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Down-regulated SOX4 Expression Suppresses Cell Proliferation, Metastasis and Induces Apoptosis in Xuanwei Female Lung Cancer Patients

Yongchun Zhou,[1] Xicai Wang,[1] Yunchao Huang,[1]* Yan Chen,[1] Guangqiang Zhao,[2] Qian Yao,[1] Congguo Jin,[1] Youguang Huang,[1] Xin Liu,[1] and Guangjian Li[2]

[1]*Key Laboratory of Lung Cancer Research of Yunnan Province, The Third Affiliated Hospital of Kunming Medical University, Kunming 650118, PR China*

[2]*Department of Cardiothoracic Surgery, Yunnan Province Tumor Hospital and The Third Affiliated Hospital of Kunming Medical University, Kunming 650118, PR China*

ABSTRACT

The transcription factor SOX4 has functional importance in foetal lung maturation and tumorigenesis in a number of cancers. However, its biological functions in the progression of lung tumorigenesis remain unclear. In this study, we found that the expression levels of SOX4 mRNA and protein were significantly higher in Xuanwei female lung cancer tissues than in benign lung lesions. The patients with high expression of the SOX4 protein had a higher pathological grade, lymph node (LN) metastasis, poor tumor differentiation and worse prognosis than those patients with low expression of SOX4. Knockdown of the SOX4 gene in the Xuanwei female lung cancer cell line XWLC-05 resulted in apoptotic morphological changes, decreased cell proliferation, invasion and migration. Furthermore, knockdown of the SOX4 gene resulted in obvious sub-G1 peaks and induction of apoptosis through upregulation of caspase-3 expression, while in cells treated with a caspase-3 inhibitor, apoptosis induced by silencing SOX4 expression was inhibited. In vivo analysis in nude mice further confirmed that knockdown of SOX4 suppressed tumor growth. In conclusion, SOX4 appears to be an important tumor suppressor gene in the regulation of Xuanwei female lung cancer cell proliferation, apoptosis and metastases, and it may be a potential target for effective lung cancer therapy. J. Cell. Biochem. 116: 1007–1018, 2015.

KEY WORDS: SOX4; LUNG CANCER; APOPTOSIS; CASPASE-3

Lung cancer is the leading cause of cancer death worldwide and has been the most serious threat to human health and life [Ferlay et al., 2010]. The death rate of farmers with lung cancer in the Xuanwei area (Yunnan Province, China) is higher than those in other areas, and the mortality of lung cancer in the Xuanwei area has continued to rise since the 1970s [Mumford et al., 1987; Youlden et al., 2008]. Previous studies have indicated that genomic deletions and mutations existed in Xuanwei female lung cancer patients. These include single nucleotide polymorphisms of the folic acid metabolism related genes CBS, MTHFR and SLC19A1; the immune related gene FCER2 [Shen et al., 2005, 2009]; and P53 gene mutations [DeMarini et al., 2001].

SOX4, a member of the SOX (SRY-related HMG-box) transcription factor family, has been shown to play important roles in many developmental processes, including embryonic cardiac development, thymocyte development, and nervous system development [Wegner, 1999]. In addition, SOX4 is involved in many cellular processes through the activation of the Wnt, Hedgehog and Notch pathways [Ruebel et al., 2008] and enhancement of β-catenin/TCF activity [Sinner et al., 2007]. In recent years, researchers have focused on the role of the SOX4 gene in tumorigenesis and identified that it was overexpressed in liver cancer [Ahn et al., 1999], breast carcinoma [Zhang et al., 2012], central nervous system neoplasms [Lee et al., 2002], and salivary gland cancers [Frierson et al., 2002]. In addition, it

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*Correspondence to: Yunchao Huang, 519# Kunzhou Road, Kunming city, Yunnan Province, 650118, China. Email: huangych2001@aliyun.com

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has been illustrated that oestrogen and progesterone had bidirectional regulation effects on the expression of the SOX4 gene; overexpression of progestational hormones in the breast cancer cell line T-47D; increased SOX4 expression; and led to proliferation and tumor metastasis [Hunt and Clarke, 1999; McGowan and Clarke, 1999]. Therefore, there is a certain correlation between the SOX4 transcription factor and female tumors. Investigators also observed that endometrial carcinoma patients with high expression of oestrogen had low expression of SOX4 and a relatively good prognosis [Huang et al., 2009]. However, other studies confirmed that higher expression of SOX4 correlated with better survival in bladder tumor patients [Aaboe et al., 2006], and it promoted prostaglandin-induced apoptosis in hepatocellular carcinoma [Ahn et al., 2002], suggesting that SOX4 has a potential tumor-suppressive function. However, the precise mechanism by which SOX4 is involved in the process of promoting or suppressing tumorigenesis remains largely unknown.

Until now, researchers have not reached a unified conclusion, and the mechanisms of SOX4 in the development of lung carcinoma are not fully understood. According to the above data, we speculate that SOX4 may play an important role in Xuanwei female lung cancer. Illustrating the relationship between SOX4 and clinical pathological indicators and further defining the mechanism of action will provide a basis for the diagnosis, prognosis and new therapeutic targets for lung cancer treatment.

MATERIALS AND METHODS

PATIENTS

The patients were all females from Xuanwei with lung cancer. We obtained 96 tumors that underwent surgical resection in the Third Affiliated Hospital of Kunming Medical University in China from 2003 to 2008. The patient characteristics, illustrated in Table I, included the following: 76 adenocarcinomas, 14 squamous cell carcinomas, 3 large cell carcinomas and 3 other types; 72 patients >55 years of age and 24 patients <55 years of age; 39 cases of stage I patients, 41 cases of stage II patients, and 16 cases of stage III patients; 58 cases of low differentiation, 26 cases of moderate differentiation, and 12 cases of high differentiation; 50 cases with lymph node metastasis and 46 patients with no lymph node metastasis; and 46 cases of primary tumors ≤3 cm and 50 cases of primary tumors ≥3 cm. The pathological diagnosis was counterchecked by two senior pathologists, and the median follow-up time was 31.5 months (range: 8–69 months). Overall survival was calculated from the time of surgery to the time of death or the date of the last follow-up. The entire survey was conducted with the approval of the Ethics Committee of Kunming Medical University.

CELL LINE

The XWLC-05 cell line was established in 2007 by the Department of Pathology, Kunming Medical University from a 68-year-old female patient with lung adenocarcinoma. Experiments demonstrated that the cells maintained the phenotypic characteristics of the original tumor and could form xenografts in nude mice that have the same morphological characteristics as Xuanwei female lung cancer. XWLC-05 cells were cultured in RPMI-1640 medium (Neuronbc Laboratories Co., Ltd. Beijing) supplemented with 10% foetal bovine serum (Thermo Scientific HyClone) in a humidified atmosphere with 5% CO_2 at 37°C.

CELL TRANSFECTION

SOX4 small hairpin RNA (shRNA) was designed and cloned into the pGFP-V-RS retroviral vector (Origene, MD). The pGFP-V-RS-SOX4

TABLE I. Correlations of SOX4 Protein Expression and Clinicopathological Features of 96 Cases of Xuanwei Female Lung Cancer Patients

		SOX4 expression			
Variant	Cases	Positive	Negative	*F*	*P*
Age					
≥55	72	40	32	0.68	0.408
<55	24	11	13		
Histology					
Adenocarcinoma	76	44	32	10.08	0.004*
Squamous cell carcinoma	14	5	9		
Large cell carcinoma	3	1	2		
Others	3	0	3		
TNM stage					
I	39	0	39	75.43	0.000*
II	41	35	6		
III	16	16	0		
Lymph node metastases					
Present	50	46	4	63.32	0.000*
Absent	46	5	41		
Differentiation					
Poorly differentiated	58	39	19	12.03	0.002*
Moderately differentiated	26	9	17		
Well differentiated	12	3	9		
Tumor diameter (cm)					
≤3cm	46	23	23	0.34	0.556
>3cm	50	28	22		

*$P < 0.05$.

shRNA sequence was AGCAAACCGCACGCCAAGCTCATCCTGGC, and the negative control shRNA (pGFP-V-RS-scramshRNA) sequence was GCACTACCAGAGCTAACTCAGATAGTACT. The plasmid pCMV6-AC-GFP-SOX4-ORF was used for SOX4 overexpression, and the plasmid pCMV-Control was used as the negative control for transfection. For cell transfections, 80% confluent cells were grown in each well of 24-well plates, and 0.8 μg of SOX4-shRNA or plasmid DNA and 2 μl of LipofectamineTM 2000 (Invitrogen, Carlsbad, CA) were incubated in 50 μl of Opti-MEM (Invitrogen, Carlsbad, CA). After a 15 to 20 min incubation, DNA-liposome complexes were added to 0.5 ml of serum- and antibiotic-free RPMI-1640. After 6 h, the medium was replaced by culture medium. After 48 h, transfected cells were selected for 14 days with 0.5 mg/ml puromycin to select for SOX4 knockdown cells or G418 (800 μg/mL, Invitrogen, Carlsbad, CA) to select for SOX4 overexpression cells.

IMMUNOHISTOCHEMISTRY

For the immunohistochemical analysis of SOX4 in clinical samples, all lung cancer and paraffin-embedded xenograft tumor tissues were cut to a thickness of 4 μm. Slides were deparaffinized in xylene twice for 10 min and rehydrated through descending concentrations of ethanol. Antigen retrieval was performed in 0.01 mol/L citrate buffer (pH 6.0) in a microwave oven for 10 min at 98–100°C. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide (in fresh methanol) for 10 min at room temperature. After washing with phosphate-buffered saline (PBS), the sections were incubated with blocking serum for one hour. Tissues were then incubated with a rabbit polyclonal SOX4 antibody (1:800, AB5803; Millipore) at 4°C overnight. As a secondary antibody, horseradish peroxidase (HRP) labelled rabbit anti-mouse IgG (Dako Envision plus System) was used. Positive staining was visualized with DAB. Slides were graded as follows: negative (0–5% cells stained); weak (5–15% cells stained); medium (15–50% cells stained); and strong (>50% cells stained). Images were captured by an Olympus BX41 light microscope. Tumor cells with nuclear immunohistochemical expression were considered to be positive cells. The percentage of positive tumor cells was counted in five separate fields and in 200 adjacent cells in the area with the highest density of positive cells for each slide. The numbers of positively labeled tumor cells were scored as follows: 0, 0%; 1, 1–25%; 2, 26–49%; 3, 50–75%; and 4, 76–100%. The intensity of staining was also evaluated and graded from 0 to 3, where 0 indicates negative or no staining, 1 indicates weak staining, 2 indicates moderate staining and 3 indicates strong staining. The two values obtained were multiplied to calculate a receptor score (maximum value, 12). For statistical analysis, the samples were grouped into negative (score ≤ 2) or positive (score >2). Slides were evaluated by two blinded observers.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QRT-PCR)

Total RNA was extracted from tissues (lung cancer tissues and adjacent non-tumor tissues) and cell groups (XWLC-05 cells group, SOX4 knockdown plasmid transfected group and SOX4 rescue group, which was co-transfected with the SOX4 knockdown plasmid and pCMV6-AC-GFP-SOX4-ORF) using TRIzol (Invitrogen) according to the manufacturer's protocols. For quantitative real-time PCR, RNA was first reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen). After that, qPCR was performed with a SYBR Green I real-time PCR kit (GenePharma, Shanghai, China) according to the manufacturer's instructions using the ABI 7300 Real-Time PCR System. GAPDH mRNA was used as an internal control. The amplification protocol was as follows: an initial 95°C for 5 min and 50 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. The analysis was performed with three independent preparations of total RNA samples harvested from each sample group. The expression level of SOX4 mRNA was normalized to that of GAPDH mRNA, and the change in expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. A two-tailed *t*-test ($P < 0.05$ was identified to indicate a statistically significant difference) was performed to identify the differentially expressed miRNAs. The following primers were used:

SOX4 forward: 5-CTTGACATGATTAGCTGGCATGATT-3;
SOX4 reverse: 5-CCTGTGCAATATGCCGTGTAGA-3;
caspase-3 forward: 5-GAGTGCTCGCAGCTCATACCT-3;
caspase-3 reverse: 5- CCTCACGGCCTGGGATTT-3;
GAPDH forward: 5-CCAAAATCAGATGGGGCAATGCTGG-3;
GAPDH reverse: 5-TGATGGCATGGACTGTGGTCATTCA-3.

WESTERN BLOTTING

The cells (XWLC-05 cells group, SOX4 knockdown plasmid transfected group and SOX4 rescue group) were washed twice with cold PBS, and total cellular protein was extracted using a modified RIPA buffer with 0.5% sodium dodecyl sulphate (SDS) in the presence of a proteinase inhibitor cocktail (Complete mini, Roche). The protein concentration was then determined by a protein assay kit (Bio-Rad), and equal amounts of protein lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% non-fat milk, followed by incubation with antibodies against SOX4 (1:1000, AB5803; Millipore) or caspase-3 (1:1000, AB3640; Millipore). A horseradish peroxidase (HRP)-conjugated secondary antibody was used and then visualized with enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia) according to the manufacturer's protocol. β-actin was used as an internal control.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

The apoptotic morphological changes of each cell group (XWLC-05 cells, negative control group and SOX4 knockdown plasmid transfected group) were detected by examination with a transmission electron microscope. The cells were harvested and fixed with 3% glutaraldehyde in a 0.1-M cacodylate buffer for 1 h. Following fixation, the samples were post-fixed in 1% OsO4 in the same buffer for 30 min. Fixed samples were rinsed and dehydrated in a graded ethanol series. Ultrathin sections were cut and stained with uranyl acetate and lead citrate by standard methods. Slides were then observed under a transmission electron microscope (JEOL, Tokyo, Japan).

APOPTOSIS AND CELL CYCLE ANALYSIS

Each cell group (XWLC-05 cells, negative control group, SOX4 knockdown group, SOX4 rescue group) was harvested after 48 h and

fixed in 70% ice-cold ethanol overnight. The cells were then washed with PBS and stained with propidium iodide (50 mg/ml) in PBS supplemented with RNase (50 mg/ml) in the dark at room temperature for 30 min. For cell apoptosis detection, FITC-conjugated terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining was performed according to the manufacturer's protocol (BD), with the exception that the reaction time was increased to 3 h at room temperature. Cells were washed twice with binding buffer, and PI solution was added. Analyses of cell cycle distribution and cell apoptosis were performed by flow cytometry in accordance with the manufacturer. Tests were performed in triplicate for each sample.

CELL PROLIFERATION ASSAY

The cell proliferation was examined by MTS. Each cell group (XWLC-05 cells, negative control group, SOX4 knockdown group, SOX4 rescue group) was seeded in 96-well plates at a density of 1×10^2 per well and cultured for 1 to 7 days. Then, 20 μl of MTS was added to each well and incubated for 4 h at 37°C. The absorbance values at 570 nm were measured using a micro-plate reader (Bio-Rad, USA). The experiment was repeated three times, and each experiment had six replicate wells. The cell proliferation rate was calculated using the following formula: Cell proliferative rate (%) = (mean absorbance in six wells of the treatment group/mean absorbance in six wells of the cell control group) ×100%.

TUMOR CELL INVASION ASSAY

The invasion assay was performed using the Transwell chamber with 8 μm pores (Corning, USA). Fifty microliters diluted matrigel (2 mg/ml, BD Biosciences, Bedford, MA) was placed on the inner surface. Isolated cells (XWLC-05 cells, negative control group, SOX4 knockdown group, SOX4 rescue group) at a concentration of 2×10^5/ml resuspended in RPMI-1640 were placed in the top chamber. RMPI-1640 with 20% FBS was added to the bottom chamber. After 24 h, non-invading cells were removed from the top of the Matrigel with a cotton-tipped swab. Invading cells at the bottom of the Matrigel were fixed in methanol and stained with Crystal violet. The invasiveness was determined by counting the penetrated cells in 5 random fields in each well using a microscope at 200× magnification. Each experiment was performed in triplicate.

WOUND ASSAY TO ASSESS CELL MIGRATION

We isolated four subpopulations of cells (XWLC-05 cells, negative control group, SOX4 knockdown group, SOX4 rescue group) for wound-healing assays (conventional scrape motility assays) and plated them in twelve-well plates (3×10^5/well) for 24 h. When the cells reached 90% confluence, sterile pipette tips were used to scratch the wound uniformly. Cell motility was assessed by measuring the movement of cells into a scraped wound. The speed of wound closure was monitored after 72 h by measuring the distance of the wound from 0 h. Each experiment was conducted in triplicate.

TUMORIGENICITY ASSAY IN NUDE MICE

Previously, we have established a severe combined immune-deficiency (SCID) mouse model using XWLC-05 cells [Zhou et al., 2012]. In the present study, animal experiments were performed on six week old Balb/c nude mice (Vital River Laboratory, Beijing, China). Mice were divided into four groups with eight mice per group. XWLC-05 and transfected cells (1×10^6 in 100 μl RPMI-1640) were implanted subcutaneously in the right flanks of nude mice. tumors were measured once per week. After 8 weeks, mice were sacrificed and tumors were removed and then subjected to immunohistochemical analysis. The tumor volume was calculated using the formula $0.5 \times \text{length} \times \text{width}^2$. All protocols were approved by the Institutional Animal Care and Use Committee and carried out according to institutional guidelines.

STATISTICAL ANALYSIS

All data in the study were evaluated with SPSS version 17 software (SPSS Inc.). Data are presented as the means ± SD. When two groups were compared, Student's t-test was used. The $\chi 2$ test was performed to determine correlations among the various parameters. The cumulative survival rate was assessed by the Kaplan–Meier method and analysed by the log-rank test. Differences were considered significant at a value of $P \leq 0.05$.

RESULTS

THE EXPRESSION OF SOX4 IN XUANWEI FEMALE LUNG CANCER

To investigate the potential roles of SOX4 in the development and progression of Xuanwei female lung cancer, RT-PCR and immunohistochemistry were performed to measure SOX4 expression in tumor samples. The RT-PCR results indicated that in 8 tumor samples the SOX4 mRNA expression level in tumor tissues (2.53 ± 0.35) was significantly higher than in the corresponding adjacent non-tumor tissue (1.43 ± 0.18) ($P = 0.003$, Fig. 1A). The immunohistochemical results in 96 paraffin-embedded tumor tissues demonstrated positive staining for SOX4 protein in the nucleus of the tumor or normal cells. Immunoreactivity for SOX4 was observed in 51 (51/96, 53.1%) tumor tissues, whereas it was only observed in 25 (25/96, 26%) adjacent non-tumor tissues (Fig. 1B). The expression of SOX4 protein was significantly higher in tumor tissues compared to the corresponding adjacent non-tumor tissues ($P < 0.01$).

To assess the prognostic value of SOX4 overexpressing lung carcinomas, we correlated the relevant clinicopathological patient data with SOX4 expression status. As shown in Table I, SOX4 expression differed depending on the tumor histological type; it was significantly higher in adenocarcinomas than other lung cancer types. Additionally, higher SOX4 expression was observed in stage III tumors than in stage I or II, and they had worse histological differentiation ($P = 0.000$ and $P = 0002$, respectively). The expression of SOX4 protein also had a significant correlation with lymph node metastasis ($P = 0.000$). No significant association between SOX4 expression and age or tumor size were observed.

Survival analysis by Kaplan–Meier survival curves and the log-rank test demonstrated that patients with higher expression of SOX4 in tumor tissue had a worse overall survival than patients with lower expression of SOX4 ($P < 0.05$). The mean survival time was 22.250 ± 4.468 months in the positive SOX4 expression group, but it was 34.762 ± 3.476 months in the negative expression group. Patients with lymph node metastasis or high pathological grade had a significantly shorter median survival time than those without

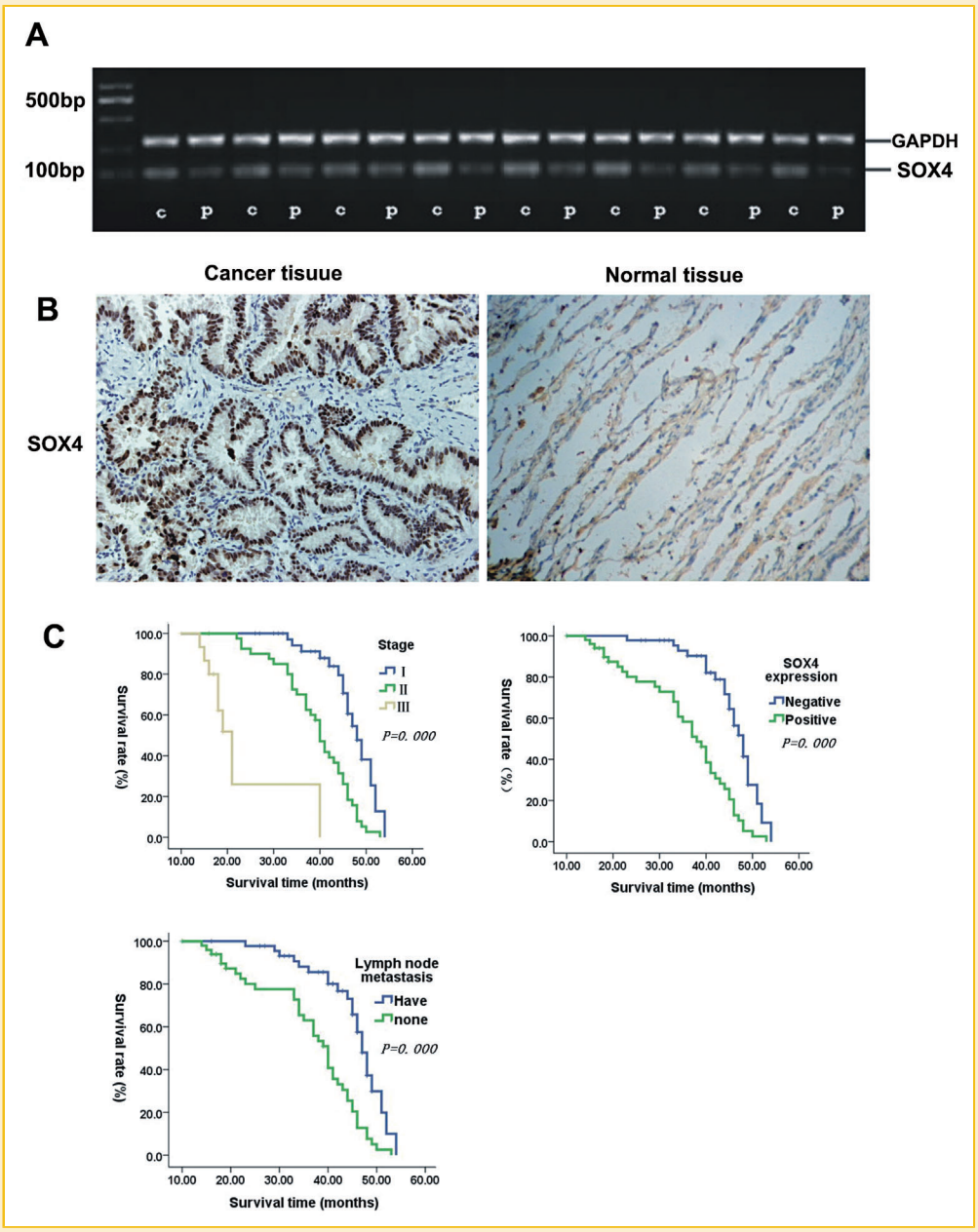

Fig. 1. The expression of SOX4 mRNA and protein in Xuanwei female lung cancer and the correlation with survival. (A) SOX4 mRNA expression in 8 Xuanwei female lung cancer samples compared to 8 adjacent non-tumor tissues from the same patient. SOX4 mRNA expression was normalized to β-actin mRNA. c represents tumor samples and p represents adjacent non-tumor tissue samples. (B) Immunohistochemistry of SOX4 in Xuanwei female lung cancer and adjacent non-tumor tissue samples. SOX4 was highly expressed in the nucleus of cancer cell specimens (left). SOX4 was not expressed in normal lung specimens (right) (×400). (C) Kaplan–Meier survival curves in Xuanwei female lung cancer showing that higher Sox4 expression levels significantly correlate with longer overall survival (median survival 22.25 vs. 34.76 months, $P = 0.000$), stage III tumors significantly correlate with shorter overall survival ($P = 0.000$) and patients with lymph node metastasis had significantly shorter median survival times than those without lymph node metastasis ($P = 0.012$).

lymph node metastasis ($P = 0.012$) or low pathological grade ($P = 0.000$), respectively (Fig. 1C).

DOWN-REGULATED SOX4 GENE EXPRESSION SHOWED APOPTOSIS MORPHOLOGY CHANGES IN XWLC-05

To further explore SOX4 function in the Xuanwei female lung tumor cells, we applied shRNA technology to knockdown SOX4 gene expression. The results showed that XWLC-05 cells and negative control (XWLC-05 cells transfected with pGFP-V-RS-scramshRNA) cells were tightly attached and exhibited polygonal morphology under light microscopy; however, cells a knocked down SOX4 gene (XWLC-05 cells transfected with pGFP-V-RS-SOX4 shRNA) became round and showed cellular shrinkage, cytoplasmic vacuolation and cell death (Fig. 2A).

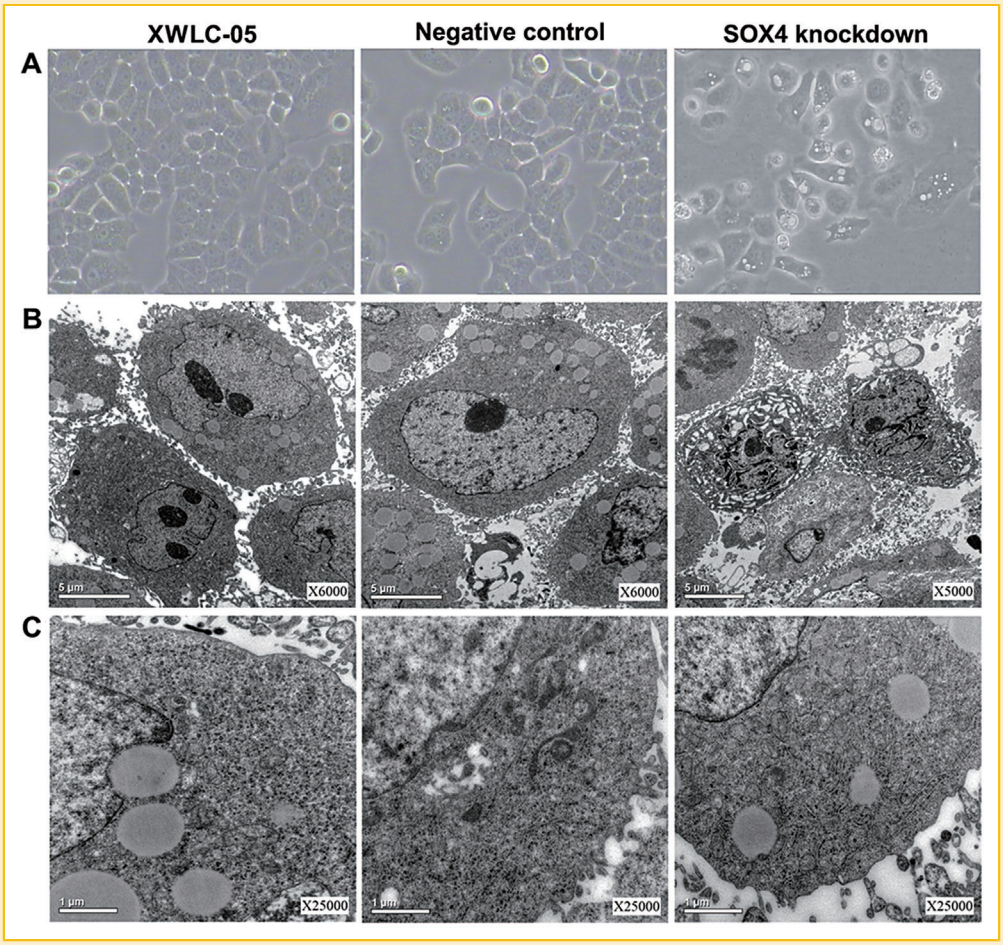

Fig. 2. Light microscopy and Transmission electron microscopy showed morphology and ultrastructure changes in XWLC-05 cells. (A) Light microscopy showed the morphology of XWLC-05 cells (left), negative control cells (XWLC-05 cells transfected with empty shRNA, middle) and XWLC-05 cells, where inhibition of the Sox4 gene expression showed apoptotic morphology (right) (200x magnification images). (B) Transmission electron microscopy showed the ultrastructure morphology of XWLC-05 cells (left) and negative control cells (middle), and there was formation of apoptotic bodies in some cells after inhibiting Sox4 gene expression (right). (C) Transmission electron microscopy showed the ultrastructure morphology of XWLC-05 cells (left) and negative control cells (middle), and the cells appeared to have an improvement in the degree of differentiation after the inhibition of Sox4 gene expression (right).

TEM observation showed that XWLC-05 cells and negative control group cells were multinuclear and contained a small amount of lipid droplets, fewer organelles in the cytoplasm, more euchromatin, less heterochromatin, more ribosomes, and decreased endoplasmic reticulum. All of these characteristics conform to poorly differentiated tumor cells. Apoptotic morphology was not observed. In the SOX4 knockdown group, after inhibiting SOX4 gene expression, some cells showed the following characteristics of early apoptosis: chromatin condensation, diminished smaller size, cytoplasmic concentration, highly coiled chromatin within the nucleus, loose endoplasmic reticulum resulting in membrane fusion, cavity formation and chromatin condensation and clotting, and gathering in the nuclear membrane to form crescent-shaped or annular bodies. In addition, there was also formation of apoptotic bodies in some cells (Fig 2B). Interestingly, after inhibition of SOX4 gene expression, XWLC-05 cells showed a significantly higher degree of differentiation than that of the blank and negative control groups, which manifested as distinct organelles and abundant bubbly and flat cystic-like endoplasmic reticulum distributed around the nucleus and cytoplasm under the plasma membrane (Fig. 2C).

EFFECTS OF SOX4 GENE EXPRESSION ON THE CELL APOPTOSIS AND PROLIFERATION OF XWLC-05 CELLS

Under light microscopy, we observed that the XWLC-05 and negative group cells had no noticeable changes in cellular growth density and morphology. MTS assays showed that knockdown of the SOX4 gene resulted in decreased cell proliferation (Fig. 3A). FCM results showed a typical sub-diploid peak before the G0/G1 phase, and the proliferation index (PI) was obviously lower than that of the negative control and blank groups ($P = 0.000$ and $P = 0.000$, respectively) (Fig. 3A). For apoptosis analysis, we observed a distinct increase in apoptosis in the SOX4 knockdown group cells compared to the XWLC-05 and negative group cells (Fig. 3B).

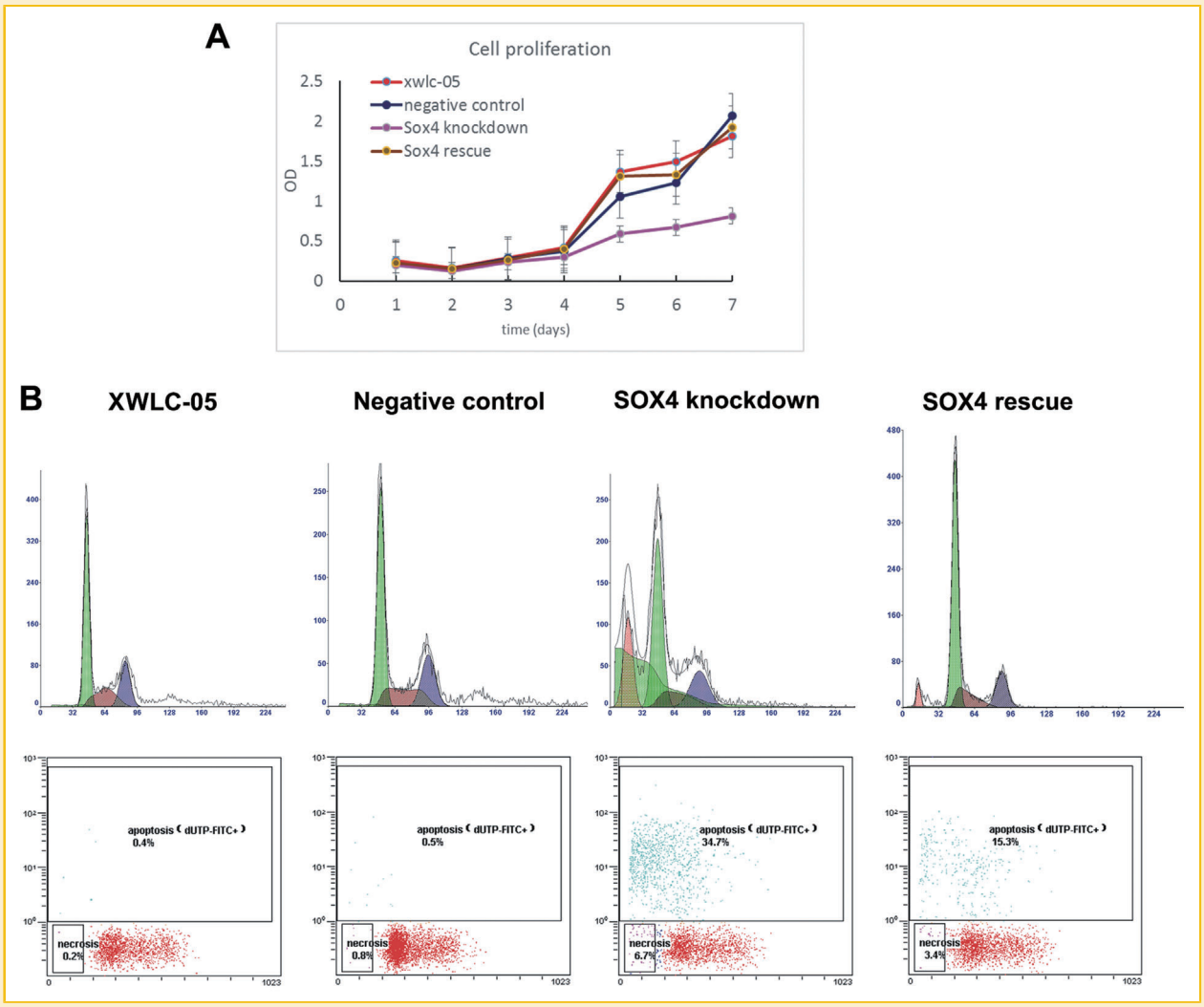

Fig. 3. Proliferation was decreased and apoptosis was induced in XWLC-05 cells by knockdown of SOX4 gene expression. (A) The proliferation of SOX4 knockdown cells was obviously lower than that of XWLC-05 cells, negative control and SOX4 rescue group cells. (B) FCM results showed a typical sub-diploid peak before the G0/G1 phase and a distinct increase in apoptosis number in XWLC-05 cells where the Sox4 gene was inhibited compared to the negative control and blank groups. These changes were recovered in the rescue group cells (XWLC-05 cells transfected with the SOX4 overexpressing plasmid after inhibiting the expression of Sox4 gene).

In addition, we used a rescue experiment to confirm whether these changes were regulated by SOX4. Cells in which the SOX4 gene was knocked down were transfected with pCMV6-AC-GFP-SOX4-ORF (SOX4 over-expression plasmid), and we observed that the SOX4 gene expression was recovered in XWLC-05 cells (rescue group). By suppressing the expression of the SOX4 gene, the average proliferation index of the cells was 31.65 ± 3.19. However, in rescue group cells, the average proliferation index significantly increased to 44.1 ± 3.16 ($P = 0.000$). The activity of rescue group cells was partially restored on the third day of exogenous expression of SOX4. Analysis of the proportion of apoptotic cells using flow cytometry revealed that the apoptosis rate in rescue group cells was reduced from $(35.1 \pm 3.48)\%$ to $(16 \pm 1.5)\%$, which was statistically significant ($P = 0.000$). Apoptosis levels in XWLC-05 cells were obviously lower than the levels in the SOX4 down-regulated group and rescue group ($P = 0.000$).

EFFECTS OF SOX4 ON CELL MIGRATION AND INVASION CAPACITY IN XWLC-05 CELLS

To examine the role of SOX4 in regulating cancer cell migration, we performed the wound healing assay. The results revealed that after 72 h, the migration distance of the SOX4 knockdown group (90.8 ± 12.0) was obviously shorter than the XWLC-05 group (290.6 ± 42.3) and the negative control group (263.7 ± 37.1) ($P = 0.000$ and $P = 0.000$, respectively) (Fig. 4A). At 48 h after knockdown of SOX4, the transwell assay showed that the number of invasive cells (55.6 ± 6.68) was less than that of the XWLC-05 group (93.1 ± 7.36) and negative control group (89.5 ± 8.68) ($P = 0.000$

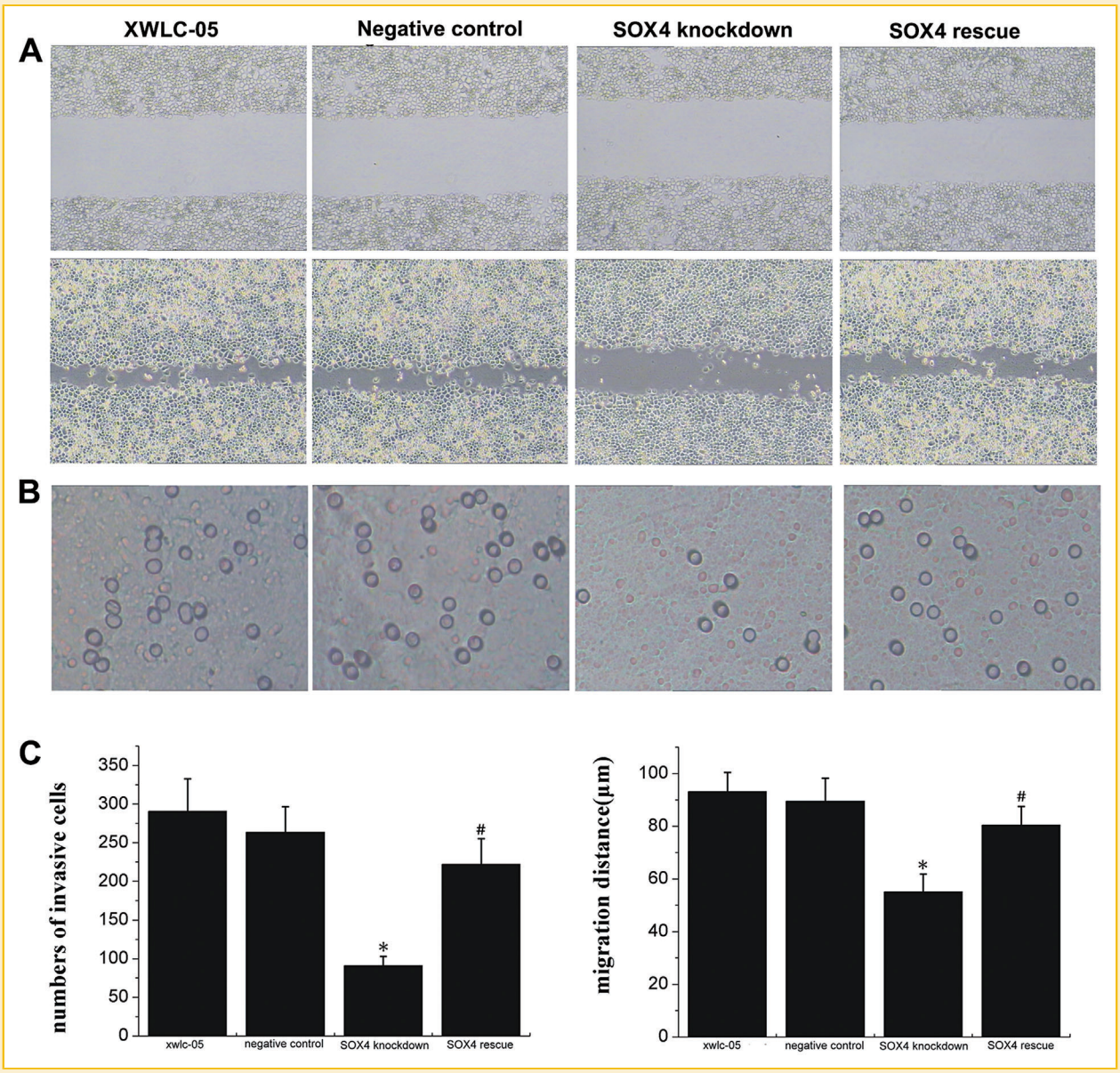

Fig. 4. Inhibition of migration and invasion in XWLC-05 cells by knockdown of SOX4 gene expression. (A) The wound healing assay showed the cell migration distances of the four different treatment group cells. (B) The transwell assay showed the transmembrane cells of the four different treatment group cells. (C) Migration distances were significantly shorter and transmembrane cells were significantly fewer in the SOX4 knockdown group cells compared to the other groups of cells. *compared with XWLC-05 and negative control group; #compared with SOX-4 knockdown group.

and $P = 0.000$, respectively) (Fig. 4B). For the rescue experiment, the averaged invasive number and the migratory ability was increased in the cells where SOX4 expression was recovered compared to the SOX4 knockdown cells ($P = 0.000$) (Fig. 4C).

SILENCING OF SOX4 INDUCED APOPTOSIS BY CASPASE-3 ACTIVATION IN XWLC-05 CELLS

The experiments showed that the down-regulation of SOX4 promoted the apoptosis of Xuanwei female lung cancer cells. Caspase-3 is a key enzyme in the apoptotic pathway. To explore the mechanisms and correlation of SOX4 and caspase-3 in the regulation of apoptosis, we detected the mRNA and protein expression in the XWLC-05 cells, the SOX4 knockdown group and the SOX4 rescue group cells. The results showed that the mRNA levels of caspase-3 in the SOX4 knockdown group were significantly higher than in the XWLC-05 cells and the SOX4 rescue group cells (Fig. 5A and B). Western blotting also showed that the caspase-3 protein was up-regulated in the SOX4 knockdown group at 48 h and 72 h. For the rescue group, caspase-3 protein expression was recovered at 48 h and 72 h (Fig. 5A and B). The above data suggested that apoptosis was promoted by the silencing of SOX4

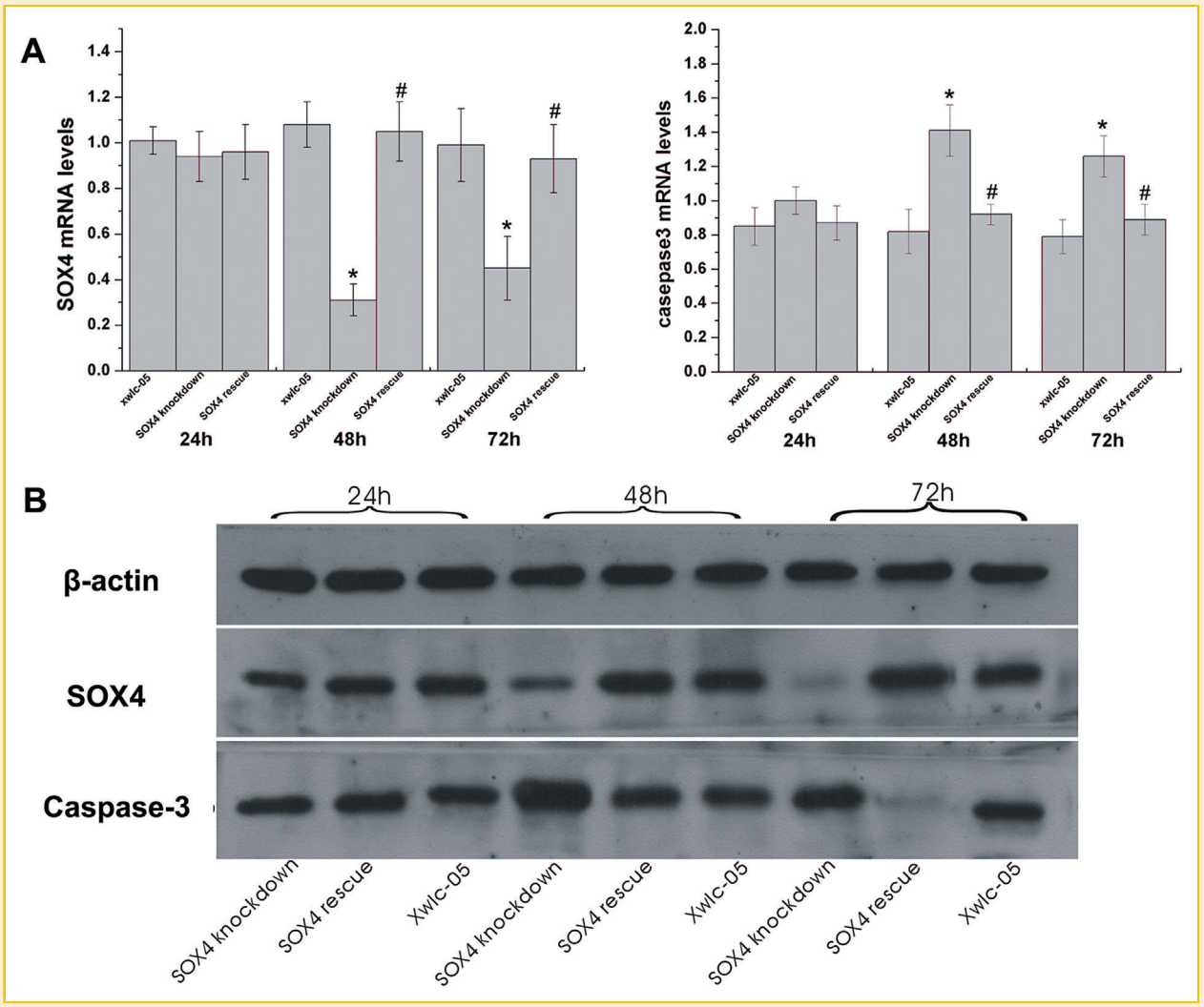

Fig. 5. The SOX4 and caspase-3 mRNA and protein expression levels in XWLC-05 cells. (A) The expression of SOX4 in XWLC-05 cells was down-regulated by shRNA (SOX4 knockdown) and up-regulated by the SOX4 overexpression plasmid (SOX4 rescue) as verified by RT-PCR. Caspase-3 mRNA was up-regulated in the SOX4 knockdown group (right). These changes were obvious 48 h after cells were transfected with SOX4 shRNA or the SOX4 overexpression plasmid. (B) a represents the SOX4 knockdown group, b represents the SOX4 rescue group, c represents the blank control group. Western blotting showed that the SOX4 protein was down-regulated and caspase-3 protein was up-regulated in the SOX4 knockdown group 48 h and 72 h after transfection. For the rescue group, SOX4 protein was recovered and caspase-3 protein was down-regulated at 48 h and 72 h. *compared with XWLC-05 group; #compared with SOX-4 knockdown group.

in XWLC-05 cells, and this was mediated through a caspase-3 dependent pathway.

IN VIVO EXPERIMENTS

To further investigate the biological effects of SOX4 on tumor growth in vivo, 1×10^6 cells were subcutaneously injected into BALB/c nude mice, which were divided into three groups that included eight mice per group. Four weeks after injection, the tumors in the SOX4 knockdown group were smaller than the XWLC-05 and negative control groups (Fig. 6A). The growth of subcutaneous xenografts were significantly suppressed after silencing the SOX4 gene when compared with the blank and negative controls ($P = 0.000$ and $P = 0.000$, respectively) (Fig. 6B). tumor volumes of mice inoculated with SOX4-silenced cells (2.3 ± 0.34 cm^3) were also significantly smaller than the negative control (3.99 ± 0.45 cm^3) and blank control groups (4.03 ± 0.42 cm^3) ($P = 0.000$ and $P = 0.000$, respectively) (Fig. 6C).

Immunohistochemical staining showed that SOX4 and Ki-67 were expressed in the cell nucleus. The expression of SOX4 and Ki-67 were significantly lower in the SOX4 down-regulated xenograft mice compared to the control group ($P < 0.05$) (Fig. 7).

DISCUSSION

SOX4 expression was decreased during foetal lung maturation, which was important for lung development and cell differentiation

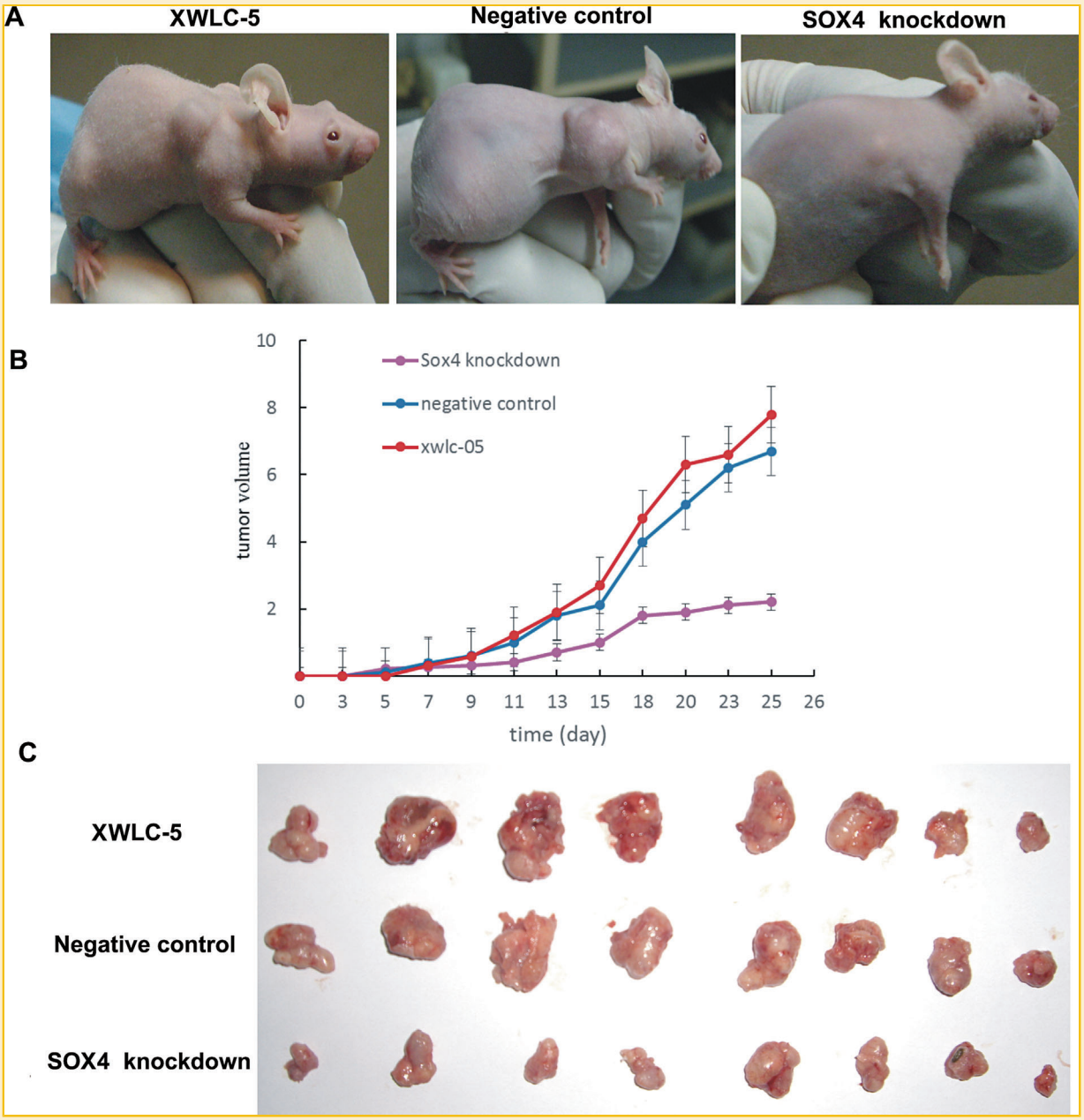

Fig. 6. The effects of SOX4 on tumor growth in vivo. (A) Four weeks after injection, xenografts in BALB/c nude mice formed by SOX4 knockdown group cells were smaller than XWLC-05 cells and negative control group cells. (B) The growth of subcutaneous xenografts was significantly suppressed in the SOX4 knockdown group when compared with the blank and negative control groups ($P = 0.000$ and $P = 0.000$, respectively). (C) Tumor volumes of mice inoculated with SOX4-silenced cells (2.3 ± 0.34 cm^3) were also significantly smaller compared to the XWLC-05 cells (4.03 ± 0.42 cm^3) and negative control cells (3.99 ± 0.45 cm^3) ($P = 0.000$ and $P = 0.000$, respectively).

[Mariani et al., 2002; Hoser et al., 2008]. In recent years, researchers found that SOX4 was abnormally expressed in lung cancer. The SOX4 mutation rate was found to be higher in non-small cell lung cancer and increasingly expressed in advanced tumor stages [Chen et al., 2007]. Castillo et al. [2012] detected the expression of SOX4 in lung cancer cells and showed that the expression levels of SOX4 together with some of the other gene family members, including SOX2 and SOX11, were significantly higher in non-small cell lung cancer cell lines compared to small cell lung cancer cell lines. Furthermore, the expression of the SOX4 gene in lung adenocarcinoma was higher than that in other types of lung cancer. In the present study, our results showed that both the mRNA and protein expression levels of SOX4 in lung tumor tissues was significantly higher than the expression levels in corresponding normal lung tissues. The expression of SOX4 in patients was closely related to tumor staging, lymph node metastasis, the degree of differentiation,

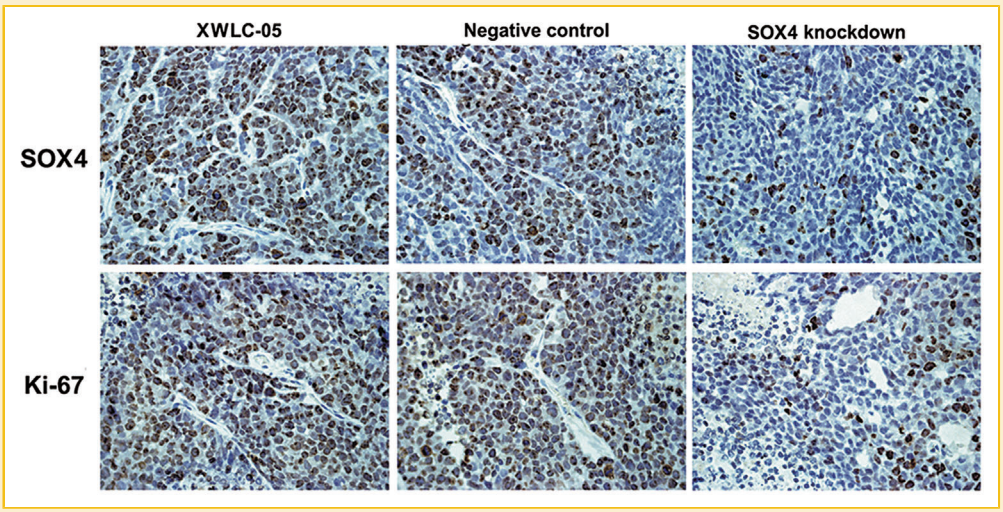

Fig. 7. Immunohistochemistry of SOX4 and Ki-67 in XWLC-05 cells, SOX4 knockdown cells and negative control group cells from tumor tissue samples. SOX4 and Ki-67 was expressed in the cell nucleus. The expression of SOX4 and Ki-67 were significantly lower in the xenografts of the SOX4 knockdown group compared to the XWLC-05 cells and negative control group.

and histological type of the tumor, which indicated that SOX4 affects the differentiation and evolution of lung cancer. The median survival time in patients with high expression of SOX4 protein was significantly shorter than in patients with lower expression of SOX4. Expression of SOX4 in lung adenocarcinoma was significantly higher than the expression in other non-small cell lung cancer types, suggesting that the expression level of SOX4 can reflect the degree of tumor malignancy and may be used as an index for judging prognosis in Xuanwei female lung cancer.

Studies found that in the process of cell differentiation and the development SOX4 gene mutations, deletions or overexpression may result in impaired differentiation and proliferation, thereby participating in tumor formation and development [Aaboe et al., 2006; Liu et al., 2006; Hur et al., 2010]. Hunt and Clarke [1999] found that the expression of SOX4 up-regulated extracellular matrix glycoprotein C, thereby increasing the cell proliferation and invasiveness in breast cancer cells. However, it has also been demonstrated that after stimulation by DNA damage, the SOX4 expression was enhanced and could specifically increase the transcriptional activity of P53. This resulted in the delay of the cell cycle and induction of cell apoptosis, which inhibited the growth of the tumor [Pan et al., 2009]. The significance of the tumor-suppressive function of SOX4 in tumorigenesis has also been emphasized by clinical research, which revealed that higher SOX4 expression correlated with better survival in bladder tumors and medulloblastoma patients [Aaboe et al., 2006; de Bont et al., 2008]. Thus, whether SOX4 is a tumor suppressor or an oncogene is presumably context-dependent and merits further investigation.

In this study, SOX4 knockdown cells showed apoptotic morphological changes. FCM analysis showed obvious sub-G1 peaks and indicated a higher apoptosis rate in SOX4-shRNA transfected cells. The cell proliferation, migration and metastasis ability were significantly reduced after inhibiting SOX4 gene expression. These biological functions were effectively recovered by overexpression of SOX4, which further confirmed the function of the SOX4 gene in Xuanwei female lung cancer cells.

Caspase family proteins are proteolytic enzymes involved in the process of cell apoptosis. They transduce the apoptotic signal through substrate degradation or directly as effector molecules of apoptosis to promote degradation of the cytoskeleton and DNA fragmentation [Wen et al., 2012; Guo et al., 2013; Hu et al., 2013]. In the present study, we observed that caspase-3 expression was up-regulated when the SOX4 gene was down-regulated. After treatment with Ac-DEVD-CHO (the caspase-3 inhibitor), the activity of caspase-3 decreased and the function of the SOX4-shRNA promoted the inhibition of apoptosis. These data indicated that the strong inhibition of apoptosis regulated by SOX4 is mainly through the caspase-3 dependent pathway.

Furthermore, we conducted in vivo experiments to confirm the role of SOX4 in Xuanwei female lung cancer. By inhibiting the expression of the SOX4 gene, the tumor volume and tumor weight of mice were significantly smaller than the negative and blank control groups, suggesting that inhibition of SOX4 expression can decrease the growth of lung cancer xenografts in nude mice. Ki-67 is an important reference index [Ahmed et al., 2012] that is used to reflect the cell proliferation activity. This study showed that the expression of Ki-67 in tumors in the SOX4 knockdown group was significantly lower than that of the control group, which further indicated that the suppression of SOX4 can significantly reduce tumor cell proliferation.

Based on our experiments, we confirmed that the SOX4 gene can not only enhance the tumor cell proliferation, invasion and migration but also can inhibit the apoptosis of Xuanwei female lung cancer cells through a caspase-3 dependent pathway. Further study of the mechanisms of SOX4 in Xuanwei female lung cancer will provide important clues for developing new lung cancer markers and therapeutic targets.

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