of Hh signaling is of great clinical interest for cancer treatment (Wu et al., 2017). Shh ligands and the palmitoylation of Shh, which is a modification critical for generating mature and activated Shh, are targets for Shh pathway inhibition (Petrova et al., 2013; Stanton et al., 2009). Hh pathway activity is higher in LSCC compared to other histological NSCLC types (Gialmanidis et al., 2009), indicating that Hh signaling is a potential target for clinical therapy for LSCC. We showed that GATA-6 transcriptionally repressed Shh expression, and N-Shh stimulation rescued the decrease in LSCC cell proliferation and migration induced by GATA-6 overexpression (Fig. 5), indicating that GATA-6 regulated LSCC cell proliferation and migration, at least in part, by regulating Shh expression. Although inhibitors that target Shh ligand and Shh palmitoylation have been identified to inhibit Hh signaling (Petrova et al., 2013; Stanton et al., 2009), our work provides a mechanistic foundation for how targeting GATA-6 can regulate Shh expression to target the activation of Hh signaling and a theoretical foundation supporting GATA-6 as a potential target for clinical therapy for LSCC.

## Declaration of Competing Interest

No potential conflicts of interest were disclosed.

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