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SOX9 regulated proliferation and apoptosis of human lung carcinoma cells by the Wnt/β-catenin signaling pathway

Y.-Z. GUO, X.-L. XIE, J. FU, G.-L. XING

Key Laboratory of Preclinical Pharmacology and Toxicology of Hainan Province, Hainan Medical College, Haikou, China

Abstract. – OBJECTIVE: Sex-determining region Y-box 9 (SOX9) is a transcription factor linked to stem cell maintenance and commonly over-expressed in solid cancers. In the present study, the effects of SOX9 on proliferation and apoptosis of human lung carcinoma cells and its mechanisms were investigated.

MATERIALS AND METHODS: Following over-expression or knock-down of SOX9 in human lung carcinoma cell line A549, cell viability was evaluated using XTT method, and cell apoptosis was measured by Flow cytometry. Caspase-3, Caspase-8, Caspase-9 and SOX9 expression was measured by RT-PCR, and Wnt, phosphorylated Wnt (p-Wnt) and β-catenin expression was detected by Western Blot.

RESULTS: Results showed that SOX9 expression was elevated in human lung carcinoma cells. Knocking down cellular SOX9 by short hairpin RNA (shRNA) decreased cell proliferation while promoted apoptosis of A549 cells. Furthermore, down-regulation of p-Wnt and β-catenin expression levels was detected in A549 cells lack of SOX9. However, over-expression of SOX9 played the opposite roles in proliferation and apoptosis of human lung carcinoma cells. To further demonstrate the functions of the Wnt/β-catenin signaling pathway in SOX9 regulated-cell functions, the inhibitor IWP-2 was used to block the activation of Wnt/β-catenin signal. No significant differences between IWP-2-treated cells and SOX9 plus IWP-2-treated cells suggested the existence of a regulatory role for SOX9 through targeting the Wnt/β-catenin pathway.

CONCLUSIONS: These findings establish the significance of SOX9 in lung cancer pathobiology and heterogeneity, with implications for targeting the Wnt/β-catenin-SOX9 signaling pathway as a rational therapeutic strategy.

Key Words:
SOX9, lung carcinoma cell, Wnt/β-catenin, proliferation, apoptosis.

Abbreviations

Sex-determining region Y-box 9 = SOX9; phosphated Wnt = p-Wnt; short hairpin RNA = shRNA; non-small cell lung cancer = NSCLC; adenocarcinoma = ADC; squamous cell carcinoma = SQC; sodium dodecyl sulfate-polyacrylamide gel electrophoresis = SDS-PAGE; polyvinylidene difluoride = PVDF; reverse transcription polymerase chain reaction = RT-PCR; short hairpin RNA = shRNA; one-way analysis of variance = ANOVA; standard deviation = SD; epithelial-mesenchymal transition = EMT; 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide = XTT.

Introduction

Lung cancer is the main malignancy and the leading cause of cancer death worldwide[1]. Non-small cell lung cancer (NSCLC) accounts for almost 80% of all lung cancers, and is classified into adenocarcinoma (ADC), squamous cell carcinoma (SQC), and large cell carcinoma[2,3]. Only 15% NSCLC patients have the 5-year survival time due to late diagnosis. So, it is crucial to identify biomarkers for their early detection and prognosis, and to obtain a better understanding of critical genes and molecular pathways involved in lung cancer development and progression due to the life-threatening nature of lung cancers[4-6].

SOX9 is a member of SOX family and functions as a stem cell-related transcription factor in lineage restriction, terminal differentiation, and maintenance of stem cell properties[7]. Increasing evidence has reported that SOX9 plays different roles in various types of malignancies including breast cancer[8], thyroid Cancer[9], renal cell carcinoma, bladder cancer, penile cancer[10], colorectal cancer[11], prostate cancer[12], cervical cancer[13], and pancreatic cancer[14]. Previous studies also repor-

Corresponding Author: Jian Fu, MD; e-mail: fujian1616@sina.com

ted that SOX9 was over-expressed in cancer tissues such as the skin, prostate, lung, colorectal, gastric, breast and brain, and contributed to tumor growth invasion and development[15-17]. In another way, SOX9 served as a tumor suppressor in some melanomas and endometrial carcinoma cells, suggesting that the role of SOX9 in tumors is tissue-dependent[18-20]. Therefore, it is of great importance to find new molecular mechanisms of SOX9 that will help improve the understanding of SOX9 in lung cancer.

The Wnt/β-catenin signaling pathway is a genetically conserved signaling pathway which is responsible for several cellular processes, including cell movement and polarity, proliferation and differentiation in different kinds of cells[21-24]. Currently, it has proven that the abnormal activation of Wnt/β-catenin signaling pathway is closely related to the development of a variety of cancers[25-27]. Several studies demonstrated that the SOX9 expression level in lung cancer cell lines was up-regulated at both mRNA and protein levels, and also elevated in NSCLC tissues[28], acute lung injury tissues[29] and human lung adenocarcinoma tissues[30]. Furthermore, SOX9 was found to positively regulate multiple WNT pathway genes including those encoding WNT receptors and the downstream β-catenin effector TCF4, indicating that SOX9 drove WNT pathway activation in prostate cancer[31]. However, the role and mechanism of SOX9 in lung cancer require further exploration. In the present work, we found that SOX9 expression was up-regulated in human lung cancer A549 cells. Furthermore, we demonstrated that knockdown of SOX9 inhibited cell proliferation and promoted cell apoptosis in lung cancer cells by directly targeting the Wnt/β-catenin signaling pathway.

Materials and Methods

Cells and Treatments

The human lung carcinoma cell line A549 (ScienCell, Carlsbad, CA, USA) was cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, New York, NY, USA) containing 10% (v/v) fetal bovine serum, 100 μg/mL streptomycin, and 100 IU/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO_2 in the air. Furthermore, the experiments were randomly divided into the following groups: control, pCMV-N1 control, non-target shRNA control, pCMV-SOX9 treatment, SOX9 shRNA treatment, IWP-2 treatment, SOX9 shRNA plus IWP-2 treatment and pCMV-SOX9 plus IWP-2 treatment. IWP-2 was purchased from Santa Cruz Biotechnology (2145 Delaware Ave Santa Cruz, CA, USA). Human SOX9 shRNA and non-target shRNA control were purchased from Genepharma, Shanghai, China, and pCMV-SOX9 was constructed according to the previous study[32].

Cell Proliferation Assay

According to the instruction, the endogenous effects of SOX9 on cell viability were evaluated using 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) Cell Viability Assay Kit (BioVision, Milpitas, CA, USA). Briefly, human lung carcinoma cells at a density of 1.0×10^5/mL were seeded in medium on 96-well plates. When cells attained 65% confluency, they were transfected with pCMV-N1, Non-target shRNA, pCMV-SOX9, SOX9 shRNA, IWP-2, SOX9 shRNA plus IWP-2 and pCMV-SOX9 plus IWP-2. At indicated times, cells in each well were added with the 100 μL fresh medium and 25 μL XTT solution. After 5 h of incubation, cell viability in each well was estimated at wavelength of 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Flow Cytometry

After human lung carcinoma cell line A549 seeded onto a 96-well plate were treated with pCMV-N1, Non-target shRNA, pCMV-SOX9, SOX9 shRNA, IWP-2, SOX9 shRNA plus IWP-2 and pCMV-SOX9 plus IWP-2 for 48 h, cell apoptotic rate was measured by Flow cytometry method according to the manufacturer instructions. Briefly, human lung carcinoma cells were collected and fixed in 70% ethanol for 30 min. Then cells were stained with 50 μg/mL FITC, Annexin V, and PI (BD Biosciences, San Jose, CA, USA), respectively. Cell apoptotic rate was analyzed using a FACScanVantage SE (BD Biosciences, San Jose, CA, USA).

Western Blot Analysis

Western Blot was used to analyze protein expression of Wnt, p-Wnt, and β-catenin according to previous study[32]. Four kinds of polyclonal antibodies anti-SOX9 (1:1000 diluted), Wnt (1:2000 diluted), p-Wnt (1:1000 diluted) and β-catenin (1:500 diluted) were purchased from Cell Signaling Technology, Danvers, MA, USA. After the membranes were incubated with antibodies, they were washed

and immunoblotted with HRP-conjugated anti-rabbit IgG antibody (diluted 1:10000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37°C for 30 min. The membranes were then exposed to X-ray film. β-actin (Biotime, Haimen, China) was used to ensure adequate sample loading for all Western blots. Band density was quantitated using Image J software (National Institutes of Health, Bethesda, MD, USA).

Quantitative RT-PCR

RNA preparation and quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to measure the mRNA expression of Caspase-3, Caspase-8, Caspase-9 and SOX9 in human lung carcinoma cells. Total cellular RNA isolation (Invitrogen, Carlsbad, CA, USA) and quantitative RT-PCR analysis (Qiagen, Valencia, CA, USA) were conducted according to the manufacturers' instructions. Specific primer sequences were synthesized in BIOSUNE Biological Technology Corp (Shanghai, China), and the sequences of the primers were shown as follows: SOX9, 5'-GGATCCCAT'GAATCTCCTG-GACCCCT-3' (forward), 5'-GAATTCTCA AG-GTCGAGTGAGCTGTGTGT-3' (reverse); Caspase-3, 5'-TTTGTTTGTGTGCTTCTGAGCC-3' (forward), 5'-GATGTTCTGGAGAGCCCCG-3' (reverse); Caspase-8, TACTACCGAAACTTG-GACC (forward), GTGAAAGTAGGTTGTGGC (reverse); Caspase-9, 5'-CGAACTCATGGCAA-GCA-3' (forward), 5'-CAAATCCTCCAGAAC-CAAT-3' (reverse) and β-actin, 5'-GGGAAATC-GTGCGTGACATTAAG-3' (forward), 5'-TGT-GTTGGCGTACAGGTCTTTG-3' (reverse).

Short Hairpin RNA (shRNA) Assay

Non-target shRNA or SOX9 shRNA were established similarly and stably transfected into cancer cells for 48 h and knockdown efficiency was evaluated by measuring SOX9 protein expression. For stable transfections, cells cultured in medium were grown to 60% confluence and then transfected with 4 μg of SOX9-specific shRNA construct or negative control shRNA using HiPerFect (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols.

Caspase-3 Activity Analysis

Caspase-3 activity was analyzed using Caspase-3 Activity Assay Kits (Beyotime Institute of Biotechnology, Nanjing, China) according to the manufacturer's instructions. Briefly, the reaction buffer and the specific enzyme DEVD-pNA were added to each cell plate and further cultured in an incubator for 1 h at 37°C. The developed colorimetric reaction was measured at 450 nm in a 96-well Biorad 680 microplate reader (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical analysis was carried out with one-way analysis of variance (ANOVA) using SPSS17.0 software (SPSS Inc., Chicago, IL, USA). Values are expressed as means ± standard deviation (SD). The mean values and standard deviations were calculated from three independent experiments. Differences were considered statistically significant at $p < 0.05$.

Results

SOX9 Expression Was Up-Regulated in Human Lung Carcinoma Cells

To investigate the role of SOX9 in human lung cancer cells, the expression of SOX9 was compared between human lung carcinoma cells and normal lung cells by Western blot and RT-PCR. As shown in Figure 1A, low SOX9 expression detected by

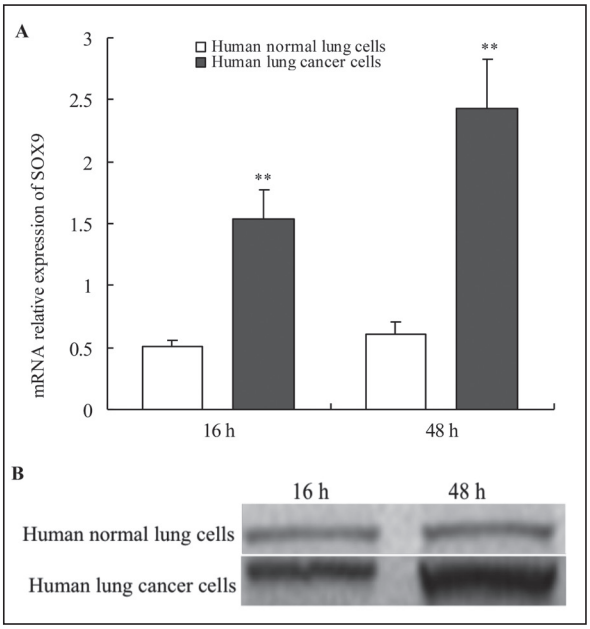

Figure 1. The expression level of SOX9 was up-regulated in human lung carcinoma cells. mRNA expression ***(A)*** and protein expression ***(B)*** of SOX9 was determined by RT-PCR and Western blot in human lung carcinoma cells and human normal lung cells. Three individual experiments were performed. Data are expressed as mean ± SD of three independent experiments in triplicate. *$p < 0.05$, **$p < 0.01$, or $p > 0.05$ *vs.* control.

RT-PCR was observed in normal lung cells, while high SOX9 expression was found in lung cancer cells. There was significant difference between two groups ($p < 0.01$). To further prove the hypothesis, Western blot assay was performed and revealed that the protein level of SOX9 was increased in lung cancer cells in comparison with the corresponding non-cancerous cells (Figure 1B). This result was consistent with the data obtained from RT-PCR. Above results indicated that SOX9 protein expression was up-regulated in lung cancer cells.

SOX9 Over-Expression Promoted the Proliferation of Human Lung Carcinoma Cells

To determine the influence of SOX9 on human lung carcinoma cell growth, SOX9 was over-expressed or knocked down and then cell OD_{450} value was detected by XTT method. It was found that the SOX9 expression level was highly upregulated in pCMV-SOX9 treated cells, but it was heavily inhibited in SOX9 shRNA treated cells (Figure 2A). Results of statistical analysis showed that, compared to the control, the OD_{450} value in the SOX9 over-expressed cells was increased while it was decreased in SOX9 knocked down cells, and the effects were time-dependent ($p < 0.05$) (Figure 2B). It was suggested that SOX9 was involved in regulating cell growth in human lung carcinoma cells.

SOX9 Over-Expression Inhibited the Apoptosis of Human Lung Carcinoma Cells

The effects of SOX9 on the apoptosis of human lung carcinoma cells were examined following

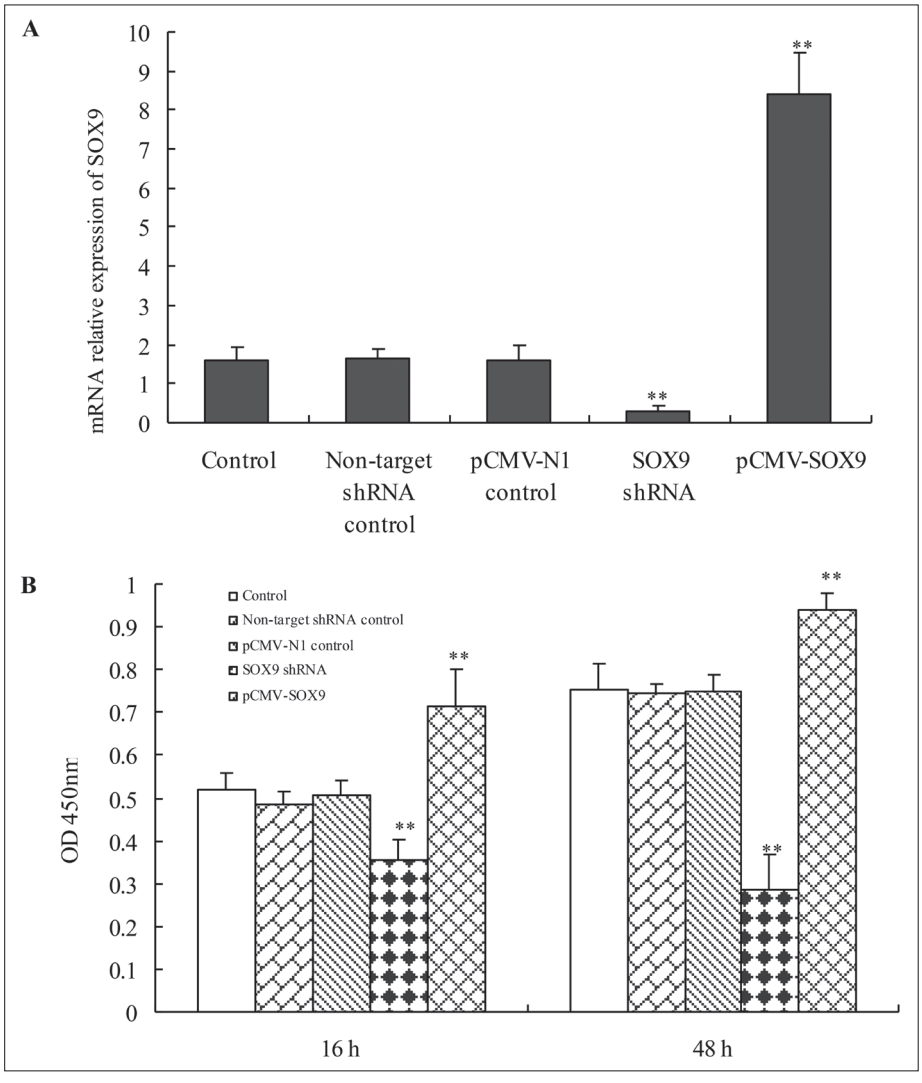

Figure 2. SOX9 over-expression promoted A549 cell proliferation. pCMV-SOX9 and SOX9 shRNA were transfected into A549 cells for 48 h, the expression of SOX9 was indicated by Western blot analysis ***(A)***. Following transfection of pCMV-SOX9 and SOX9 shRNA into A549 cells for 16 h and 48 h, cell viability was determined by XTT assay ***(B)***. Data are expressed as mean ± SD of three independent experiments in triplicate. $*p < 0.05$, $**p < 0.01$, or $p > 0.05$ *vs.* control.

SOX9 over-expression or knockdown. As demonstrated in Figure 3A, cells over-expressing SOX9 showed a significant decrease in the number of apoptotic cells compared with the control cells. The cell apoptotic rate in the control group was 7.36 ± 0.65%, which decreased to 2.12 ± 0.44% in the pCMV-SOX9 treatment. By contrast, after silencing SOX9, the apoptotic rate of A549 cells was promoted showing as 42.58 ± 3.26%. In addition, the activity of Caspase-3, mRNA expression of Caspase-3, Caspase-8, Caspase-9 were examined in pCMV-SOX9-treated or SOX9 shRNA-treated A549 cells. Compared with the control, both mRNA expression of Caspase-3, Caspase-8, Caspase-9 (Figure 3B) and the activity of Caspase-3 (Figure 3C) were significantly lowered in pCMV-SOX9-treated cells but they were increased in SOX9 shRNA-treated cells ($p < 0.05$). Above data suggested that SOX9 over-expression inhibited A549 cell apoptosis.

SOX9 Over-Expression Activated the Wnt/β-Catenin Signaling Pathway in Human Lung Carcinoma Cells

To understand the molecular mechanism of SOX9 involved in lung cancer cells, the alterations in the Wnt/β-catenin signaling pathway were investigated in pCMV-SOX9 or SOX9 shRNA-treated A549 cells. Protein expression of the Wnt/β-catenin signal targeted genes including Wnt, β-catenin, p-Wnt in A549 cells treated with pCMV-SOX9 or SOX9 shRNA for 24 h was assessed using Western Blot (Figure 4A). It was suggested that there were no changes in protein expression of Wnt. A

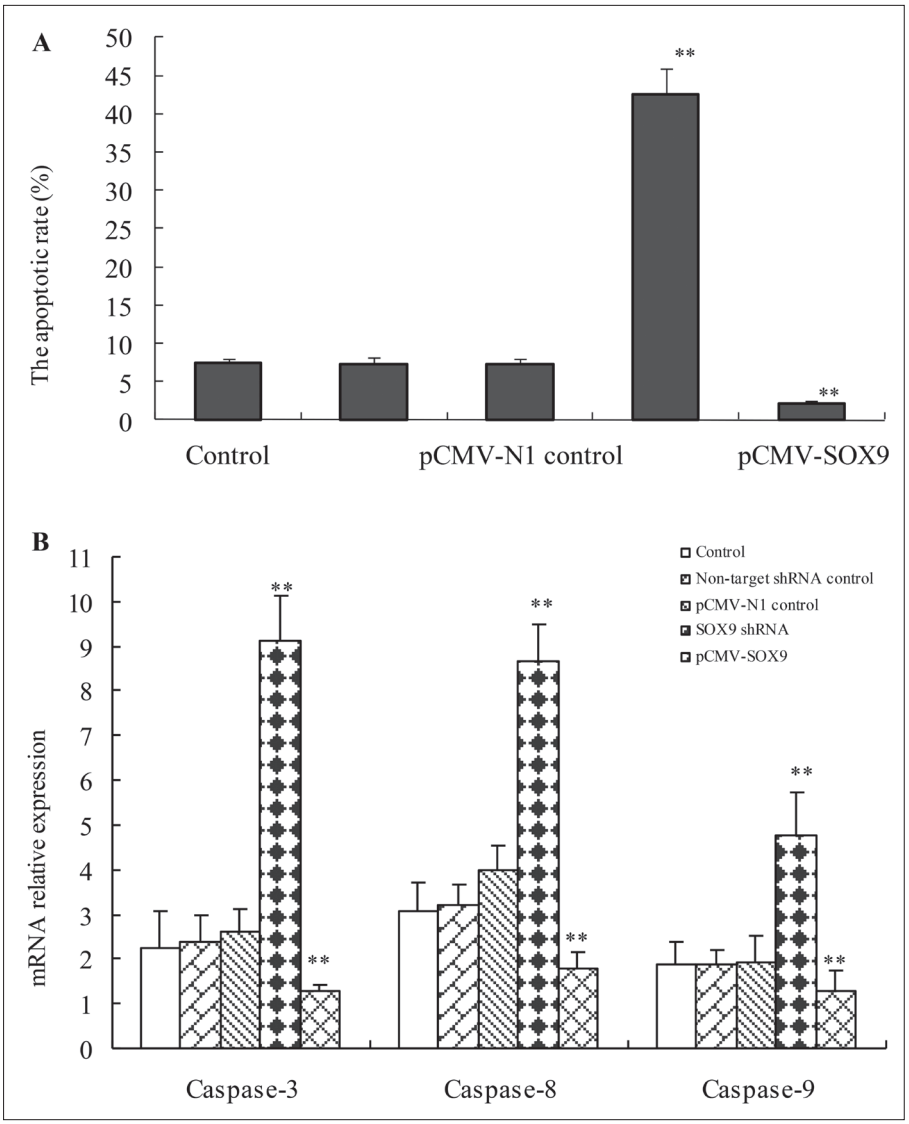

Figure 3. SOX9 over-expression inhibited A549 cell apoptosis. pCMV-SOX9 and SOX9 shRNA were transfected into A549 cells for 48 h, and the apoptotic rates ***(A)***, mRNA expression of Caspase-3, Caspase-8 and Caspase-9 ***(B)*** and the activity of Caspase-3 ***(C)*** was determined by different methods. Data are expressed as mean ± SD of three independent experiments in triplicate. *$p < 0.05$, **$p < 0.01$, or $p > 0.05$ *vs.* control.

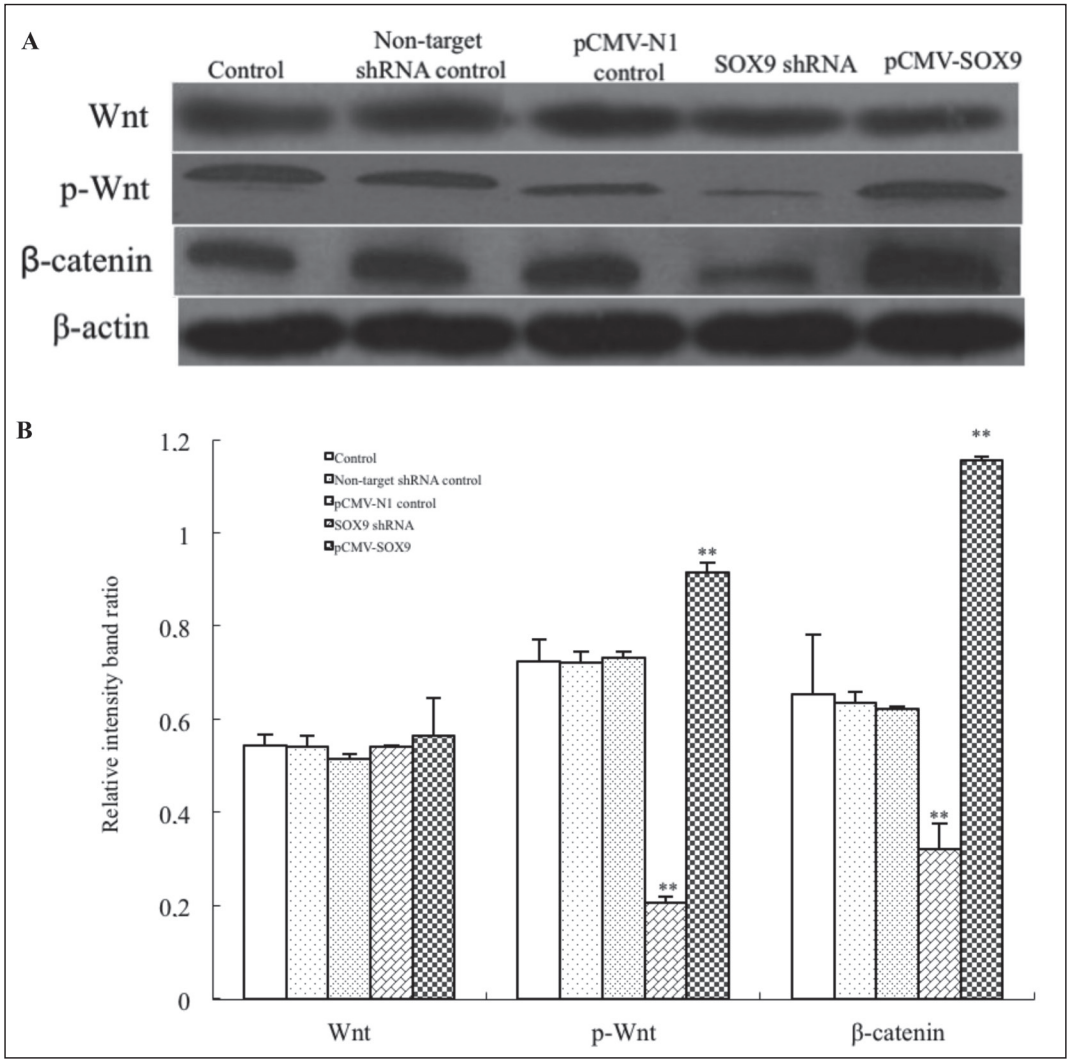

Figure 4. SOX9 over-expression activated the Wnt/β-catenin signaling pathway in A549 cells. Human lung cancer A549 cells were treated with pCMV-SOX9 and SOX9 shRNA for 24 h, then protein expression of Wnt, β-catenin and p-Wnt was measured by Western Blot ***(A)***. Band ratio was analyzed using Image J software ***(B)***. Data are expressed as mean ± SD of three independent experiments in triplicate. *$p < 0.05$, **$p < 0.01$, or $p > 0.05$ *vs.* control.

marked increase of β-catenin and p-Wnt was found in SOX9 over-expressed A549 cells. In contrast, an evident decrease of β-catenin and p-Wnt was observed in SOX9 silenced A549 cells (Figure 4B). Accordingly, it was indicated that SOX9 over-expression could activate the Wnt/β-catenin signaling pathway in A549 cells.

The Wnt/β-Catenin Signaling Pathway Was Involved in SOX9-Changed Cell Apoptosis in Human Lung Carcinoma Cells

To investigate whether SOX9 exerted its functions through the Wnt/β-catenin signaling pathway, this signaling pathway was blocked using inhibitor IWP-2 in human lung carcinoma cells. Western blot analysis confirmed the significantly decreased protein expression of p-Wnt by IWP-2 in A549 cells (Figure 5A). Compared to the negative control, following SOX9 over-expression, cell viability was promoted and the apoptotic rate was significantly prohibited in A549 human lung carcinoma cells. Moreover, knocking down SOX9 conducted its functions opposite to SOX9 over-expression. It was shown that inhibition of the Wnt/β-catenin signaling pathway suppressed cell proliferation (Figure 5B) and promoted cell apoptosis (Figure 5C). SOX9-mediated cell proliferation and apoptosis was attenuated after cells were treated with

inhibitor IWP-2 in A549 cells, and there was no difference between SOX9 over-expressing treatment, SOX9 knocking down treatment and IWP-2 treatment. Taken together, results above indicated that the regulatory roles of SOX9 on cell proliferation and apoptosis were mediated by the Wnt/β-catenin signaling pathway in A549 human lung carcinoma cells.

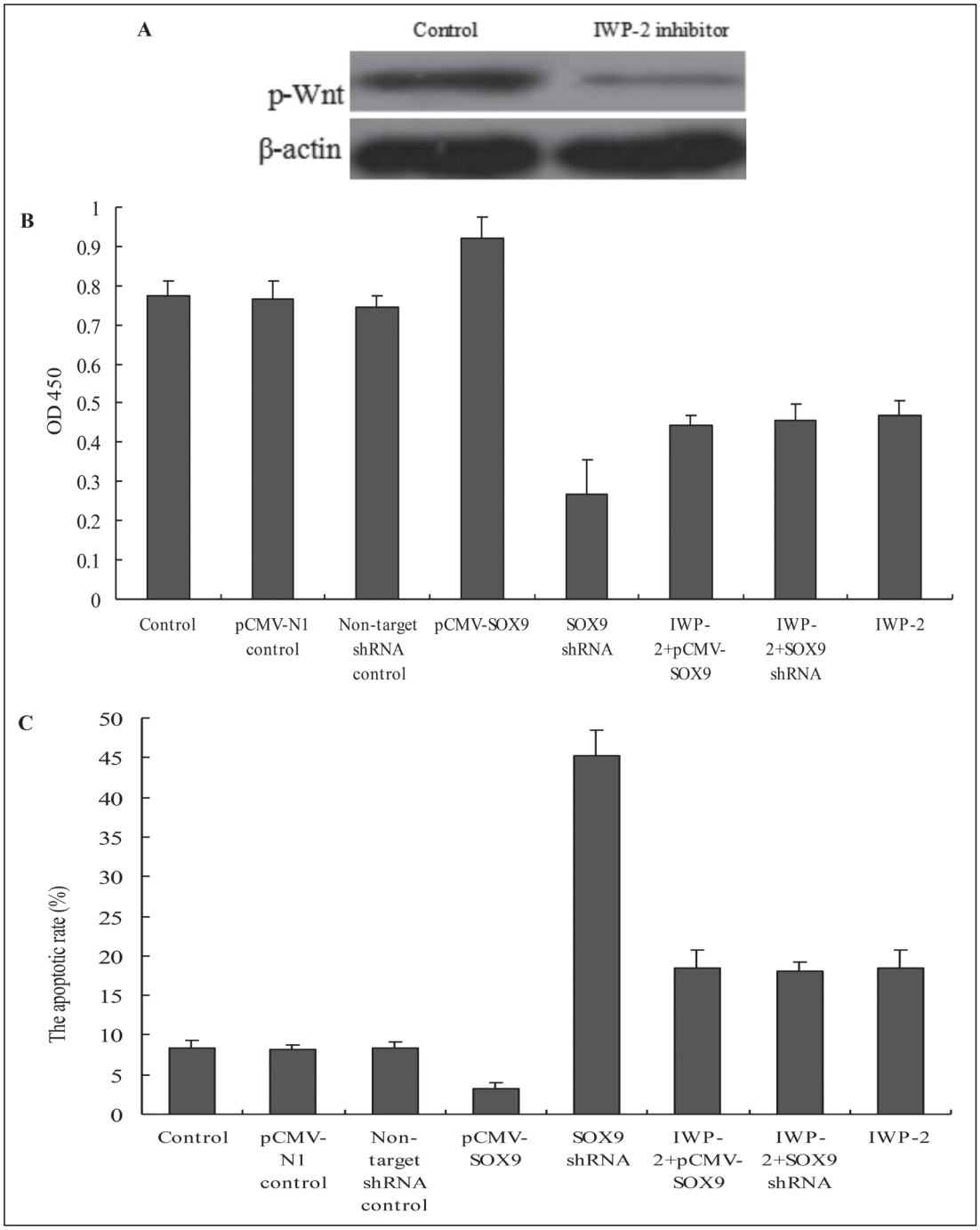

Figure 5. The Wnt/β-catenin signaling pathway was required for cell apoptosis in SOX9-treated A549 cells. Cells were seeded in 6-well plates and then treated with IWP-2 in A549 cells for 48 h, Western blot was performed to determine the blocking efficiency of the inhibitor IWP-2 ***(A)***. IWP-2 was added in A549 cells for 6 h, and then pCMV-SOX9 and SOX9 shRNA were transfected into cells for 48 h. Cell viability was determined by XTT method ***(B)***, and the apoptotic rates were measured by Flow cytometry ***(C)***. Data are expressed as mean ± SD of three independent experiments in triplicate. $*p < 0.05$, $**p < 0.01$, or $p > 0.05$ *vs.* control.

Discussion

SOX9 belonging to the SOX family of transcription factors is required for many developmental processes of cancers[8,10-12]. In this study, we used SOX9 to stimulate human lung carcinoma cells and demonstrated that SOX9-knocking down cells possessed the ability to self-renew while SOX9-overexpressing cells lost the ability to proliferate. In addition, by inhibiting the Wnt/β-catenin pathway, we revealed that the proliferation ability of SOX9-overexpressing or knocking down cells had no difference. These results clearly showed that SOX9 played an important role in human lung cancer cells through targeting the Wnt/β-catenin pathway.

To date, abnormal expression of SOX9 has been reported to be linked to cancer development. SOX9 is a key regulator of developmental processes and its expression in cancer tissues is usually evaluated by RT-PCR, Western blot, and immunohistochemistry. Numerous studies proved that SOX9 was significantly over-expressed in various cancers such as thyroid, prostate, breast, colorectal, colon, renal cell carcinoma and bladder cancers[10], indicating that SOX9 might have an important value as a biomarker when evaluating risk and treatment of cancers[33]. Conversely, SOX9 expression was progressively decreased in cervical carcinoma in situ and especially in invasive cervical carcinoma[13]. In cultured cells, abnormal expression of SOX9 was also found. For instance, SOX9 expression was up-regulated in papillary thyroid cancer tissues and cell lines[9]. SOX9 was highly expressed in liver cancer stem cells and that high levels of SOX9 predicted a decreased probability of survival in hepatocellular carcinoma patients[34]. SOX9 was over-expressed and associated with the growth, invasion, and metastasis of gastric cancer cells[35]. Over-expression of SOX9 was detected in HEK293, A549, SW480, and T47D cancer cells[36]. In addition, SOX9 was expressed at a higher frequency in primary prostate cancer *in vivo* and in prostate cancer cell lines LNCaP, CWR22, PC3, and DU145[37]. This paper also provided the evidence that SOX9 was over-expressed in human lung cancer cell line A549.

The previous work demonstrated that knockdown of SOX9 inhibited cell proliferation, invasion, and the epithelial-mesenchymal transition (EMT) process via suppressing the Wnt/β-catenin signaling pathway in papillary thyroid cancer cell line TPC-1 and BCPAP[9]. SOX9 silencing in gastric cancer cells enhanced apoptosis and senescence by targeting the WNT canonical pathway[38]. Down-regulation of SOX9 by siRNA contributed to the decreased cellular proliferation in prostate cancer cells[37]. SOX9 silencing significantly reduced cell proliferation and invasion in breast cancer cells[39]. Moreover, Sox9 was required for maintaining proliferation, self-renewal, and tumorigenicity in liver CSCs. Over-expression of exogenous SOX9 in liver non-CSCs restored self-renewal capacity[34]. Over-expression of SOX9 in cervical carcinoma cell lines SiHa and C33A inhibited cell growth *in vitro* and tumor formation *in vivo*. In agreement, the silencing of SOX9 promoted cell growth in HeLa cells and tumor formation in mice[13]. SOX9 might dramatically increase cell growth, migration and invasion in human lung ADC A549 cell line[30]. This research demonstrated that SOX9 knockdown inhibited cell proliferation and boosted cell apoptosis in human lung cancer cells. However, SOX9 over-expression exerted the functions in human lung cancer cells contrary to SOX9 silencing.

The Wnt/β-catenin pathway has been implicated in several cancers, and its aberrant up-regulation is associated with the development and progression of prostate cancer[25-27]. The reports have shown the involvement of the Wnt/β-catenin pathway in controlling the proliferation and apoptosis of cancer cells[40]. Blocking Wnt signal using inhibitors obviously prohibited cell proliferation and tumor growth of colorectal cancer[41]. Inhibition of the Wnt signaling pathway was able to significantly increase the apoptosis induced by 5-FU/DDP in the colon cancer cell lines SW480 and SW620[42]. Our findings strongly proved that SOX9-overexpression could activate the Wnt/β-catenin pathway and SOX9-Wnt/β-catenin axis acted as an important regulator in cell apoptosis of human lung cancer cells.

Conclusions

We showed the involvement of SOX9 in the regulation of cell proliferation and apoptosis of human lung cancer cells. Further, SOX9 over-expression was reported to activate the Wnt/β-catenin pathway to complete its role in A549 cell apoptosis. The interaction of SOX9 and its target Wnt/β-catenin pathway would help us to understand lung cancer development and to identify potential new targets for lung cancer therapy.

Declarations Availability of Data and Material

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Authors' Contributions

Jian Fu was the guarantor of integrity of the entire study and was responsible for manuscript review. Yanzi Guo designed this study and took experimental studies. Xueli Xie and Guilan Xing took cell culture and Western blot assay.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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