

LHX6 acts as a novel potential tumour suppressor with epigenetic inactivation in lung cancer

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LIM homeobox domain 6 (LHX6) is a putative transcriptional regulator that controls the differentiation and development of neural and lymphoid cells. However, the function of *LHX6* in cancer development remains largely unclear. Recently, we found that *LHX6* is hypermethylated in lung cancer. In this study, we analysed its epigenetic regulation, biological functions, and related molecular mechanisms in lung cancer. Methylation status was evaluated by methylation-specific PCR and bisulfite genomic sequencing. *LHX6* mRNA levels were measured in relation to the methylation status. The effects of *LHX6* expression on tumourigenesis were studied *in vitro* and *in vivo*. *LHX6* was readily expressed in normal lung tissues without methylation, but was downregulated or silenced in lung cancer cell lines and tissues with hypermethylation status. Treatment of lung cancer cells with the demethylating agent 5-aza-2'-deoxycytidine restored *LHX6* expression. Moreover, *LHX6* hypermethylation was detected in 56% (52/93) of primary lung cancers compared with none (0/20) of the tested normal lung tissues. In lung cancer cell lines 95D and H358, forced expression of *LHX6* suppressed cell viability, colony formation, and migration, induced apoptosis and G1/S arrest, and inhibited their tumorigenicity in nude mice. On the other hand, knockdown of *LHX6* expression by RNA interference increased cell proliferation and inhibited apoptosis and cell cycle arrest. These effects were associated with upregulation of *p21* and *p53*, and downregulation of *Bcl-2*, *cyclinD1*, *c-myc*, *CD44*, and *MMP7*. In conclusion, our results suggest that *LHX6* is a putative tumour suppressor gene with epigenetic silencing in lung cancer.

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Subject Category: Cancer

Lung cancer is one of the most common malignancies and the leading cause of cancer-related death worldwide.¹ Although traditional cytology and imaging technology have important roles in the diagnosis of lung cancer, 67% of newly diagnosed lung cancer patients are still detected at an advanced stage.² Effective early diagnosis and targeted therapies to reduce mortality would benefit from a better understanding of the key molecular changes in normal cells, which lead to precancerous lesions and malignant tumour cells.

Lung carcinogenesis is a multi-phase process involving the accumulation of multiple genetic and epigenetic changes.^{3–6} Alterations in normal DNA methylation patterns are fundamental to the tumorigenic process that contributes to all of the typical hallmarks of a cancer cell.^{7,8} Hypermethylation of tumour suppressor genes is associated with epigenetically mediated gene silencing. Recently, an increasing number of genes inactivated by CpG island hypermethylation have been recognised as important components involved in multistep cancer development in humans.^{9–13} Characterisation of novel functional genes associated with promoter methylation may help us to understand the molecular mechanisms of alterations in tumour-suppressive pathways involved in lung carcinogenesis, and find better potential targets for the diagnosis and treatment of lung cancer.

The human *LIM homeobox domain 6 (LHX6)* gene is located on 9q33.2 and encodes a LIM homeodomain transcription factor involved in embryogenesis, more specifically in head development.¹⁴ *LHX6* activity is required for normal tangential and radial migration of GABAergic interneurons and specification of neuronal subtypes in the neocortex and hippocampus.¹⁵ *LHX6* and *LHX8* co-ordinately induce neuronal expression of Sonic hedgehog that controls the generation of interneuron progenitors.¹⁶ In addition, *LHX6* hypermethylation acts as a sensitive methylation marker in head and neck carcinomas and has an important role in early diagnosis of cervical cancer.^{17–19} Recently, we identified *LHX6* hypermethylation in lung cancer. However, the role of *LHX6* in lung cancer remains unclear. In the present study, we investigated the epigenetic regulation and biological function of *LHX6* and its molecular basis in lung cancer.

Results

LHX6 is downregulated or inactivated in lung cancer tissues and cell lines. First, we performed reverse transcription-polymerase chain reaction (RT-PCR) to detect *LHX6* mRNA expression in 10 primary lung cancer tissues and their corresponding adjacent non-tumour tissues.

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Abbreviations: 5-aza-dC, 5-aza-2'-deoxycytidine; *LHX6*, *LIM Homeobox domain 6*; BGS, bisulfite genomic sequencing; MSP, methylation-specific polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction

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As shown in Figure 1a, *LHX6* expression was downregulated in lung cancer tissues compared with that in the adjacent normal tissues. Next, we examined *LHX6* expression in nine lung cancer cell lines and the normal human bronchial epithelial (HBE) cell line by RT-PCR and quantitative RT-PCR. As shown in Figure 1b and Supplementary Figure S1, *LHX6* expression was reduced or silenced in eight (90%) cell lines, but was readily detected in H1395 cells and the normal cell line HBE. These results suggest aberrant gene silencing of *LHX6* in lung cancer.

LHX6 hypermethylation is associated with transcriptional silencing. To clarify whether expression of the *LHX6* gene was regulated by DNA hypermethylation, we first analysed the methylation status of *LHX6* in human cell lines by methylation-specific polymerase chain reaction (MSP). MSP region information is shown in Supplementary Figure S2a.

The reliability of the MSP results was verified by DNA sequencing (Supplementary Figure S2b). As shown in Figure 1c, we found that all lung cancer cell lines in our study showed hypermethylation, whereas HBE cells exhibited an unmethylated status. However, the methylation pattern was heterogeneous in the CpG island region. Fully methylated *LHX6* was detected in cell lines A549, 95D, and H1975, whereas *LHX6* was partly methylated in SPC-A-1, H358, H1650, LTEP, H1395, and H460 cell lines, which showed downregulation of *LHX6* expression. The methylation status was inversely correlated with the *LHX6* expression level in all lung cancer cell lines and HBE cells except in H1395 cells. These results suggest that *LHX6* hypermethylation is associated with transcriptional downregulation or silencing.

Next, we detected the *LHX6* methylation status in 93 cases of human primary lung cancer and 20 cases of normal

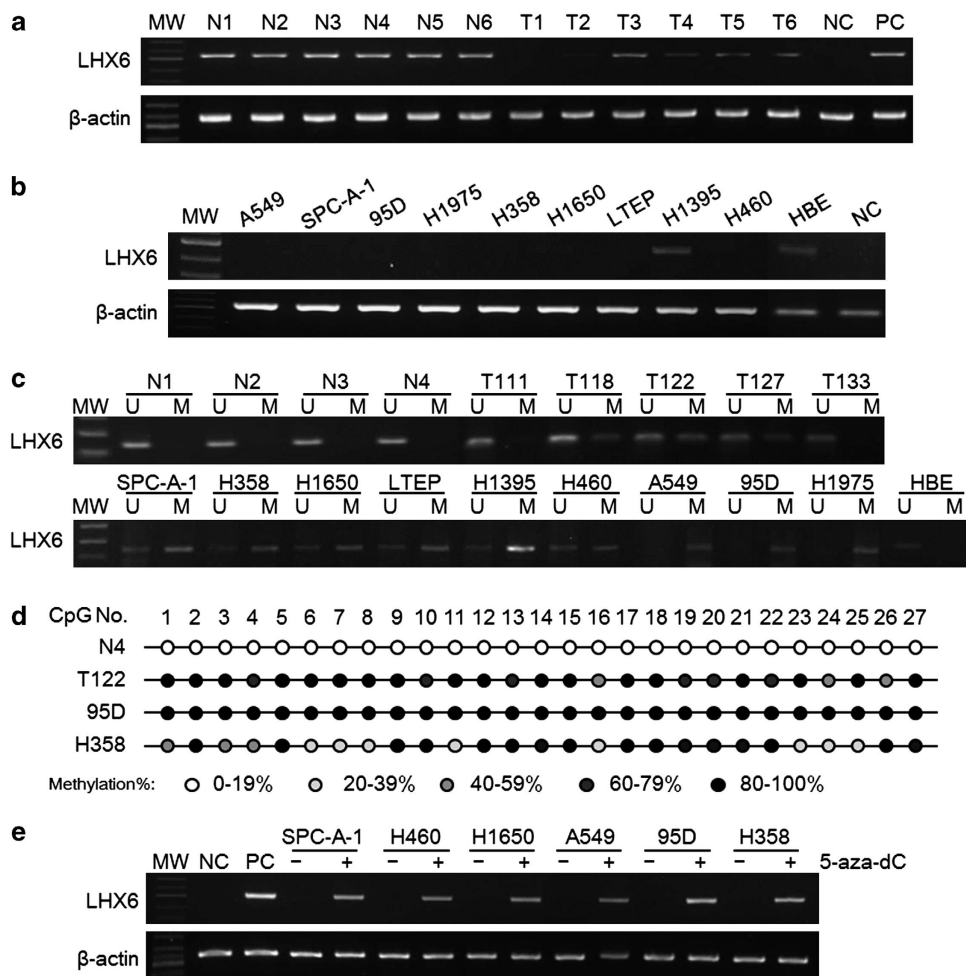


Figure 1 Epigenetic silencing of *LHX6* in lung cancer tissues and cell lines. (a) Representative results of *LHX6* mRNA expression in tissues. *LHX6* mRNA expression was significantly decreased in primary lung cancer tissues compared with that in the corresponding normal tissues as detected by RT-PCR. (b) mRNA expression of *LHX6* in lung cancer cell lines was determined by semi-quantitative RT-PCR with β -actin as the internal control. *LHX6* was weakly or not expressed in all lung cancer cell lines except for H1395. (c) Representative MSP results of the *LHX6* methylation status in human tissues and cell lines. *LHX6* was unmethylated in human normal lung tissues, but frequently hypermethylated in human lung cancer tissues and cell lines. MW, molecular weight; M, methylated product; U, unmethylated product; N, normal tissue; T, lung cancer tissue. (d) BGS analysis confirmed the methylation status of *LHX6* in human tissues and cell lines. *LHX6* was fully methylated in 95D cells, partially methylated in H358 and T122 cells, and unmethylated in normal tissue. (e) *LHX6* mRNA expression was restored in lung cancer cell lines after treatment with the demethylation agent 5-aza-dC. ‘-’ indicates DMSO control; and ‘+’ indicates 5-aza-dC

lung tissue. Using MSP, we found *LHX6* hypermethylation in 52 (including 43 samples with partial methylation and 9 samples with fully methylation) out of 93 (56%) lung cancer samples and no *LHX6* hypermethylation (0/20) in normal lung tissues (representative results are shown in Figure 1c). Next, we analysed the association of the *LHX6* methylation status and clinicopathological characteristics in 62 patients with lung cancer. However, as shown in Supplementary Table S1, there was no correlation between *LHX6* methylation and clinicopathological features such as age, gender, histological type, pathological stage, or differentiation status.

We performed bisulfite genomic sequencing (BGS) to provide a detailed map of the DNA methylation pattern within the CpG island region of the *LHX6* gene. The size of the PCR product was 324 bp and included the transcriptional start site (−99 to +225 bp; Supplementary Figure S2a). Representative results are shown in Figure 1d. In 27 CpG sites of this region, *LHX6* was fully hypermethylated in 95D cells. In H358 cells and lung cancer tissue T122, *LHX6* was partially methylated. As expected, *LHX6* was unmethylated in HBE cells expressing *LHX6*. These results are in agreement with those of MSP, and suggest that DNA methylation may be responsible for loss of *LHX6* expression.

5-aza-2'-deoxycytidine restores *LHX6* expression. To further test the hypothesis that loss of *LHX6* expression is caused by hypermethylation of the *LHX6* gene, lung cancer cell lines were treated with the demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC). Representative results of semi-quantitative RT-PCR and quantitative RT-PCR analyses are illustrated in Figure 1e and Supplementary Figure S3, respectively. As expected, lung cancer cell lines treated with 5-aza-dC, which initially showed high levels of *LHX6* methylation, were induced to express *LHX6*. Quantitative RT-PCR analyses showed that the ratio of *LHX6* to β -actin transcripts was increased several fold in the 5-aza-dC-treated group compared with that in the control group. These results strongly indicated that hypermethylation is responsible for defective expression of *LHX6* in lung cancer.

***LHX6* inhibits cell growth *in vitro* and *in vivo*.** To evaluate the functional significance of *LHX6* in lung cancer, we examined growth suppression by overexpression of *LHX6* in 95D and H358 cells that showed loss of *LHX6* expression and CpG island hypermethylation. The transfection efficiency is shown in Supplementary Figure S4. Re-expression of *LHX6* in these cell lines was evidenced by RT-PCR and western blot analyses (Figure 2a). The results of the cell proliferation assay indicated that ectopic expression of *LHX6* significantly inhibited the growth of 95D and H358 cells compared with that in the control groups (Figure 2b). To confirm the inhibitory effect of *LHX6* on the growth of lung cancer cell lines, we used a colony formation assay using 95D and H358 cells. Compared with control vector-transfected cells without *LHX6* expression, we found a greatly reduced number of colonies formed by *LHX6*-transfected 95D and H358 cells ($P < 0.01$; Figure 2c). These data further demonstrated that *LHX6* inhibits lung cancer cell proliferation.

We confirmed the effect of *LHX6* on cell proliferation by examining *LHX6* overexpression in a tumorigenesis assay using nude mice. Tumour growth of H358 cells overexpressing *LHX6* was significantly reduced compared with that of control cells (Figure 2d). The mean tumour weight was significantly less in nude mice injected with *LHX6*-overexpressing H358 cells compared with that in mice injected with control vector-transfected cells ($P < 0.01$; Figure 2d). These results suggest that *LHX6* is a tumour suppressor gene that negatively regulates tumour growth both *in vitro* and *in vivo*.

***LHX6* induces apoptosis and cell cycle arrest.** We next determined whether inhibition of tumour cell growth by *LHX6* is related to apoptosis and cell cycle arrest. First, we performed an apoptosis assay by flow cytometry (Figure 3a). We found that ectopic expression of *LHX6* caused a significant increase in early apoptosis and late apoptosis compared with that of control vector-transfected 95D (Figure 3a(1)) and H358 (Figure 3a(2)) cells. In addition, morphological observation and Hoechst 33258 staining were performed to definitively establish the effect of *LHX6* expression on apoptosis (Supplementary Figure S5).

Next, the effect of *LHX6* expression on the cell cycle was evaluated by flow cytometry (Figure 3b). Among 95D cells without exogenous *LHX6* expression, the percentages of cells in G1, S, and G2 phases were 43.26 ± 1.10 , 40.39 ± 0.44 , and $16.35 \pm 0.66\%$, respectively. In contrast, among 95D cells with re-expression of *LHX6*, the percentages of cells in G1, S, and G2 phases were 51.96 ± 1.00 , 34.86 ± 0.52 , and $13.18 \pm 0.48\%$, respectively (Figure 3b(1)). This trend was also found in H358 cells. The percentages of H358 cells in G1, S, and G2 phases were 52.71 ± 0.47 , 32.95 ± 1.22 , and $14.35 \pm 0.74\%$, respectively. In contrast, among H358 cells with re-expression of *LHX6*, the percentages of cells in G1, S, and G2 phases were 62.48 ± 1.19 , 26.70 ± 0.76 , and $10.83 \pm 0.43\%$, respectively (Figure 3b(2)). These results demonstrate a reduction in the percentages of cells in S and G2 phases, and retention of 95D and H358 cells with restoration of *LHX6* expression in G1 phase ($P < 0.05$).

***LHX6* expression induces cell migration.** The effect of *LHX6* expression on lung cancer cell motility was investigated by a wound healing assay. *LHX6* transfectants spread along wound edges much slower than that of control vector transfectants (Figure 4). Quantitative analyses at 48 h confirmed a significant reduction in wound closure by *LHX6*-transfected 95D (Figure 4a) and H358 (Figure 4b) cells compared with that of empty vector-transfected control cells.

Knockdown of *LHX6* promotes cell proliferation. To confirm the inhibitory effect of *LHX6* expression on cell growth, we knocked down *LHX6* expression in the *LHX6*-expressing cell line HBE by siRNA. Real-time quantitative RT-PCR showed that *LHX6* expression was significantly decreased by *LHX6* siRNA (Figure 5a). We found that knockdown of *LHX6* promoted the growth of HBE cells by a CCK-8 cell viability assay (Figure 5b). Accordingly, knockdown of *LHX6* significantly increased colony formation by

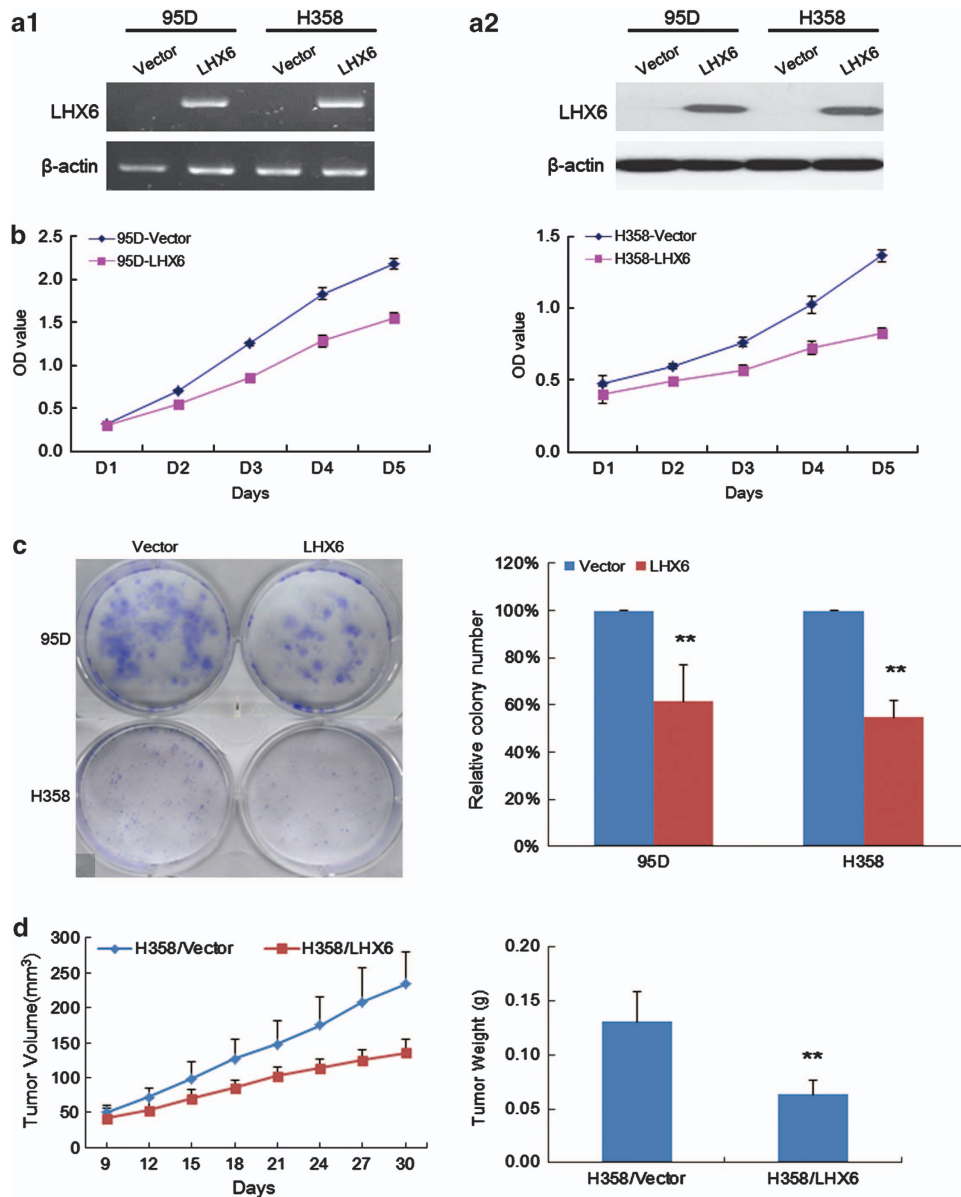


Figure 2 LHX6 inhibits cell growth. (a) Re-expression of LHX6 mRNA and protein in transfected cell lines 95D and H358 was confirmed by RT-PCR (a1) and western blotting (a2), respectively. (b) LHX6 significantly inhibited the proliferation of 95D and H358 cells as shown by CCK-8. These data are shown as the mean ± S.D. of three independent experiments. (c) Colony formation assays were performed to confirm the effect of LHX6 on cell growth. Quantitative analysis of colony numbers is shown in the right panel. Compared with empty vector transfectants, a significant reduction of colony numbers was observed for LHX6 transfectants. (d) Inhibition of tumour growth by LHX6 expression *in vivo*. Growth curves of tumours in nude mice showed that LHX6 transfectants grew slower than empty vector transfectants. ** $P < 0.01$

more than 60% compared with that of cells transfected with control siRNA (Figure 5c). These results further indicated that LHX6 acts as a potential tumour suppressor gene in lung cancer.

Next, we further examined the effect of LHX6 knockdown on cell cycle regulation. Among HBE cells transfected with negative control siRNA, 70.92 ± 2.37% were in G1 phase, 19.18 ± 1.76% were in S phase, and 9.91 ± 0.61% were in G2 phase. In contrast, among LHX6 siRNA-transfected HBE cells, 61.65 ± 1.99% were in G1 phase, 21.57 ± 0.85% were in S phase, and 16.79 ± 1.14% were in G2 phase ($P < 0.05$) (Figure 5d). Thus, LHX6-targeted siRNA decreased the number of cells in G1 phase, whereas significantly increasing

the number of cells in G2 phase, suggesting that loss of LHX6 expression increases cellular proliferation.

Finally, we examined the effect of LHX6 knockdown on apoptosis by flow cytometry. Our results showed that both early apoptotic cells (7.26 ± 1.05% versus 4.96 ± 0.63%) and late apoptotic cells (23.00 ± 1.41% versus 19.75 ± 1.63%) among LHX6 siRNA-transfected HBE cells decreased significantly compared with those among control siRNA-transfected HBE cells ($P < 0.05$) (Figure 5e).

Molecular targets of LHX6 in cell lines. To gain insights into the molecular mechanisms of the tumour-suppressive effect of LHX6, LHX6-modulated downstream target genes

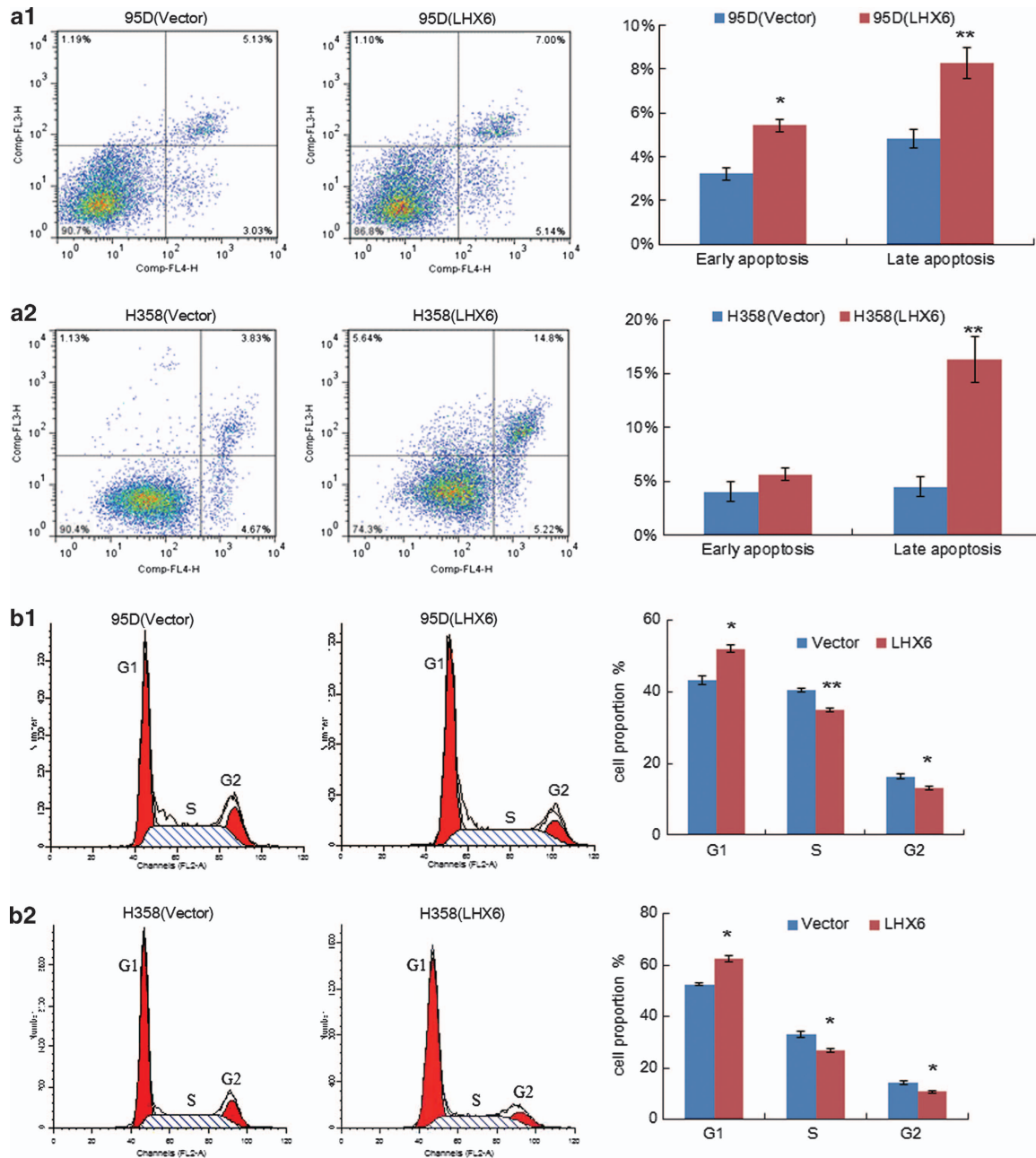


Figure 3 Effect of *LHX6* expression on apoptosis and the cell cycle. (a) Flow cytometric assays with Annexin V-APC and 7-AAD double staining of 95D (a1) and H358 (a2) cells. Left panel shows representative dots plots and right panels show the quantitative analysis. (b) Cell cycle analysis by flow cytometry. The distribution of empty or *LHX6* expression vector-transfected 95D (b1) and H358 (b2) cells in cell cycle phases. The percentages are represented by bar graphs. Each experiment was repeated three times. * $P < 0.05$; ** $P < 0.01$

were characterised by quantitative RT-PCR analysis of H358 cells stably transfected with *LHX6*. As shown in Figure 6a, *LHX6* increased expression of the pro-apoptotic gene *p53* and downregulated the anti-apoptotic gene *Bcl-2*. *LHX6* also enhanced expression of *p21*, a cell cycle regulator. *LHX6* exerted an anti-proliferative effect by downregulating expression of cell proliferation genes *cyclinD1* and *c-myc*, which are both important G1 phase regulators. Moreover, inhibition of cell migration by *LHX6* resulted from downregulation of *CD44* and *MMP7* expression. On the other hand, loss of *LHX6* expression had the opposite effect on regulation of these

genes (Figure 6b). The results suggest that *LHX6* acts as tumour suppressor by regulating the expression of important genes involved in proliferation, apoptosis, and migration pathways (Figure 6c).

Discussion

Several lines of evidence support the hypothesis that epigenetic changes by DNA methylation have an important role in lung carcinogenesis.^{20–23} Recently, epigenetic silencing of many novel genes that function as putative tumour

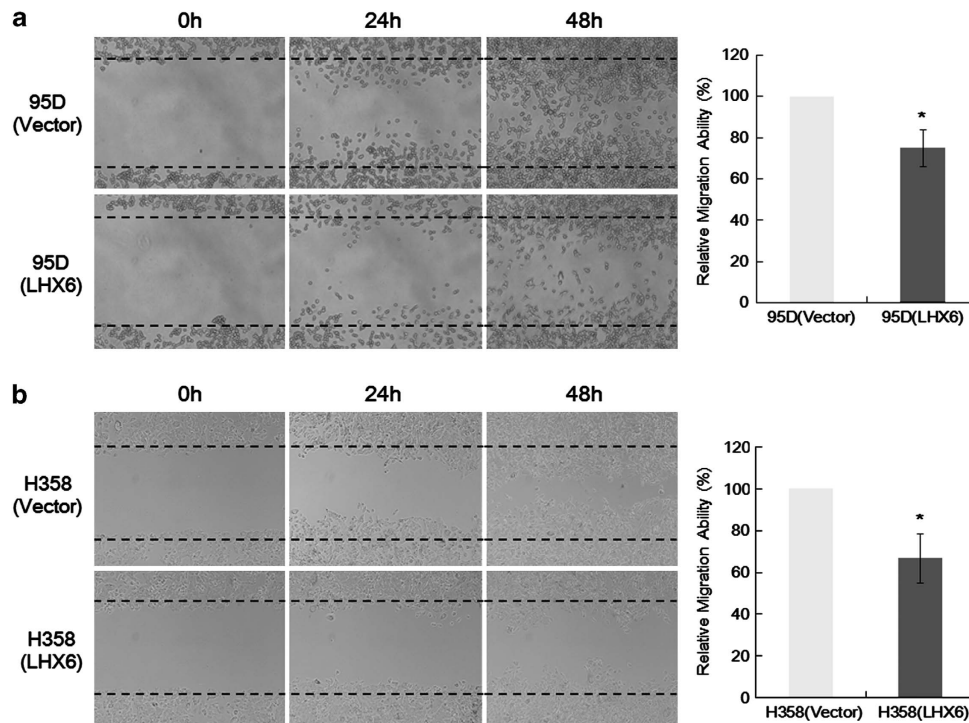


Figure 4 Cell motility as determined by a wound healing assay. The ability of *LHX6* transfectants to spread along wound edges in 95D (a) and H358 (b) cells was significantly reduced compared with that of control cells. Quantification of the migrated cells is shown in the right panel. All data represent the mean \pm S.D. of three independent experiments. * $P < 0.05$

suppressor genes has been reported to contribute to human lung cancer.^{9–11,21,23} In this study, we identified a typical CpG island located in the transcription region of the *LHX6* gene, which showed a hypermethylation status in lung cancer. To our knowledge, this is the first study of epigenetic regulation of *LHX6* expression and its function in lung cancer.

In this study, we found that *LHX6* hypermethylation is frequent and cancer specific in lung cancer. Our results suggest that *LHX6* methylation is associated with tumorigenesis. In accordance with our study, methylation levels of the *LHX6* promoter are also strongly associated with cervical carcinogenesis and cancer development.^{18,19} This epigenetic alteration of the *LHX6* gene begins at a relatively early stage, suggesting its potential as a biomarker for early diagnosis and prevention of head and neck carcinomas, and cervical cancer.^{17,18}

We also found that the reduction of *LHX6* expression was related to CpG island methylation of *LHX6*. Furthermore, a high dose of 5-aza-dC stimulated *LHX6* re-expression in most of the lung cancer cell lines, suggesting that CpG island methylation is the predominant regulatory mechanism of *LHX6* inactivation in lung cancer. However, compared with 5-aza-dC alone, exposure to various concentrations of 5-aza-dC with trichostatin A increases *LHX6* expression more significantly in cervical cell lines.^{18,19} Thus, other epigenetic modifications such as histone modifications may be involved in silencing *LHX6* expression in lung cancer. Accordingly, studies on the human *LHX3* gene have shown that cell-specific expression of two different transcripts of *LHX3* is driven by epigenetic and genetic regulatory systems.²⁴ Considering that *LHX3* and *LHX6* belong to the same family,

it is plausible to assume that human *LHX6* expression may be regulated in a similar manner. In the future, the mechanism of *LHX6* regulation requires further study.

LHX6 is a putative transcriptional regulator that controls the differentiation and development of neural and lymphoid cells, particularly in the central nervous system.^{14,15} In addition to its established role in muscle development, little is known about the function of *LHX6*, especially in cancer. Recently, several other *LHX* genes have also been implicated in tumorigenesis.^{25–29} *LHX4* may act as a tumour suppressor by downregulation of alpha-fetoprotein expression in hepatocarcinogenesis.²⁵ Inactivation of *LHX8* expression reduces the transcription of pro-apoptotic genes *bax*, *caspase-2*, and *caspase-3* in mouse oocytes.²⁶ Epigenetic alteration of the *LHX9* gene is involved in glioma cell invasiveness and migration.²⁷ Hypermethylation-mediated reduction of *LMX1A* expression inhibits tumorigenesis, epithelial-to-mesenchymal transition, and stem-like properties of the epithelia in gastric and ovarian cancers.^{28,29} In addition, several novel epigenetically silenced genes have been found to suppress cell growth in numerous types of cancer.^{30–35} These studies have suggested that *LHX6* may have a potential tumour suppressor role in lung cancer.^{25–35}

Thus, the tumour suppressor role of *LHX6* in lung cancer was investigated both *in vitro* and *in vivo*. Ectopic expression of *LHX6* in the silenced 95D and H358 cell lines showed a significant growth suppressive effect by inhibition of cell proliferation and colony formation. A decrease of tumour growth by *LHX6* was confirmed by the reduction of tumourigenesis in nude mice. On the other hand, siRNA-mediated knockdown of *LHX6* in HBE cells resulted in significantly

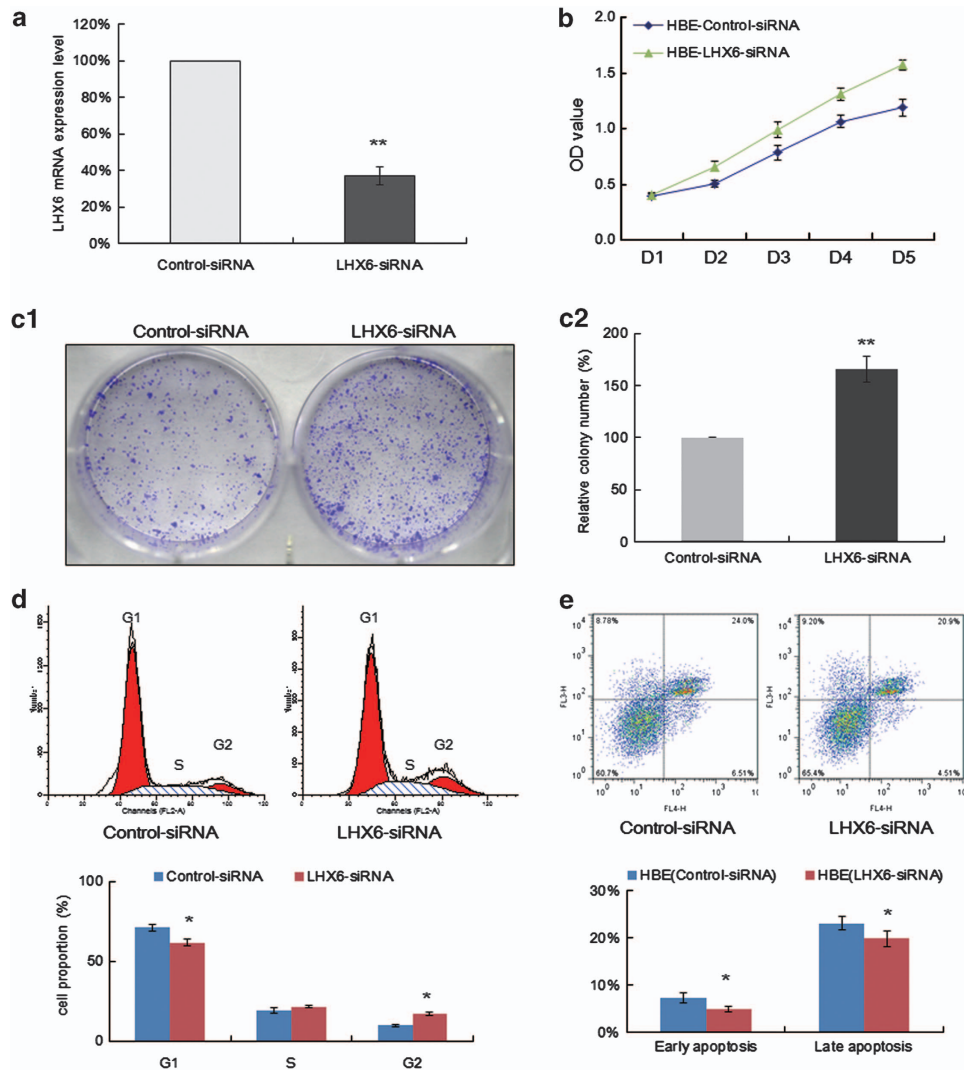


Figure 5 Effect of *LHX6* knockdown on cell growth. (a) *LHX6* mRNA-targeting siRNA and a non-targeting control siRNA were transfected into *LHX6*-expressing HBE cells. The efficiency of *LHX6* knockdown was examined by real-time RT-PCR. (b) siRNA-mediated knockdown of *LHX6* induced cell proliferation as shown by CCK-8. (c) Colony formation assays confirmed the effect of *LHX6* expression on cell growth. Colony numbers were increased by knockdown of *LHX6*. Colonies were photographed under a phase-contrast microscope (left, c1). Average numbers of colonies are represented by the bar graph (right, c2). Each experiment was repeated three times. (d) Cell cycle analysis by flow cytometry. The distribution of siRNA negative control- and *LHX6* siRNA-transfected HBE cells in cell cycle phases. The percentages are represented by the bar graph. Each experiment was repeated three times. (e) The effect of *LHX6* knockdown on apoptosis was analysed by flow cytometry. * $P < 0.05$; ** $P < 0.01$

increased cell proliferation. These results indicate that *LHX6* functions as a tumour suppressor in lung carcinogenesis. Similar to our findings, overexpression of *LHX6* in cervical cancer cells causes a reduction of cell proliferation and suppresses the tumorigenic phenotype, further demonstrating the growth suppressive effects of *LHX6* expression and suggesting that it may be a novel tumour suppressor gene in cervical cancer.¹⁹

LHX6 is a LIM homeobox transcription factor expressed during embryogenesis. However, the molecular mechanisms that regulate *LHX6* transcriptional activity are unknown. Previously, *LHX6* and an interaction with *PITX2* were found to regulate cell proliferation in the cervical loop and promote cell differentiation in the anterior region of the incisor.³⁶ Recently, Zhang *et al.*³⁷ demonstrated that *LHX6* directly interacts with *PITX2* to inhibit *PITX2* transcriptional activity

and activation of multiple promoters. *PITX2* directly interacts with β -catenin to synergistically regulate *LEF-1* expression, which regulates the activity of several growth control genes.^{38,39} These findings demonstrate new molecular mechanisms of *LHX6* for normal craniofacial and tooth development. In our study, we also found that *LHX6* decreased the expression of Wnt signalling target genes *cyclinD1*, *c-myc*, *CD44*, and *MMP7*. Our results suggest that *LHX6* may be an important negative regulator of the WNT signalling pathway that is involved in many biological processes including embryogenesis and carcinogenesis.⁴⁰ However, further definition and investigation of *LHX6*-regulated genes are critical to understand the pathways through which *LHX6* exerts its tumour-suppressive activity.

In conclusion, we have identified *LHX6* as a novel functional tumour suppressor gene that is silenced by CpG island

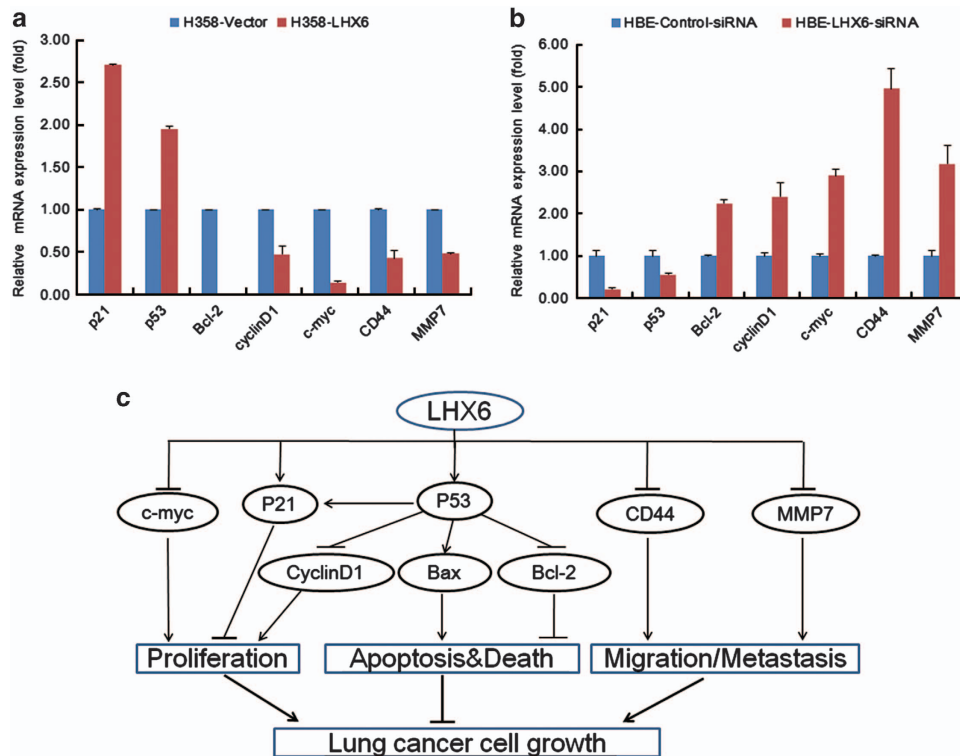


Figure 6 Effect of LHX6 on its downstream genes in cancer pathways. (a) Ectopic expression of *LHX6* enhanced the expression of *p21* and *p53*, and decreased the expression of *Bcl-2*, *cyclinD1*, *c-myc*, *CD44*, and *MMP7*. (b) Expression of *p21* and *p53* decreased, whereas the expression of *Bcl-2*, *cyclinD1*, *c-myc*, *CD44*, and *MMP7* increased in HBE cells with knockdown of *LHX6*. (c) Schematic diagram for the mechanisms of *LHX6* as a tumour suppressor

methylation in lung cancer. *LHX6* contributes to the suppression of tumorigenesis by decreasing cell proliferation and inducing apoptosis and cell cycle arrest by regulating several important signalling pathways. *LHX6* methylation may serve as a potential epigenetic biomarker for early diagnosis of patients with lung cancer.

Materials and Methods

Cell lines and tissue samples. Lung cancer cell lines (A549, SPC-A-1, 95D, H1975, H358, H1650, LTP, H1395, and H460) and an immortalised HBE cell line were obtained from the American Type Culture Collection (Manassas, VA, USA) and Cell Biology Institute of Chinese Academy of Science (Shanghai, China). All cell lines were cultured in RPMI-1640 medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% foetal bovine serum (Gibco BRL) and incubated in a humidified atmosphere with 5% CO₂ at 37 °C.

A total of 93 primary lung cancer tissues and 20 normal lung tissues were obtained during biopsy before any therapeutic intervention at the Affiliated Xi'an Hospital of the Third Military Medical University. The tumours were classified according to the WHO histological typing of lung tumours, and staged following the TNM classification of malignant tumours defined by the International Union against Cancer. All experiments and procedures were approved by the Clinical Research Ethics Committee of the Third Military Medical University.

RNA extraction, RT-PCR, and real-time quantitative RT-PCR analyses. Total RNA was extracted from cells and tissues with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesised from 1 µg total RNA using a PrimeScript RT reagent Kit with gDNA Eraser (Takara, Otsu, Japan). RT-PCR and real-time quantitative RT-PCR analyses were performed as described previously with the housekeeping gene β -actin used as an internal control.^{41–43} Primer sequences are listed in Supplementary Table S2.

DNA extraction, MSP, and BGS. Genomic DNA from cell lines and tissues was isolated by a DNA extraction kit (Promega, Madison, WI, USA) and chemically modified using an EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. Primer pairs used for MSP and BGS are listed in Supplementary Table S2. For MSP, the protocol was carried out as reported previously.^{44–46} For BGS, the PCR products were purified and inserted into a pGEM-T easy vector (Promega). Ten colonies were chosen randomly for sequencing.

Demethylation agent 5-aza-dC treatment. Demethylation was performed as reported previously.⁴¹ Briefly, lung cancer cells were seeded at a density of 1×10^6 cells per ml in 10-cm dishes. After 24 h of culture, cells were treated with 10 µM 5-aza-dC (Sigma, St. Louis, MO, USA) for 3 days. Control cells were treated in parallel with DMSO for each time point. The medium was replaced every day. Cells were harvested and mRNA expression of *LHX6* was analysed by RT-PCR and quantitative RT-PCR.

Construction of the *LHX6* overexpression vector. cDNA corresponding to the full-length human *LHX6* gene was verified by sequencing and subcloned into the mammalian expression vector pIRES2-EGFP (Invitrogen). Lung cancer cell lines were transfected using X-treme Gene HP DNA transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. Clones with stable transfection of *LHX6* or empty vector were established with G418 (Invitrogen) selection for 2–3 weeks. Gene and protein expression of *LHX6* was confirmed by RT-PCR and western blot analyses.

Knockdown of *LHX6* by siRNA. Four siRNA sequences and a negative control sequence of the siRNA annealing sequence were designed, synthesised, and then subcloned into pcDNA6.2 GW/EmGFP siRNA vectors (Invitrogen). The HBE cell line with *LHX6* expression was transfected with the vectors carrying *LHX6* or negative control siRNAs. Knockdown efficiency was evaluated at 48 h after transfection by real-time quantitative RT-PCR. The siRNA with the highest knockdown efficiency was used for functional studies. Stably transfected cells with *LHX6* knockdown were selected with 0.6 mg/ml Blastidicin S HCl (Invitrogen) for colony formation assays.

Cell viability assay. Briefly, 8×10^3 cells per well were seeded in 96-well plates and transiently transfected with pIRES2-EGFP-LHX6, LHX6 siRNA, or control vectors. After 1–5 days of transfection, cell viability was evaluated by Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Data were expressed as the optical density measured at a wavelength of 450 nm. Experiments were carried out in triplicate.

Colony formation assay. For the overexpression assay, 95D and H358 cells (1×10^5 cells/well) were seeded in 24-well plates and transfected with pIRES2-EGFP-LHX6 or empty vectors. For the knockdown assay, HBE cells were transfected with LHX6 siRNA or control vectors. At 2 days post transfection, cells were passaged at a 1:20 ratio into six-well plates containing medium with G418 or Blastidicin S HCl. After culturing for 14–21 days, cells were fixed with 4% paraformaldehyde and then stained with crystal violet. Colonies with more than 50 cells per colony were counted. All experiments were conducted three times in triplicates.

Hoechst 33258 staining. Hoechst 33258 staining was carried out at 48 h post transfection. The cells were fixed with 4% paraformaldehyde for 15 min, washed with PBS, and then stained with Hoechst 33258 (Beyotime, Jiangsu, China) for 5 min. After two washes with PBS, morphological changes of apoptotic nuclei were observed under a fluorescence microscope with excitation and emission at 346 and 460 nm, respectively.

Flow cytometry. Cells with overexpression or knockdown of LHX6 were fixed in 70% ethanol and stained with 15 μ g/ml propidium iodide at 4 °C in the dark. The cells were then analysed by a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Cell cycle profiles were determined using ModFitLT software (Becton Dickinson, San Diego, CA, USA). Apoptosis was determined by dual staining with an Annexin V-APC and 7-AAD Apoptosis Detection kit (Keygen, Nanjing, China) according to the manufacturer's instructions, and then analysed by FlowJo software (TreeStar, San Carlos, CA, USA).

Wound healing assay. Cell migration was assessed by a scratch wound assay. Briefly, 95D and H358 cells (5×10^5 cells/well) stably transfected with pIRES2-EGFP-LHX6 or empty vector were cultured in six-well plates. At 90% confluence, three scratch wounds across each well were made using a P-200 pipette tip. At 0, 24 and 48 h of culture, images were taken of the wound closure areas. The experiment was performed in triplicate wells and repeated three times.

In vivo tumorigenicity. H358 cells (5×10^6 cells in 0.2 ml PBS) stably transfected with pIRES2-EGFP-LHX6 or empty vector were injected subcutaneously into the dorsal flank of 4-week-old female Balb/c nude mice ($n = 4$ per group). Tumour volume (mm^3) was estimated by measuring the longest (a) and shortest (b) diameter of the tumour and calculated as $ab^2/2$. All experimental procedures were approved by the Animal Ethics Committee of the Third Military Medical University.

Western blot analysis. Total protein was extracted from cell lines, and the protein concentration was measured by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples (40 μ g) were separated by 10% SDS-PAGE and then transferred to an equilibrated polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). After incubation at 4 °C overnight with a primary antibody against LHX6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then reacting with the secondary antibody, the proteins were detected by enhanced chemiluminescence (Amersham Corporation, Arlington Heights, IL, USA).

Statistical analysis. Statistical analyses were performed with SPSS 13.0 software (SPSS, Chicago, IL, USA). Data were expressed as the mean \pm S.D. Results were evaluated using the *t*-test, Fisher's exact test, and Mann-Whitney *U*-test. A *P*-value < 0.05 was considered significant.

Conflict of Interest

The authors declare no conflict of interest.

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