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Original Article

ZFX knockdown inhibits growth and migration of non-small cell lung carcinoma cell line H1299

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Abstract: ZFX (zinc finger transcription factor, X chromosome-linked) contributes to the maintenance of different types of stem cells and the progression of various cancers. We have previously reported that ZFX knockdown inhibits proliferation of glioma *in vitro* and *in vivo*. Since overexpression of ZFX in lung cancer tissue correlates with lymph node metastasis, we hypothesized that ZFX may play a role in lung cancer. In this study, we identified ZFX as a promoter of lung cancer growth and migration in a NSCLC (non-small cell lung carcinoma) cell line H1299. ZFX knockdown caused proliferation inhibition determined by MTT assay and colony formation assay, G0/G1 arrest of cell cycle and slightly increased proportion of apoptotic cells assessed by flow cytometry assay, decreased population of migrating cells showed by wound-healing assay, increased cell senescence evidenced by senescence-associated β-galactosidase staining. ZFX knockdown also led to decreased proportion of tumor bearing mice and reduced mean tumor volume in a subcutaneous tumor model. In addition, western blot showed that ZFX knockdown down regulated a set of proteins involved in proliferation, survival and motility. Altogether, these results suggest that ZFX may be a potential therapeutic target for NSCLC.

Keywords: ZFX, non-small cell lung carcinoma, growth, migration, senescence

Introduction

Lung cancer is the leading cause of cancer deaths in the world. As estimated by the American Cancer Society, 228,190 new cases of lung cancer are expected and 159,480 persons are projected to die from lung cancer in America in 2013 [1]. Though many treatments including surgical resection, chemotherapy, radiation and targeted therapy can prolong the survival time and the life quality in patients with lung cancer, the overall survival rate of 5 years after diagnosis is dismally 15% [2, 3]. Non-small cell lung carcinoma (NSCLC) constitutes 85% of the cases [2]. The molecular mechanism of the initiation and progression of NSCLC is poorly understood so far and warrants urgent description for developing intelligent therapeutic strategies.

The zinc finger transcription factor ZFX plays a key role in controlling the self-renewal of embryonic and adult stem cells [4, 5]. ZFX together with c-Myc and cofactors Cnot3 and Trim28 form a network that regulates the transcription of genes involved in cell cycle, cell death and cancer in mouse embryonic stem cells [6]. Its expression was upregulated in a variety of cancers. ZFX contributes to the rapid proliferation and apoptosis inhibition in some cancer cell lines such as glioblastoma, laryngeal squamous cell carcinoma (LSCC) and prostate cancer, and tumorigenesis of glioblastoma in nude mice [7-12]. It has also been reported that ZFX is overexpressed in disseminated gastric cancer cells in bone marrow. This high expression of ZFX correlates with lymph node metastasis [7, 10], suggesting its likely role in the regulation of cancer cell motility. In this study, lentivirus vector mediated RNAi was used to investigate the role of ZFX in NSCLC. When this manuscript was being prepared, another similar work based on the same hypothesis was published [10]. In that work, lentivirus-mediated short hairpin RNA

interference (shRNA) was also used to study the role of ZFX in NSCLC in 95D cells. The authors found that mRNA level of tumors were higher than their adjacent normal tissues from patients. They also found knockdown of ZFX by shRNA inhibited cell viability, reduced colony formation, and arrested cell cycle in G0/G1 phase in 95D cells. But they didn't perform the in vivo tests. Here, we found that ZFX knockdown inhibited the proliferation and migration, and induced the senescence in NSCLC cell line H1299, which was consistent with the above mentioned work. Whereas the expression of some proteins involved in cell cycle, apoptosis, and motility were changed. In addition, tumor formation ability of ZFX deprived H1299 cells were attenuated in a subcutaneous nude mice model.

Materials and methods

Cell culture and reagents

NSCLC cell lines 95D, A549, H1299, H460, SPC-A1 (Institute of Biochemistry and Cell Biology, Shanghai, China) were cultured in RMPI 1640 (Invitrogen, Carlsbad, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) at 37°C with 5% CO_2 in a humidified atmosphere.

We used antibodies specific for ZFX (Abcam, Cambridge, UK), Akt, c-Myc, MMP-2, p-Akt (Ser-473), p-ATM (S1981), p-ERK1/2 (Thr202/Tyr204), p-STAT3 (Tyr705), Survivin, ZFX, horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signaling Technology, Beverly, USA), Bcl-2, ERK1/2, Ki-67, STAT3 (Bioworld Technology, Nanjing, China), Cyclin B1, Cyclin D1, (Boster, Wuhan, China), Alexa Fluor 568 goat anti-mouse (Invitrogen) and GAPDH (Kangchen, Shanghai, China). Annexin V Apoptosis Detection Kit APC (eBioscience, San Diego, USA), lentiviral vector pLKD-CMV-GFP-U6-shRNA (Neuron Biotech, Shanghai, China), Lipofectamine 2000, Trizol (Invitrogen), propidium iodide (PI, Sigma-Aldrich, St. Louis, USA), protease inhibitor cocktail, RNase A (Thermo Scientific, Waltham, USA), phosphatase inhibitor (Sangon, Shanghai, China), RT M-MLV (Promega, Beijing, China), SYBR Premix Ex Taq (Takara, Dalian, China), PMSF, senescence β-galactosidase staining kit (Beyotime, Nantong, China) were used in this study.

Lentivirus-mediated shRNA knockdown

Lentiviral shRNA plasmids targeting ZFX and a scrambled shRNA plasmid were transfected into 293T cells using Lipofectamine 2000 together with the gag/pol packaging vector and the VSVG encoding plasmid in order to produce the lentiviruses. The knockdown efficiency of different lentiviral shRNA clones in cells was then determined by western blot analysis. The most efficient shRNA sequences were as follows: shZFX, CCGGGTCGGAAATTGATCCTTGTAACTCGAGTTACAAGGATCAATTTCCGACTTTTTTG; scrambled shRNA, CCGGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTTG.

Quantitative real-time PCR

RNA was extracted, reverse transcribed and analyzed by quantitative real-time PCR using the indicated primer sets as previously described [12].

Western blot

After collection, cells were lysed in lysis buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 1% NP-40, 150 mM NaCl, 1 mg/ml SDS, 0.25 mg/ml sodium deoxycholate) supplemented with 1×PMSF, 1×phosphatase inhibitor and 1×protease inhibitor cocktail. Western blot was carried out as previously described [12].

Immunofluorescence

Cells were plated onto glass coverslips in cultured medium for 24 h, then fixed with 4% paraformaldehyde, incubated with blocking buffer (3% BSA and 0.1% Triton in PBS) for 1 h, stained with ZFX antibody (cell signaling, #5419) overnight, followed by secondary antibody conjugated to Alexa-568 and Hoechst 33342. Fluorescence signals of different channels were observed using the Nikon eclipse Ti-S inverted microscope.

MTT assay

To measure the proliferation rate, cells were seeded onto 96-well plates in 150 μl of RMPI 1640 with 10% FBS, at a density of 1500 cells/well. Each day for the next five days, 100 μg of MTT was added to the corresponding well. After an additional 4 h in culture, 150 μl of DMSO

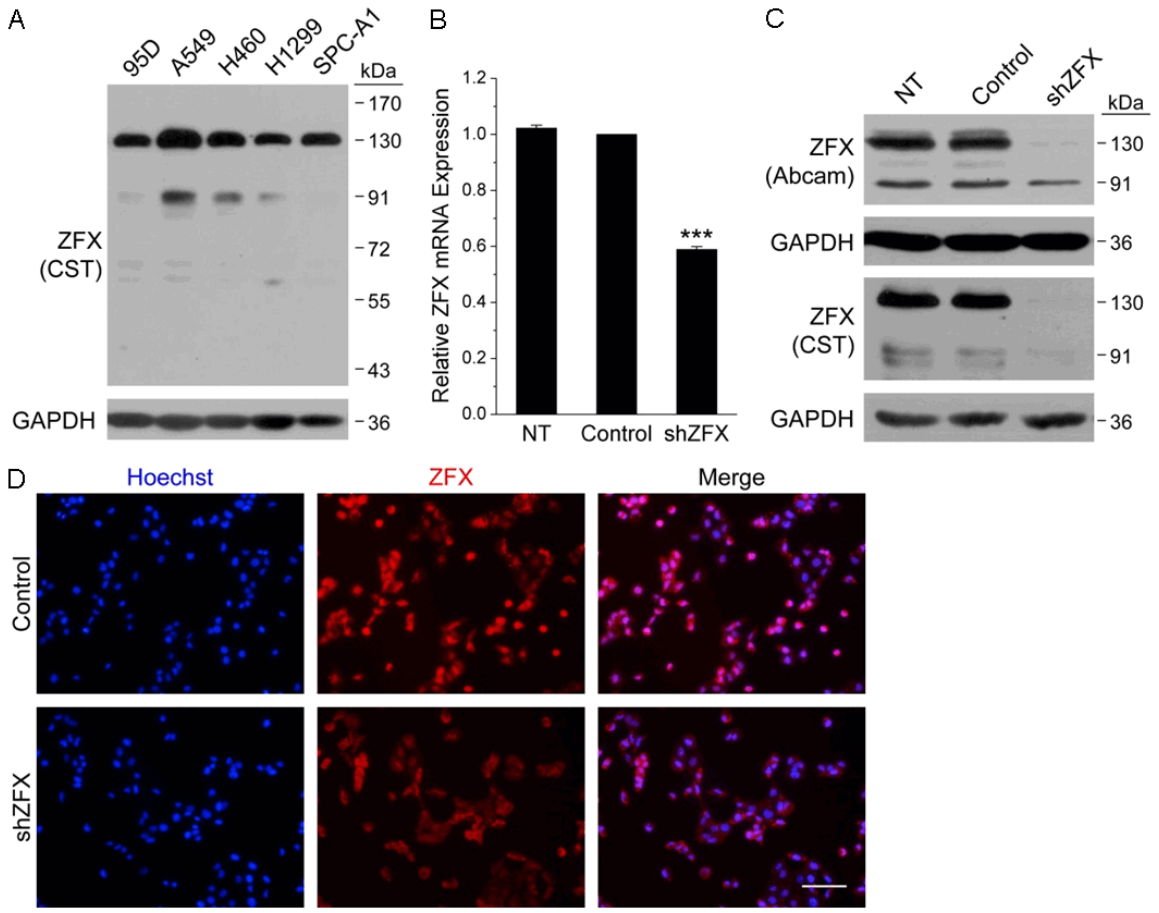

Figure 1. ZFX is expressed in human NSCLC cell lines and lentivirus-mediated RNAi downregulated ZFX expression in H1299 cells. A: Western blot analysis of ZFX protein levels in five human NSCLC cell lines. Protein sizes are indicated on the right-hand side. GAPDH was used as the loading control. B: Total RNA was isolated from non-transfected H1299 cells (NT) and from cells permanently infected with a scrambled shRNA plasmid (Control) or a ZFX shRNA plasmid (shZFX). Real-time quantitative PCR was then used to measure relative ZFX mRNA levels. C: H1299 cells from each of the three treatment groups were lysed and subjected to western blot analysis with anti-ZFX antibodies from Abcam or cell signal (CST). GAPDH was used as the loading control. D: Immunofluorescence images showing the expression level and subcellular localization of ZFX (red). Nuclei were counterstained with Hoechst (blue). Scale bar, 100 μM.

was added and optical density measured at 490 nm in a multiwell spectrophotometer.

Colony formation assay

Cells were seeded onto 6-well plates at a density of 300 cells/well in triplicate. After 10 days in culture, cells were fixed with methanol and stained with 0.5% crystal violet. The colony formation rate was calculated by dividing the number of colonies by the number of seeded cells.

Flow cytometry assay

Cells in the log phase of growth were harvested a week after lentivirus infection. Cell cycle distribution and apoptosis were determined by flow cytometry as perviously described [12].

Senescence-associated β-galactosidase staining

Cultured cells were fixed and stained according to the supplied instructions with a senescence β-galactosidase staining kit.

Wound-healing assay

Five days after infection, confluent monolayers of cells were scratched with a 20 μl pipette tip to induce a wound. The wounded edges were imaged using a Nikon Ti-S inverted microscope.

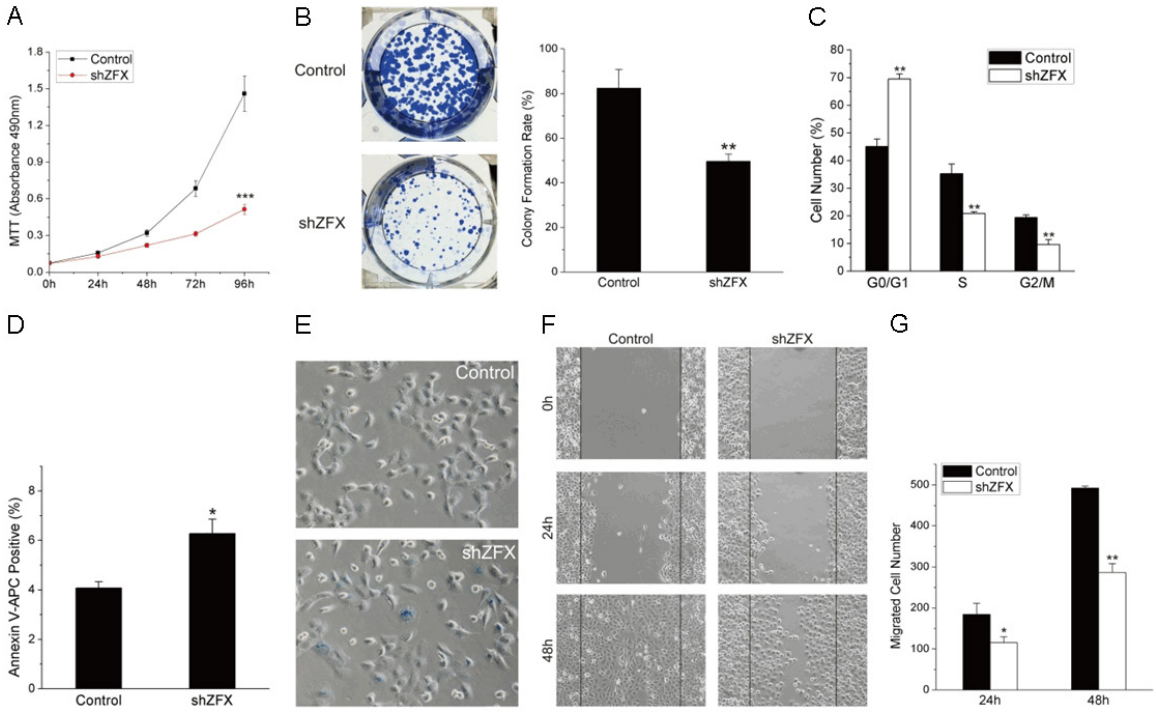

Figure 2. ZFX knockdown inhibited proliferation, survival and migration and induced apoptosis and senescence of H1299 cells in vitro. A: H1299 cells infected with control or shZFX were seeded on 96-well plates and proliferation rates were measured by MTT assay. Error bars represent SD; n=5. B: H1299 cells were seeded onto 6-well plates in triplicate, with 300 cells per well. Cells were stained with crystal violet and imaged (top panel) 10 days later to assess colony formation rates. Quantification of colony formation rates in each group is shown as histograms (bottom panel). Error bars represent SD; n=3. C and D: Cell cycle distribution and apoptosis of H1299 cells were analyzed by flow cytometry. The number of cells found in G0/G1, S and G2/M phases (C) and the Annexin V-APC positive apoptotic cells (D) are shown as percentages. Error bars represent SD; n=3. E: Senescent H1299 cells were detected by senescence-associated β-galactosidase staining (×200). Scale bar, 100 μm. F and G: Confluent monolayers of H1299 cells were scratched and imaged at 0 h, 24 h and 48 h. Black lines, position of the wounded edges at 0 h. The number of cells across the line at 24 h or 48 h in E was counted and shown as histograms (F). *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$ compared with control.

Images were collected with a ×10 objective 0, 24 and 48 h after wounding. Images were quantified by measuring the number of cells across the initial wounded edge (black line) at each time point.

Xenografted tumor model

For subcutaneous implantation, 12 six-week-old male BALB/c nude mice (Sippr-bk, Shanghai, China) were randomly divided into a ZFX knockdown group (n=6) and a control group (n=6). Mice were anesthetized with 70 mg/kg pelltobarbitalum natrium, and 2.5×10^6 H1299 cells infected with shZFX or scrambled shRNA (2 days after infection) were injected into the right flank near the upper extremity. Tumor length and width were measured with calipers every five days. Tumor volume was calculated using the formula: volume = length × width × width / 0.5. Seventy days after implantation, mice were anesthetized for imaging and sacrificed.

Statistical analysis

All values were expressed as mean±standard deviation of the mean (SD). Statistical significance between groups was measured by Student's t-test, with statistical significance defined as: *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$.

Results

Lentivirus mediated RNAi downregulated ZFX expression in H1299 cells

A monoclonal antibody specific for ZFX (cell signaling, #5419) was used to detect the ZFX protein level in five NSCLC cell lines (95D, A549,

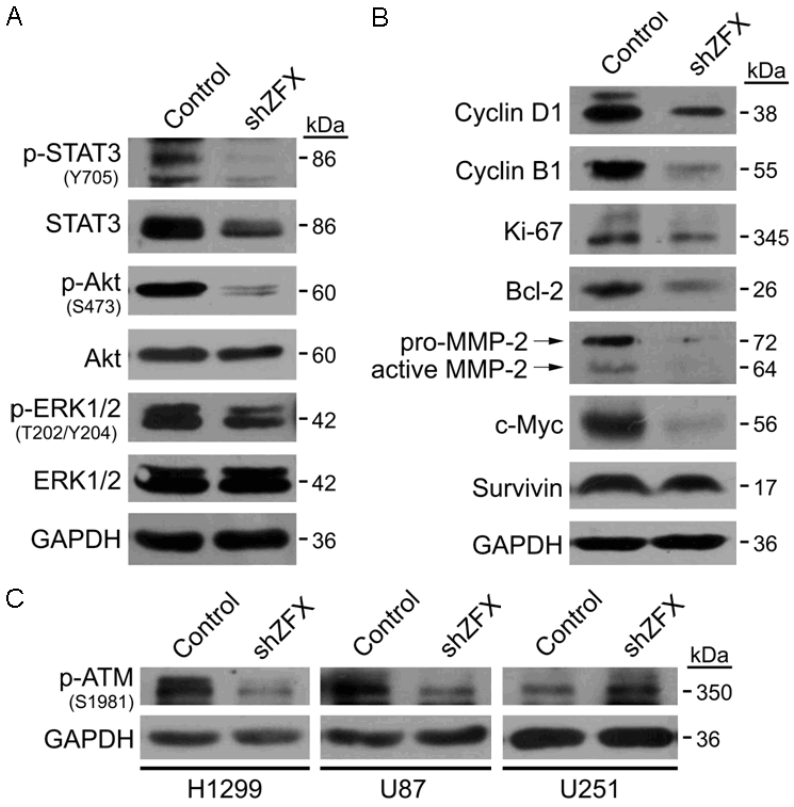

Figure 3. ZFX knockdown altered levels or activities of proteins related to proliferation, survival and motility. A: Western blot showing phosphorylated and total levels of STAT3, Akt and ERK1/2 in H1299 cells treated as indicated. B: Western blot showing levels of Cyclin D1, Cyclin B1, Ki-67, Bcl-2, MMP-2, c-Myc and Survivin in H1299 cells treated as indicated. C: Western blot showing phosphorylated levels of ATM in H1299, U87 and U251 cells treated as indicated. All experiments were repeated twice. GAPDH was used as the loading control.

H460, H1299, SPC-A1). The N-glycosylated 130 kDa subtype of ZFX was highly expressed in all these cell lines, whereas the non-N-glycosylated 91 kDa subtype was lowly expressed (**Figure 1A**). H1299 cells were selected to infect with lentivirus packaging ZFX shRNA-GFP (shZFX) plasmid or scrambled shRNA-GFP (control) plasmid those have been used in our pervious study [12]. The mRNA level of ZFX in ZFX shRNA interfered cells (shZFX cells) was significantly downregulated (**Figure 1B**). For protein level, the ZFX-130 kDa band was downregulated no matter detected by the antibody from abcam (ab85483) or from cell signaling (cell signaling, #5419). However the ZFX-91 kDa band was significantly downregulated only detected by the antibody from cell signaling (**Figure 1C**). Immunofluorescence showed that expression of ZFX in cell nucleus was weaker in shZFX cells than that in control cells (**Figure 1D**). These results demonstrated the efficiency of the shRNA to downregulate the expression of ZFX.

ZFX knockdown inhibited proliferation, survival and migration and induced apoptosis and senescence of H1299 cells in vitro

The MTT assay and the colony formation assay were used to determine the effect of ZFX knockdown on the proliferation and survival of H1299 cells, respectively. ZFX knockdown resulted in a 63.20% reduction in proliferation rate and a 39.76% reduction in colony formation rate (**Figure 2A** and **2B**). Cell cycle distribution and levels of apoptosis were further determined by flow cytometry. The percentage of H1299 cells expressing shZFX in G1 phase was increased by 24.37% compared with control (**Figure 2C**). The apoptotic level of shZFX cells was only slightly increased, from 4.08% to 6.28% (**Figure 2D**). This is distinct from studies showing dramatic apoptosis change in p53 deficient cell lines (Hep-2 [8], PC-3 [9], DU145 [9], U251 [11] and U373 [12]. Moreover, the senescence-associated β-galactosidase staining level was higher in shZFX cells than in control cells (**Figure 2E**). Migration ability was determined by wound-healing assay. Knockdown of ZFX in H1299 cells moderately lowered the migration rate (**Figure 2F** and **2G**). These results suggested that ZFX regulated the proliferation, survival, migration and senescence of H1299 cells *in vitro*.

ZFX knockdown altered expression levels or activities of proteins related to proliferation, survival and motility

Akt and MAPK signaling were shown to be regulated by ZFX in glioma cells before [12]. In NSCLC cell line H1299, the phosphorylation level of Akt was compromised following ZFX

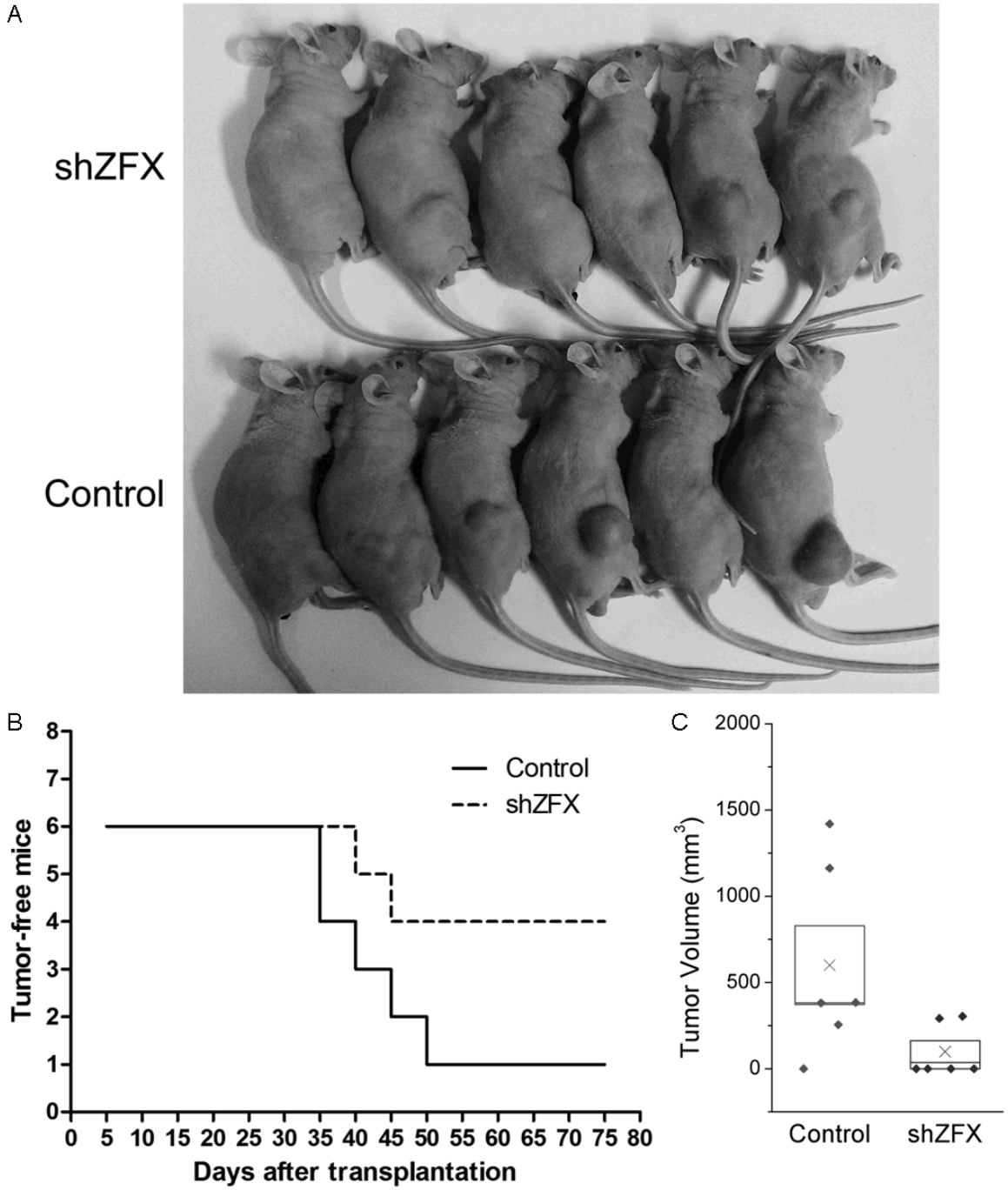

Figure 4. ZFX regulates growth of H1299 cells in a subcutaneous mouse model. A: Photograph showing the tumor-bearing and tumor-free mice of control and shZFX groups 70 days after transplantation. B: Kaplan-Meier curve showing the number of tumor-free mice of control and shZFX groups after transplantation. C: Box plots of average tumor volume in control and shZFX groups. In each group, the box represents SEM (n=6) and the line within the box indicates the median value. Tumor volume of each mouse is indicated by the symbol "◊" and mean of each group is indicated by the symbol "×".

knockdown. ZFX deficiency also led to the reduced level of STAT3 (**Figure 3A**). Besides, decreased levels of cell cycle regulators Cyclin D1 and Cyclin B1, proliferation marker Ki-67, apoptosis inhibitor Bcl-2, cell motility regulator MMP-2 and proto-oncogene c-Myc and Survivin were in accordance with the pronounced regression of proliferation, survival and motility

of shZFX H1299 cells (**Figure 3B**). Furthermore, ZFX knockdown led to the inhibition of ATM phosphorylation in apoptotic-resistant H1299 cells and U87 glioma cells while it had no effect in apoptotic-sensitive U251 glioma cells. These results advised ZFX regulated the expression of proteins related to lung cancer progression and the activity of some signaling pathways.

ZFX knockdown inhibited growth of H1299 cells in vivo

To investigate the role of ZFX in lung cancer development in vivo, control-infected or shZFX-infected H1299 cells were injected subcutaneously into the flank region of nude mice and tumor growth was monitored every 5 days. The tumor formation incidence, the tumor emergence time and the tumor volume of shZFX-infected cells were significantly reduced at the end of the experiment (**Figure 4A** and **4B**). Seventy days after transplantation, the mean tumor volume of control-infected cells was 600.53 mm^3 (interquartile range = 287.18-969.93 mm^3, n=6), but that of shZFX-infected cells was 99.29 mm^3 (interquartile range = 0-218.97 mm^3, n=6) (**Figure 4C**). These results suggested that ZFX could promote the lung cancer development in vivo.

Discussion

Previous studies uncovered the important role of the transcription factor ZFX in the proliferation and survival of a variety of cancer cells, including LSCC, prostate cancer cells and glioblastoma cells [8, 9, 11, 12]. In agreement with these findings, ZFX knockdown in NSCLC cell line H1299 by lentivirus-mediated RNA interference inhibits the viability and colony formation through cell cycle G0/G1 phase arrest and senescence. Additionally, ZFX knockdown resulted in the inhibition of migration of H1299 cells. Since H1299 cell line is derived from lymph node and ZFX mRNA expression associates with lymph node metastasis [10], the importance of ZFX in the motility and metastasis of NSCLC should be elucidated explicitly in future work. Downregulation of ZFX was associated with lower levels of Cyclin D1, Cyclin B1, Ki-67, Bcl-2, MMP-2 as well as c-Myc and Survivin which may contribute to the attenuated malignant phenotypes of H1299 cells.

Akt and MAPK signal pathways were influenced in glioma cells undergoing ZFX knockdown. Meanwhile, Akt was still a downstream element of ZFX in lung cancer, indicating its crucial role in mediating ZFX-dependent progression of different types of cancers. Interestingly, both phosphorylated STAT3 and total STAT3 were downregulated in ZFX knockdowned H1299 cells. STAT3 activation is frequently observed in cancers, including lung cancer. Transcriptional inactivation of STAT3 will lead to inhibition of proliferation and invasion and enhancement of apoptosis in lung cancer cells [13]. The mechanism through which ZFX regulates the expression of STAT3 and the role of STAT3 in mediating ZFX-dependent progression in lung cancer is worth studying.

Early studies reported that ZFX knockdown induces apoptotic cancer cell death [8, 9, 11]. In the current and recent works of ours, however, ZFX knockdown do not markedly alter the apoptosis level in H1299 and a glioma cell line U87, but alternatively promote senescence [12]. In addition, H1299 cells arrested in G0/G1 phase of the cell cycle undergo replicative senescence, whereas U87 cells arrested in G2 phase sustain premature senescence [14]. Besides, ATM phosphorylation is lower in apoptotic-resistant H1299 (p53 null) and U87 (p53 wild-type) cells but unchanged in apoptotic-sensitive U251 (p53 mutant) cells. ATM is a central member in the DNA damage response signaling and functions differently in apoptosis regulation depending on the cell environment. It can induce apoptosis via p53 dependent and independent pathways or activate prosurvival signaling upon genotoxic therapies [15, 16]. Low levels of DNA double-strand break caused by doxorubicin in ATM-deficient human fibroblast do not induce cell apoptosis but premature senescence [17]. Thus, it needs to be demonstrated that whether ATM determines the cell fate or just reflects the extent of DNA damage upon ZFX knockdown, and whether ZFX knockdown-induced apoptosis is independent of p53 status.

In summary, ZFX is ubiquitously expressed in NSCLC cell lines. Downregulation of ZFX attenuates tumorigenicity of H1299 cells in vitro and in a subcutaneous tumor model. Furthermore, phosphorylation of Akt and ATM and expression of STAT3 are inhibited in ZFX deficient H1299 cells, which suggested their potential roles in mediating ZFX regulated tumorigenicity of lung cancer cells. Thus, inhibition of ZFX is a potential treatment of NSCLC.

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

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