Inhibition of SOX4 induces melanoma cell apoptosis via downregulation of NF-κB p65 signaling

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Abstract. SOX4 (SRY Box 4) has attracted attention due to its important effects on cell growth, proliferation and apoptosis, among other cellular processes. However, the role of SOX4 in melanoma cell apoptosis remains unclear. In the present study, we investigated whether inhibition of SOX4 induces melanoma cell apoptosis, and explored the possible mechanism involving the NF-κB signaling pathway. SOX4 was knocked down using a lentivirus in melanoma A2058 and SK-MEL-5 cell lines. Cell proliferation was measured by MTT assay. Apoptosis was determined by flow cytometry. Western blotting was performed to detect the protein levels of SOX4, p65 and apoptosis-related proteins, such as PARP, Bcl-2, Bax and survivin. Quantitative real-time PCR (qRT-PCR) was used to examine the mRNA levels of SOX4 and p65. To determine whether SOX4 is able to bind to the promoter of p65, a CHIP-PCR assay was performed. Our data demonstrated that SOX4 knockdown significantly induced apoptosis in melanoma cells, which was accompanied by increases in cleaved PARP and Bax, and decreases in Bcl-2 and survivin. The expression of p65 was also decreased in SOX4-knockdown melanoma cells. The CHIP-PCR assay indicated that SOX4 was able to bind to the promoter region of p65. We also observed that apoptosis in SOX4-knockdown and p65-overexpressing A2058 cells was much lower than that in SOX4-knockdown alone cells. This revealed that the overexpression of p65 partially reversed SOX4 downregulation-induced apoptosis. In conclusion, our results demonstrated that inhibition of SOX4 markedly induced melanoma cell apoptosis via downregulation of the NF-κB signaling pathway, which thus may be a novel approach for the treatment of melanoma.

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Introduction

SOX4 (SRY Box 4) is a 47-kDa protein and contains a conserved signature sequence in the high-mobility group (HMG). SOX4 is highly expressed in almost all types of cancer in humans, and plays an important role in the development of tumors (1). Recent studies have uncovered the key functions of the SOX4 gene as a regulator of cancer cell proliferation, apoptosis, invasion and metastasis.

SOX4 is considered to be a tumor-suppressor gene or an oncogene in different types of tumors. For example, SOX4 inhibits the cell proliferation of polymorphous glioblastoma (GBM) (2). However, high expression of SOX4 in nasopharyngeal carcinoma promotes tumor growth and metastasis (3). Whether SOX4 promotes apoptosis or represses apoptosis in different tumors remains a controversial question. Liu *et al* revealed that silencing of SOX4 by small interfering RNA transfection induced apoptosis in prostate cancer cells (4). Yoon *et al* found that SOX4 knockdown (KO) induced apoptosis by activating caspase-3 and caspase-7 in head and neck squamous cell carcinoma (HNSCC) cells (5). Hur *et al* reported that SOX4 overexpression led to a significant suppression of p53-induced Bax expression, and subsequent repression of p53-mediated apoptosis induced by γ-irradiation (6). Pramoonjago *et al* discovered that SOX4 knockdown resulted in the apoptosis of adenoid cystic carcinoma (ACC) cells (7) and Bilir *et al* also reported that SOX4 knockdown enhanced the effects of a wnt pathway inhibitor (iCRT-3) on apoptosis in breast cancer cells (8). Other researchers have reported the opposite findings regarding the role of SOX4 in apoptosis. Pan *et al* revealed that SOX4 promoted cell cycle arrest and apoptosis in human lung non-small cell carcinoma H460 cells (9). Aaboe *et al* confirmed that SOX4 strongly impaired bladder carcinoma cell viability and promoted apoptosis (10). Li *et al* revealed that SOX4 was more highly expressed in primary-stage (AJCC I and II) than in advanced-stage (AJCC III and IV) melanoma cases (11). Jafarnejad *et al* reported that SOX4 was underexpressed in metastatic melanoma compared with dysplastic nevi and primary melanoma; furthermore, knockdown of SOX4 enhanced melanoma cell invasion (12).

Table I. PCR primer data.

Gene name	Forward primer	Reverse primer
SOX4	ACAGCGACAAGATCCCTTTC	CGGACTTCACCTTCTTCCTG
P65	AGCACAGATACCACCAAGACC	CGGCAGTCCTTTCCTACAAG

However, the role of SOX4 in the apoptosis of melanoma cells remains unknown.

NF-κB is a classical signaling pathway that is involved in the survival, proliferation and apoptosis of tumor cells. Watanabe *et al* revealed that inhibition of the expression and/or activity of NF-κB induced apoptosis in melanoma cells (13). In this study, the role of SOX4 in the apoptosis of melanoma A2058 and SK-MEL-5 cells was investigated, and the underlying mechanisms were determined. We demonstrated that inhibition of SOX4 markedly induced melanoma cell apoptosis via downregulation of the NF-κB signaling pathway, thus, indicating a novel target for melanoma treatment.

Materials and methods

Cell culture and lentiviral transfection. The human melanoma A2058 and SK-MEL-5 cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Carlsbad, CA, USA), supplemented with 1% penicillin/streptomycin (Sigma-Aldrtich; Merck KGaA, Darmstand, Germany) and 10% fetal bovine serum (FBS; Thermo Fisher Scientific) at 37˚C. The melanoma cells ($8x10^4$/well) were plated into 6-well plates and incubated overnight. When the melanoma cells had reached ~70% confluence, they were infected with SOX4 shRNA (Shanghai GeneChem Co., Ltd., Shanghai, China) and/or p65-overexpressing lentivirus (Shanghai GeneChem Co., Ltd.) in FBS-free medium containing 6 μg/ml Polybrene (Sigma-Aldrtich; Merck KGaA). We used blank vector lentivirus as the control. Twenty-four hours later, the FBS-free medium was replaced with 10% FBS culture medium. SOX4 and p65 expression in cells was confirmed by western blot analysis and a real-time PCR assay 72 h after lentiviral infection.

MTT assay. Cells ($5x10^3$) were seeded in 96-well plates. At 24, 48 or 72 h post-transfection, 20 μl MTT solution (5 mg/ml) was added to each well and incubated for 4 h. The medium was subsequently removed and 150 μl dimethyl sulfoxide (DMSO) was added. The optical density (OD) was detected at 600 nm with a microplate spectrophotometer (BD Biosciences, San Jose, CA, USA).

Western blot assay. A2058 and SK-MEL-5 cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) for total protein extraction. The cells were lysed on ice for 15 min, after 12,000 rpm centrifugation for 10 min. The proteins in the supernatant were extracted. We carried out nuclear protein extraction, according to the manufacturer's protocol (Beyotime Institute of Biotechnology). The samples were separated by 12% SDS-PAGE, then transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking of the membranes in 5% skimmed milk, the membranes were incubated with specific antibodies against SOX4 (1:100 dilution; cat. no. ab85204; Abcam, Cambridge, UK), Bcl-2 (1:1,000 dilution; cat. no. ab32124; Abcam), Bax (1:1,000 dilution; cat. no. ab32503), survivin (1:5,000 dilution; cat. no. ab76424; Abcam), p65 (1:1,000 dilution, cat. no. 3039; Cell Signaling Technology, Beverly, MA, USA) and PARP (1:1,000 dilution; cat. no. 9542; Cell Signaling Technology). An ECL detection system was used to detect the protein bands (Amersham Pharmacia Biotechnology, Tokyo, Japan).

Apoptosis assay. Apoptosis was determined based on Annexin V/7AAD staining using an Apoptosis Detection kit (BD Biosciences, San Diego, CA, USA), according to the manufacturer's instructions. Cells were harvested at 72 h post-transfection, and $2x10^4$ cells were collected and suspended in 100 μl 1X binding buffer, mixed with 5 μl Annexin V-PE and 5 μl 7AAD, in darkness for 20 min, and then 400 μl of 1X binding buffer was added to stop the staining reaction. Data were acquired using a FACSCalibur™ (BD Biosciences). Apoptosis was analyzed using FlowJo software v6.0 (Tree Star, Inc., Ashland, OR, USA).

RNA extraction and real-time PCR assay. Total RNA was extracted from melanoma cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific). Real-time PCR was performed using a SYBR® Premix Dimer Eraser™ kit (Takara Biotechnology, Co., Ltd., Dalian, China) in a 25-μl reaction system. Denaturation was performed at 95˚C for 30 sec, annealing at 60˚C for 30 sec, and elongation at 72˚C for 30 sec over 39 cycles. An ABI Prism 7900HT fast RT-PCR system (Applied Biosystems; Thermo Fisher Scientific) was used to perform the real-time PCR assay. The primers were designed as shown in Table I.

Chromatin immunoprecipitation (CHIP). The CHIP assay was performed according to the manufacturer's protocol (Millipore, Billerica, MA, USA). At 72 h after lentiviral infection, the A2058 cells were cross-linked using 1% formaldehyde (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature and stopped with glycine buffer, followed by sonication. DNA was sheared to fragments of 200-1,000 bp in length. Anti-SOX4 antibody was added at 4˚C overnight with rotation and IgG was used as a control. The immunoprecipitated DNA fragments were detected on 2% agarose gels and images were analyzed with an LAS 4000 luminescent image analyzer (Fujifilm, Tokyo, Japan). The CHIP primers were generated by Shanghai Sangon Co., Ltd. (Shanghai, China) (Table II).

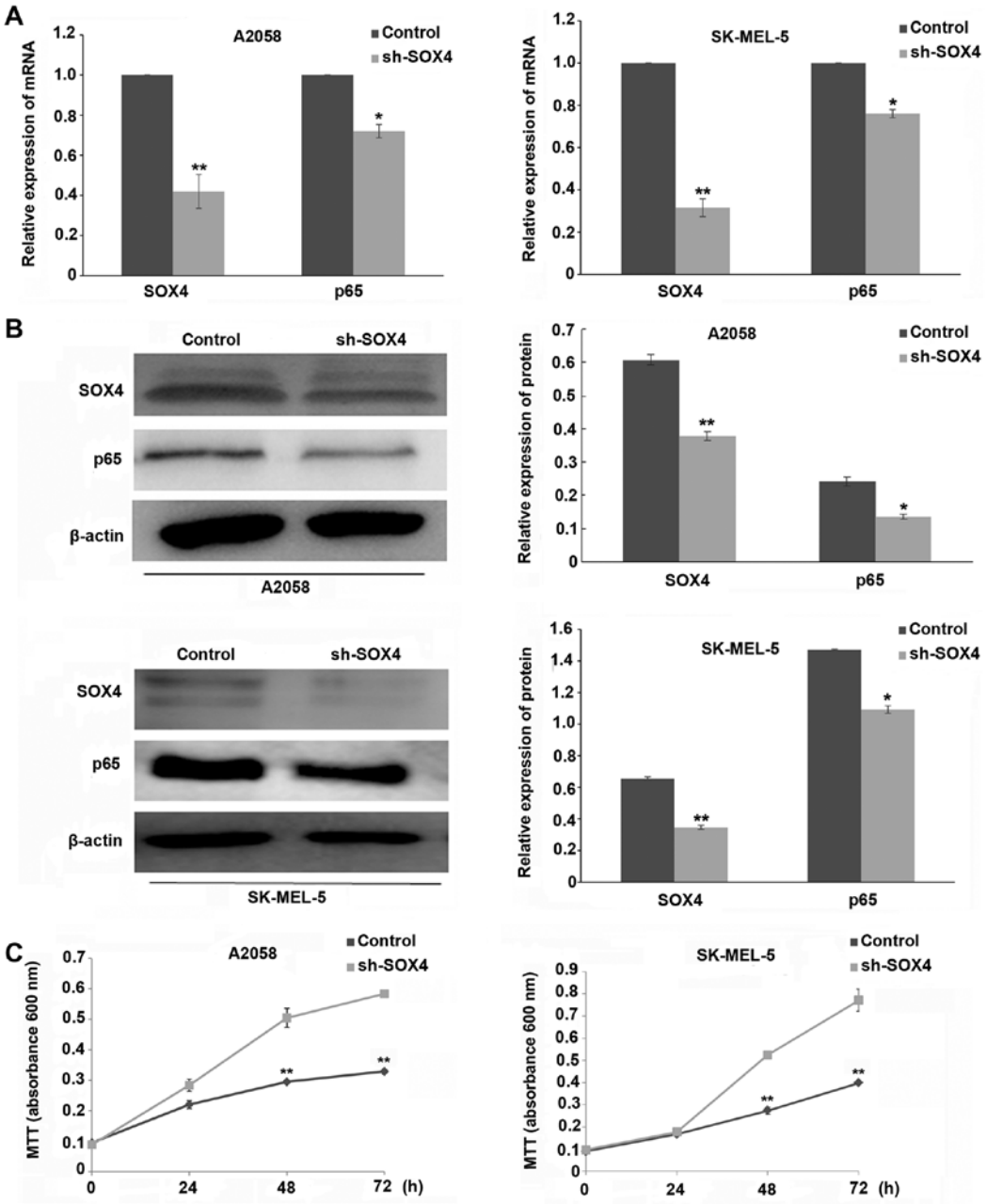

Figure 1. SOX4 downregulation inhibits p65 expression and cell proliferation in melanoma A2058 and SK-MEL-5 cell lines. (A) Real-time PCR assay for SOX4 and p65 mRNA, after SOX4 shRNA lentiviral transfection for 48 h. (B) Western blot analysis of SOX4 and p65 protein, after SOX4 shRNA lentiviral transfection for 72 h. (C) Cell proliferation was determined by MTT assay after SOX4 shRNA lentiviral transfection for 24, 48 and 72 h. Data are shown as the mean ± standard deviation. Each experiment was performed in triplicate. *P<0.05, **P<0.01 indicate statistical significance. sh-SOX4 represents SOX4 shRNA.

Table II. p65 PCR primer data.

p65	Forward primer	Reverse primer
+15 to -104	cgcgcacttggccccgac	cgcgcctgcgcgct
-113 to -202	acaaagtgagtaatcg	gtcggggccaagtgcgc
-1703 to -1810	cttgagcccaggagtttg	ggcgtgagccaccacgc
-1490 to -1603	ccacttctttacaaa	atctctgctcactgcag
-221 to -318	cctgcgcggggcgggc	taggggatttcagggc
-1300 to -1405	atacaatacaatacaata	taacttttaaattaatac
-990 to -1100	gtgctaactctattttcac	gacttttttattttctctga
-831 to -931	catcctcctttggggat	tctgtcatgtgacccc
-680 to -780	acacaggcgggggca	aatcccggagcctcg
-350 to -441	ggtaggacattttaacg	ggccttctgctccgcaga

Statistical analysis. All data were obtained from three separate experiments and are presented as the mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA). When comparing differences between two groups, we used a Student's t-test. Differences were considered to be statistically significant when P-values were <0.05.

Results

SOX4 downregulation inhibits p65 expression and cell proliferation in melanoma cells. As shown in Fig. 1A and B, the expression of SOX4 at both the mRNA and protein levels was significantly decreased in SOX4 shRNA-transfected A2058

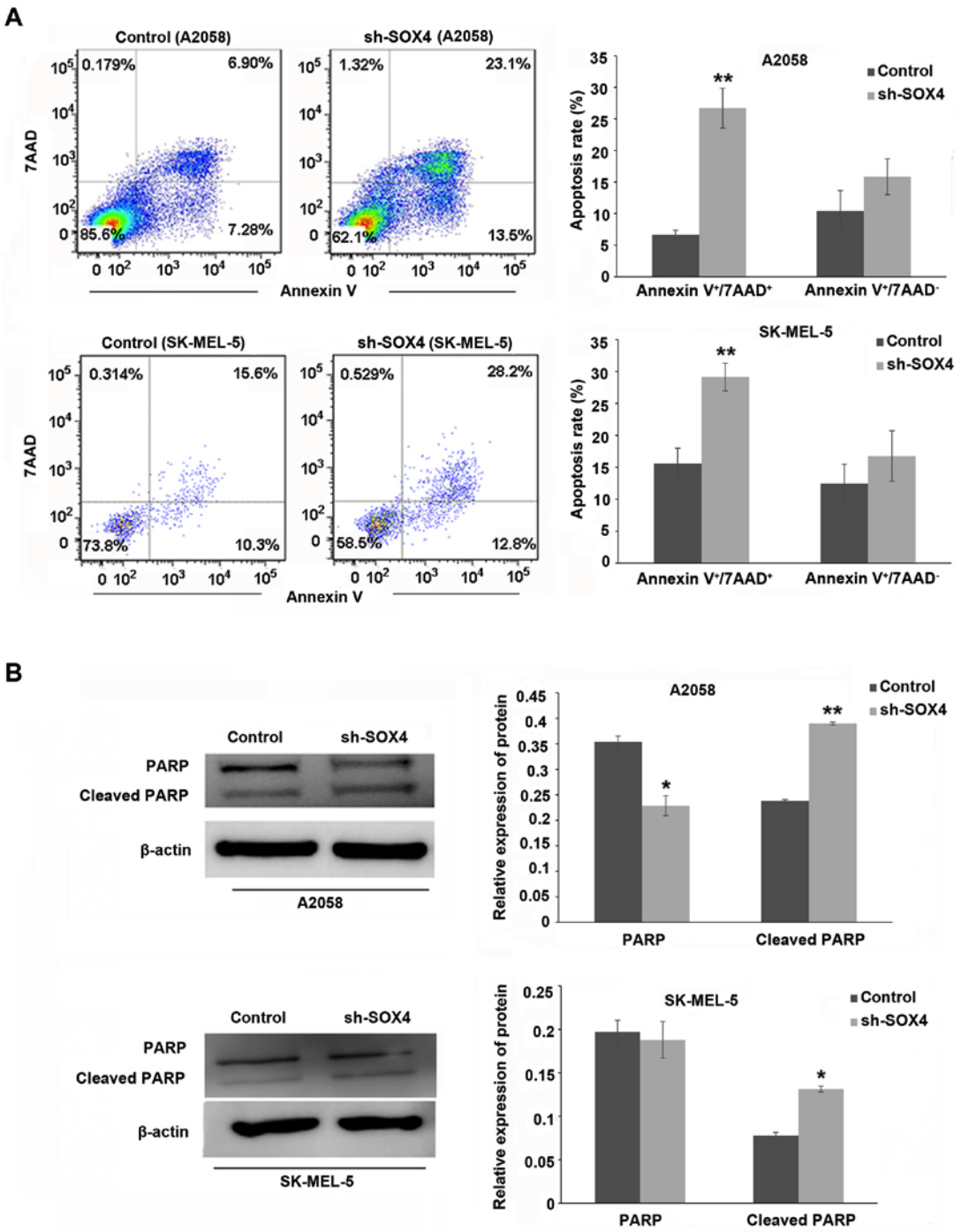

Figure 2. SOX4 downregulation promotes melanoma cell apoptosis. (A) Flow cytometry assay. SOX4 shRNA lentivirus-transfected A2058 and SK-MEL-5 cells were stained with Annexin V/7AAD, and then analyzed by flow cytometry. (B) Western blot analysis of PARP and cleaved PARP protein after SOX4 shRNA lentiviral transfection for 72 h in A2058 and SK-MEL-5 cells. *P<0.05, **P<0.01 indicate statistical significance. sh-SOX4 represents SOX4 shRNA.

and SK-MEL-5 cells, compared to the scrambled shRNA control group (P<0.01). As demonstrated in Fig. 1C, downregulation of SOX4 markedly inhibited the proliferation of A2058 and SK-MEL-5 cells at 48 or 72 h post-SOX4 shRNA transfection (P<0.01). We also observed that SOX4 downregulation inhibited the expression of NF-κB p65 at both the mRNA and protein levels (P<0.05) (Fig. 1A and B).

SOX4 downregulation promotes melanoma cell apoptosis. As shown in Fig. 2A, SOX4 downregulation significantly increased the level of apoptosis in the A2058 and SK-MEL-5 cells at 72 h post-SOX4 shRNA transfection (P<0.01). The expression of cleaved PARP was increased, while that of pro-PARP was decreased (Fig. 2B; P<0.01, P<0.05).

SOX4 downregulation decreases the expression of Bcl-2 and survivin, and increases the expression of Bax; meanwhile, SOX4 was able to bind to the promoter region of p65. Bcl-2 and survivin belong to an anti-apoptotic protein family, while Bax belongs to a pro-apoptotic protein family. As shown in Fig. 3A and B, SOX4 downregulation markedly inhibited the expression of Bcl-2 and survivin in the A2058 (P<0.05, P<0.01,

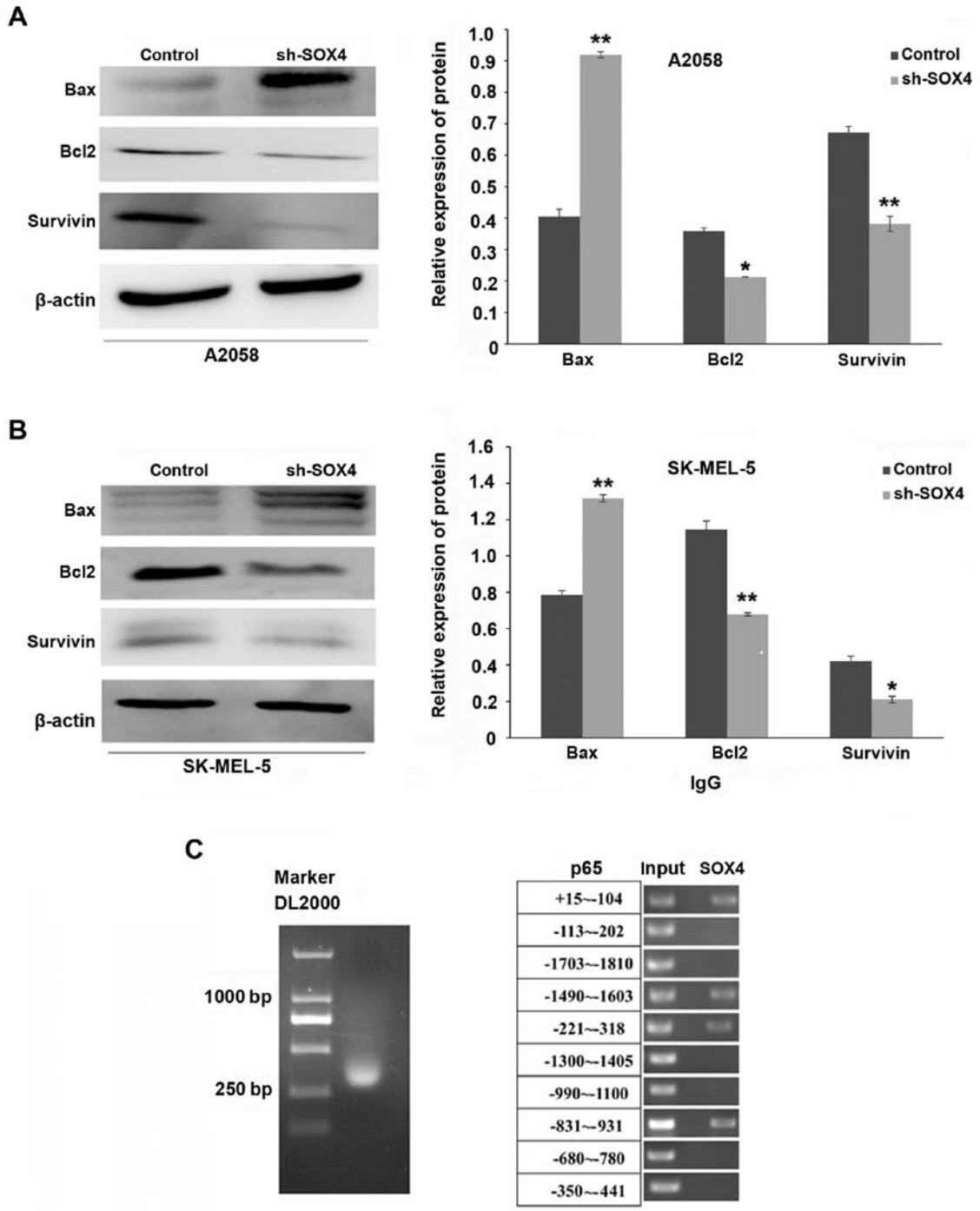

Figure 3. SOX4 downregulation decreased the expression of Bcl-2 and survivin, and increased the expression of Bax; meanwhile, SOX4 was able to bind to the promoter region of p65. Western blot analysis of Bax, Bcl-2 and survivin proteins in (A) A2058 and (B) SK-MEL-5 cell lines. (C) CHIP-PCR assays were performed with A2058 cells. Chromatin fragments were pulled down with anti-SOX4, using IgG as a control. Semi-quantitative PCRs were performed using specific p65 promoter primers. The assay was repeated twice. *P<0.05, **P<0.01 indicate statistical significance. sh-SOX4 represents SOX4 shRNA.

respectively) and SK-MEL-5 (P<0.01, P<0.05, respectively) cells, while the expression of Bax was increased (P<0.01). To determine whether SOX4 could bind to the promoter region of p65, a CHIP-PCR assay was performed. As shown in Fig. 3C, SOX4 bound to the p65 promoter region at the following positions: +15 to -104, -1490 to -1603, -221 to -318 and -831 to -931 bp.

p65 overexpression partially reverses SOX4 downregulation-induced apoptosis. As shown in Fig. 4A and B, the expression of p65 mRNA and protein was significantly increased in the p65-overexpressing and SOX4-knockdown melanoma cells, compared to SOX4-knockdown alone cells (P<0.01). As demonstrated in Fig. 4C and E, p65 overexpression partially reversed SOX4 downregulation-induced decreases in cell proliferation and increases in apoptosis (P<0.01, P<0.05). The decreases in pro-PARP and survivin were also partially reversed in p65-overexpressing and SOX4-knockdown melanoma cells, compared to the SOX4-knockdown alone cells (Fig. 4D).

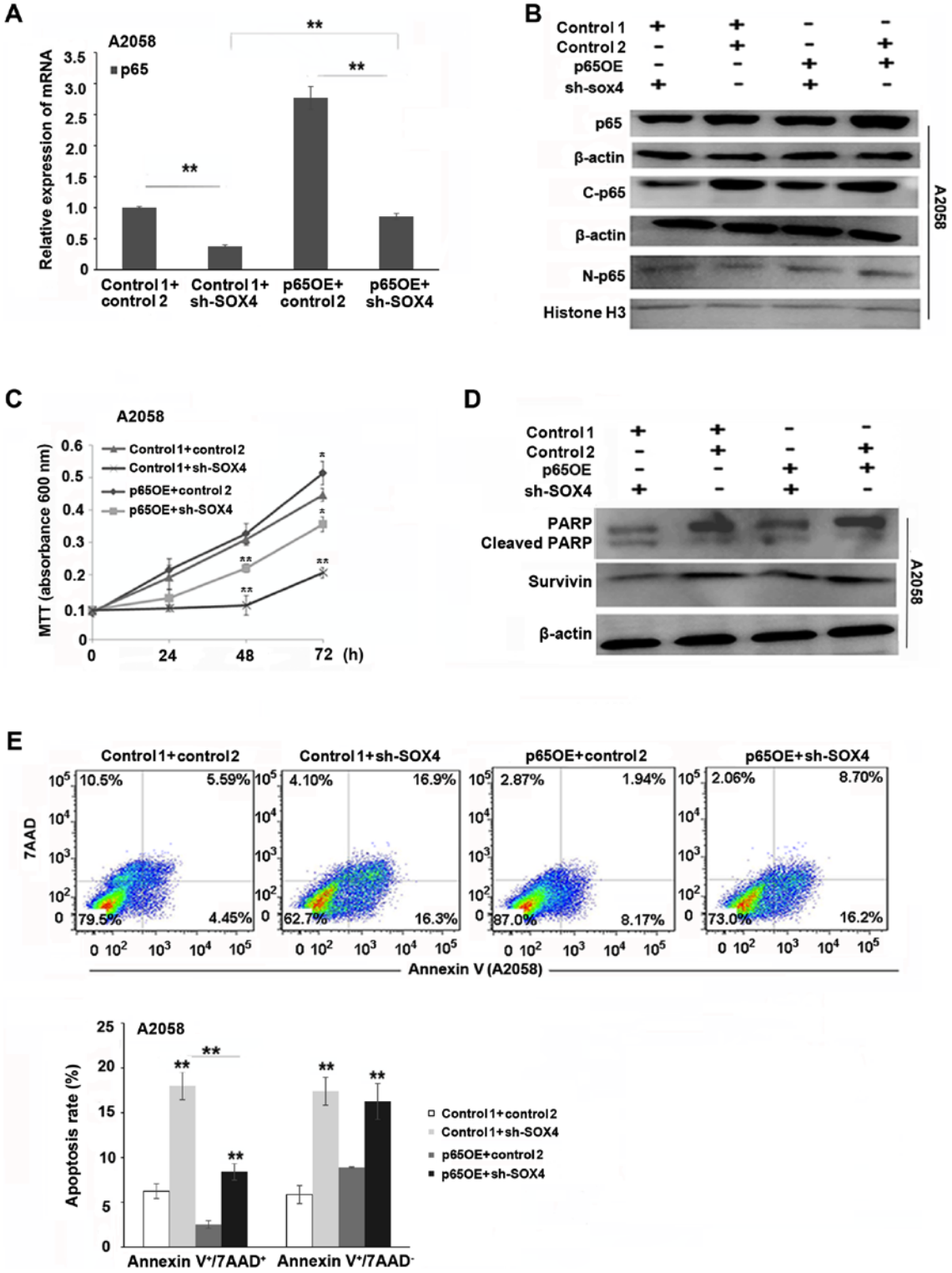

Figure 4. p65 overexpression partially reverses SOX4 downregulation-induced apoptosis. Melanoma A2058 cells were co-transfected with p65-overexpressing and SOX4 shRNA lentiviruses for 72 h. (A) Real-time PCR assay for p65 mRNA. (B) Western blot analysis for p65. (C) Cell proliferation was determined by MTT assay after transfection for 24, 48 and 72 h. Data are shown as the mean ± standard deviation. (D) Western blot analysis of PARP, cleaved-PARP and survivin. (E) Apoptosis analysis. The cells were stained with Annexin V/7AAD and then analyzed by flow cytometry. Each experiment was performed in triplicate. *P<0.05, **P<0.01 indicate significance. p65-OE represents p65 overexpression. Control 1 represents p65-OE vector control. sh-SOX4 represents SOX4 shRNA. Control 2 represents sh-SOX4 vector control. N-p65 represents p65 in nuclear protein; C-p65 represents p65 in cytoplasmic protein.

Discussion

Several studies have demonstrated that SOX4 is highly expressed in nearly all of the major cancers in humans, including breast, lung, brain, prostate, colorectal, bladder and ovarian cancers, which indicates a central role in the development of multiple tumors (14). Previous studies have demonstrated that SOX4 effectively drives cells towards apop-

tosis (15,16). In bladder carcinoma cells, the pathway through which SOX4 promotes apoptosis is still not understood. This indicated that SOX4 may induce apoptosis independently of caspase-3. Downstream target genes of SOX4 are involved in signal transduction (MAP2K5), angiogenesis (NRP2), and cell cycle arrest (PIK3R3) (10). However, other studies have reached the opposite conclusion. Zhou *et al* revealed that downregulation of SOX4 expression induced apoptosis in lung cancer patients through upregulation of caspase-3 expression (17). The role of SOX4 in the apoptosis of melanoma cells remains unknown. In the present study, we demonstrated that downregulation of SOX4 markedly inhibited melanoma cell proliferation and promoted cellular apoptosis.

It has been reported that SOX4 may interact with many signaling pathways, such as Wnt (8) or p53 (18), to influence cellular apoptosis. Low SOX4 expression can also facilitate peritoneal macrophage apoptosis by activating caspase-3 (19). Hur *et al* demonstrated that there is an important structural domain in SOX4 related to apoptosis; when the glycine in the domain is replaced by serine, apoptosis is inhibited (20). Nonetheless, the mechanisms of SOX4 inhibition-induced apoptosis in melanoma cells require further investigation.

NF-κB is one of the most important transcription factors that play a crucial role in the suppression of apoptosis, as well as the induction of cell proliferation and inflammation, which is closely associated with cancer development (21-24). NF-κB activation is involved in the inhibition of apoptosis by upregulating the expression of anti-apoptotic proteins, including Bcl-2, Mcl-1 and survivin (25). Tao *et al* revealed that triptolide induced melanoma A375 apoptosis by inhibiting NF-κB signaling pathways (26). However, NF-κB has not only been proven to suppress apoptosis, but has also been proven to induce apoptosis. Schneider *et al* reported that NF-κB activation promoted neuronal cell death in focal cerebral ischemia (27). In this experiment, we observed that inhibition of SOX4 downregulated the expression of NF-κB p65, and NF-κB p65-targeted genes, such as Bcl-2 and survivin, were also decreased.

Apoptosis is the process of programmed cell death and plays an important role in cancer development. Apoptosis involves the balance between pro-apoptotic and anti-apoptotic proteins. Tumor cells can resist apoptosis by expressing the anti-apoptotic protein Bcl-2, while decreasing the expression of the pro-apoptotic protein Bax (28). Survivin is a member of the IAP family that inhibits cellular apoptosis, and is expressed in human melanoma, including primary melanoma and metastatic melanoma, but not expressed in normal melanocytes (29). Survivin has been found to inhibit the activity of caspase-9 by binding to HBXIP (30). Our study demonstrated that the anti-apoptotic proteins Bcl-2 and survivin were downregulated, while the pro-apoptotic proteins Bax and cleaved-PARP were upregulated, in SOX4-knockdown melanoma cells.

In order to determine whether SOX4 binds to the promoter of p65, a CHIP-PCR assay was performed. Our data revealed that SOX4 was able to bind to the p65 promoter at positions +15 to -104, -1490 to -1603, -221 to -318 and -831 to -931 bp. However, the CHIP-PCR assay only indicated that there was a connection between SOX4 and NF-κB p65, and it could not confirm whether the connection was direct or indirect. Further research needs be performed to confirm a direct binding event. We also revealed that p65 overexpression partially reversed SOX4 knockdown-induced increases in apoptosis, which further suggested that inhibition of SOX4 induces melanoma cell apoptosis partially through the downregulation of NF-κB p65 signaling.

In conclusion, we demonstrated that inhibition of SOX4 markedly induced melanoma cell apoptosis via downregulation of the NF-κB signaling pathway, which thus may serve as a novel approach for the treatment of melanoma.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

QC, HX and JFW conceived and designed the study. QC, JD, LX performed the experiments. QC and JD wrote the paper. XL and ZL analyzed the data. FGZ, JFW, JHX and QC reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Institutional Review Board of the Department of Laboratory Animal Science of Fudan University (Shanghai, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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