SOX4 promotes melanoma cell migration and invasion though the activation of the NF-κB signaling pathway

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Abstract. SOX4 has been reported to be abnormally expressed in many types of cancer, including melanoma. However, its role in cell proliferation and metastasis remains controversial. In this study, SOX4 was downregulated or overexpressed in A375, A2058 and A875 melanoma cells by siRNA or lentivirus transfection, respectively. Cell metastasis was observed by Transwell assay. In an aim to elucidate the underlying mechanisms, we determined the expression of nuclear factor-κB (NF-κB) by real-time PCR assay and western blot analysis. Our data indicated that SOX4 knockdown inhibited melanoma cell migration and invasion. In the melanoma cells in which SOX4 was downregulated, the expression levels of NF-κB/p65, matrix metalloproteinase (MMP)2 and MMP9 were suppressed at both the mRNA and protein levels. Conversely, the overexpression of SOX4 promoted melanoma cell migration and invasion. In the melanoma cells in which SOX4 was overexpressed, the expression levels of NF-κB/p65, MMP2 and MMP9 were increased at both the mRNA and protein level. On the whole, our findings indicate that SOX4 promotes melanoma cell migration and invasion through the activation of the NF-κB/p65 signaling pathway. Thus, SOX4 may prove to be a potential therapeutic target for the treatment of melanoma.

Introduction

According to the Centers for Disease Control and Prevention (CDC) in the USA, malignant melanoma is the most aggressive and lethal skin cancer (1,2). Its incidence is increasing (3). Patients with metastatic melanoma have an extremely poor prognosis (4). Recent studies have reported that the abnormal activation of pathways, such as WNT, mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), maybe crucial events responsible for the invasion and metastasis of melanoma (5-8). NF-κB is an important signaling pathway which is highly expressed in a number of tumors. Hu *et al* proved that NF-κB inhibitor could inhibit the migration of human uveal melanoma cells (9). Wu *et al* also found s low expression of NF-κB p65 protein accompanied by the decreased migration and invasion of A375.S2 cells (10). These studies show that the expression of NF-κB in melanoma is closely related to the migration and invasion of tumors. The SOX4 gene, a transcription factor and s member of the SOX family, regulates transcription through numerous methods and mediates both gene activation and repression. It is overexpressed in a wide variety of malignancies, such as lung cancer (11), breast cancer (12,13) and prostate cancer (14), and is closely associated with cancer migration and invasion. However, in a limited subset of tumors, SOX4 has been reported to act as a tumor suppressor (15). Some studies have shown that the increased expression of SOX4 correlates with prolonged patient survival and slower cancer invasion and metastasis, including bladder cancer, melanoma and gallbladder cancer (16-18). Over the past 10 years, scholars have focused on the role of SOX9 and SOX10 in melanoma. It is not surprising that both SOX9 and SOX10 are expressed during various stages of melanoma progression and in established melanoma cell lines (19).

However, few scholars have investigated the association between SOX4 and melanoma. In this study, we examined SOX4 expression in melanoma. The expression of SOX4 was knocked down or upregulated by small interfering RNA (siRNA) or lentivirus transfection, respectively to examine the effects of SOX4 on the invasion and migration of melanoma cells. We also aimed to elucidate the underlying mechanisms. We found that SOX4 promoted melanoma cell migration and invasion, and these that these effects were partly mediated though the activation of the nuclear factor-κB (NF-κB)/p65 signaling pathway.

Materials and methods

Cell culture, siRNA and lentivirus transfection. The human melanoma cell lines. A375, A875 and A2058. were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Sigma, Poznan, Poland) at 37˚C in a

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5% CO_2 incubator. SOX4-specific siRNA (si-SOX4; sc-38412) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and si-SOX4 was transfected into the cells at a final concentration of 5 or 10 nM using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Scrambled siRNA (sc-37007; Santa Cruz Biotechnology, Inc.) was used as a negative control. The cells were harvested at 24 or 48 h following transfection. The melanoma cells ($8x10^4$/well) were plated into a 6-well plate and incubated overnight. The medium was changed with FBS-free medium containing 6 μg/ml polybrene and SOX4 shRNA viral or overexpression lentivirus (both from GeneChem, Shanghai, China). A blank vector lentivirus was used for the control group. After 24 h, fresh medium with 10% FBS was added to the cells and the cells were incubated for an additional 48 h. SOX4 expression in cells following transfection was confirmed by western blot analysis and real-time PCR.

In this study, we used both siRNA and shRNA. This was done as the use of siRNA in some cases can lead to mismatches with the RNA target, and the knockdown of genes other than the intended target may make the results difficult to interpret. In order to eliminate the off-target effects of siRNA, we also used sox4 shRNA viral transfection.

Western blot analysis. For total protein extraction, the A375, A875 and A2058 cells were lysed in RIPA buffer (Beyotime, Jiangsu, China) for 15 min and centrifuged for 10 min at 12,000 rpm. The supernatant was harvested and stored at -80˚C. For nuclear protein extraction, the A2058 cells were suspended in hypotonic buffer (20 mM Tris-HCL pH 7.9, 1.5 mM $MgCl_2$ and 10 mM KCL). Following incubation on ice for 15 min, 12 μl 10% (v/v) NP-40 were added, and the combination was vortexed for 10 sec and kept on ice again for a further 10 min, followed by centrifugation at 14,000 rpm at 4˚C for 1 min. The supernatants, which were considered to be the cytoplasmic extracts, were harvested. The pellets were washed once with 100 μl hypotonic buffer, and resuspended in high salt buffer (20 mM Tris-HCL pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$ and 0.2 mM EDTA). Following 30 min of incubation on ice, the lysates were centrifuged at 14,000 rpm at 4˚C for 10 min; the resultant supernatants were kept as nuclear extracts. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. Specific proteins were sequentially blotted with primary antibodies, including SOX4 (ab80261; Abcam, Cambridge, UK) p65 (#8242), matrix metalloproteinase (MMP)2 (#13132) and MMP9 (#2270) (Cell Signaling Technology, Beverly, MA, USA). After washing, the blots were incubated with HRP-conjugated secondary antibody (#7074; Cell Signaling Technology) Immunocomplexes were detected using the ECL detection system (Amersham Pharmacia Biotechnology, Tokyo, Japan). Densitometric analysis of the western blot analysis results was carried out using ImageJ software.

RNA extraction and real-time PCR. RNA from the melanoma cell lines was extracted using TRIzol reagent (Invitrogen). The resulting RNA was reverse transcribed into cDNA using PrimeScript™ 1st strand cDNA Synthesis kit (Takara, Dalian, China) and amplified using specific primers. The primers were composed by Sangon Co. (Shanghai, China) (Table I). The RNA concentration and quantity were assessed using a NanoDrop Spectrophotometer. Real-time PCR was performed on a 25 μl reaction system using the SYBR® Premix DimerEraser™ kit according to the manufacturer's instructions (Takara). Denaturation was performed at 95˚C for 30 sec, annealing at 60˚C for 30 sec, and elongation at 72˚C for 30 sec for 39 cycles. PCR was carried out in triplicate and analyzed using the ABI Prism 7900HT Fast RT-PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA).

Cell migration and invasion assay. Cell invasion assay and migration assay were both measured by counting the numbers of cells that invaded through a well with 8.0-μm pores (Costar, Cambridge, UK). Cell invasion assay was performed using a well coated with Matrigel™ (1:5 dilution; BD Biosciences, San Jose, CA, USA), while the migration assay was performed using a well without Martigel coating. The cells were starved with serum-free DMEM for 12 h, $3x10^4$ cells in serum-free DMEM were then added to the upper chamber and DMEM with 10% serum was added to the lower chamber. After 24 or 48 h of incubation, the cells on the surface of the filter membrane were removed with cotton swabs. The invading cells on the lower surface of the filter membrane were fixed in 4% paraformaldehyde, stained with 0.5% crystal violet (46364; Sigma-Aldrich, St. Louis, MO, USA), and counted in 5 random squares using a microscope (Olympus CX22, Olympus, Tokyo, Japan). Each experiment was performed in triplicate.

Immunological staining assay. The cells were transfected with si-SOX4 or scrambled siRNA, After 48 h, the cells were fixed with 4% paraformaldehyde on the slides and incubatedin 0.2% Triton X-100 for 2 min. The slides were then blocked with 5% BSA at room temperature for 30 min, followed by incubation with rabbit anti-p65 antibody (#8242; Cell Signaling Technology)at 4˚C overnight. The cells were then stained with a secondary Alexa Fluor 594-labeled goat anti-rabbit IgG (1:500; ab150080; Abcam) and counterstained with 1 μg/ml 4,6-diamino2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich). The cells were visualized using an Olympus fluorescence microscope.

Statistical analysis. All data are presented as the means ± standard deviation. Data analysis was performed by one-way analysis of variance (ANOVA). For comparisons between 2 groups, a Student's t-test was used. Differences with P-values <0.05 were considered to be statistically significant.

Results

Downregulation of SOX4 induced by SOX4 siRNA inhibits the migration of melanoma cells. We examined the effects of the siRNA-mediated knockdown of SOX4 in melanoma cell lines (A375, A2058 and A875) *in vitro*. Transfection with SOX4-specific siRNA resulted in a significant decrease in the SOX4 mRNA and protein levels in the A375, A2058 and A875 cells, as compared with the negative control ($P<0.01$ and $P<0.05$) (Figs. 1A and 2A-C). We then examined the effects of SOX4 knockdown on cell migration. The downregulation

Table I. Sequences of primers used for PCR.

Gene name	Forward primer	Reverse primer
SOX4	ACAGCGACAAGATCCCTTTC	CGGACTTCACCTTCTTCCTG
p65	AGCACAGATACCACCAAGACC	CGGCAGTCCTTTCCTACAAG
MMP2	TATGGCTTCTGCCCTGAGAC	CACACCACATCTTTCCGTCA
MMP9	AGTCCACCCTTGTGCTCTTC	ACTCTCCACGCATCTCTGC

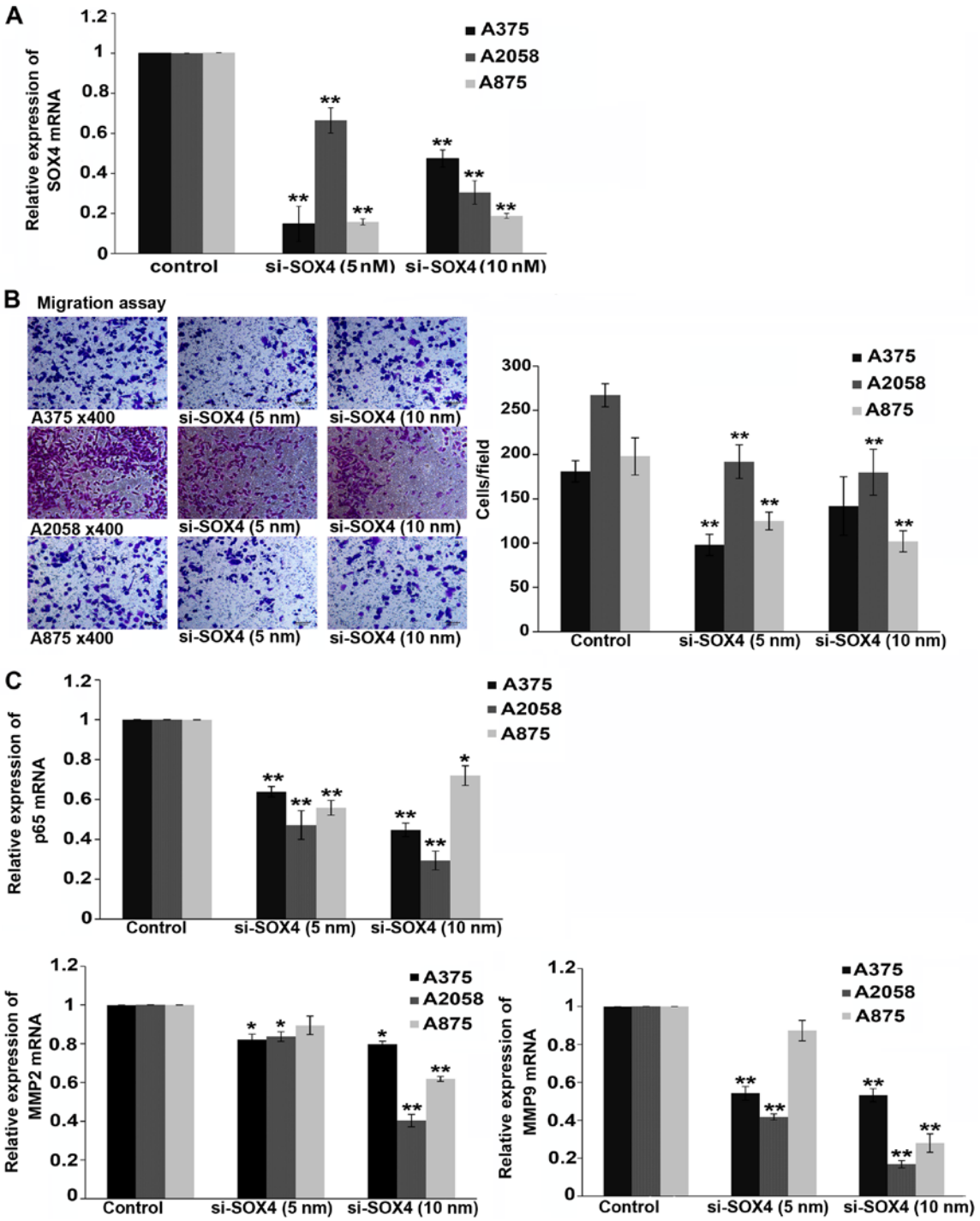

Figure 1. Downregulation of SOX4 induced by SOX4 siRNA suppresses melanoma cell migration through the inhibition of p65, MMP2 and MMP9 mRNA expression. A375, A2058, and A875 cells were transfected with si-SOX4 (5 and 10 nM) or scrambled siRNA. (A) Real-time PCR assay for SOX4 mRNA expression following transfection with siRNA for 24 h. (B) Transwell migration assay: the invading cells were stained with 0.5% crystal violet, and counted in 5 random squares using a microscope. Each experiment was performed in triplicate. (C) Real-time PCR assay for p65, MMP2 and MMP9 mRNA expression following transfection with SOX4 siRNA transfection for 24 h. *P<0.05 and **P<0.01 compared to the control.

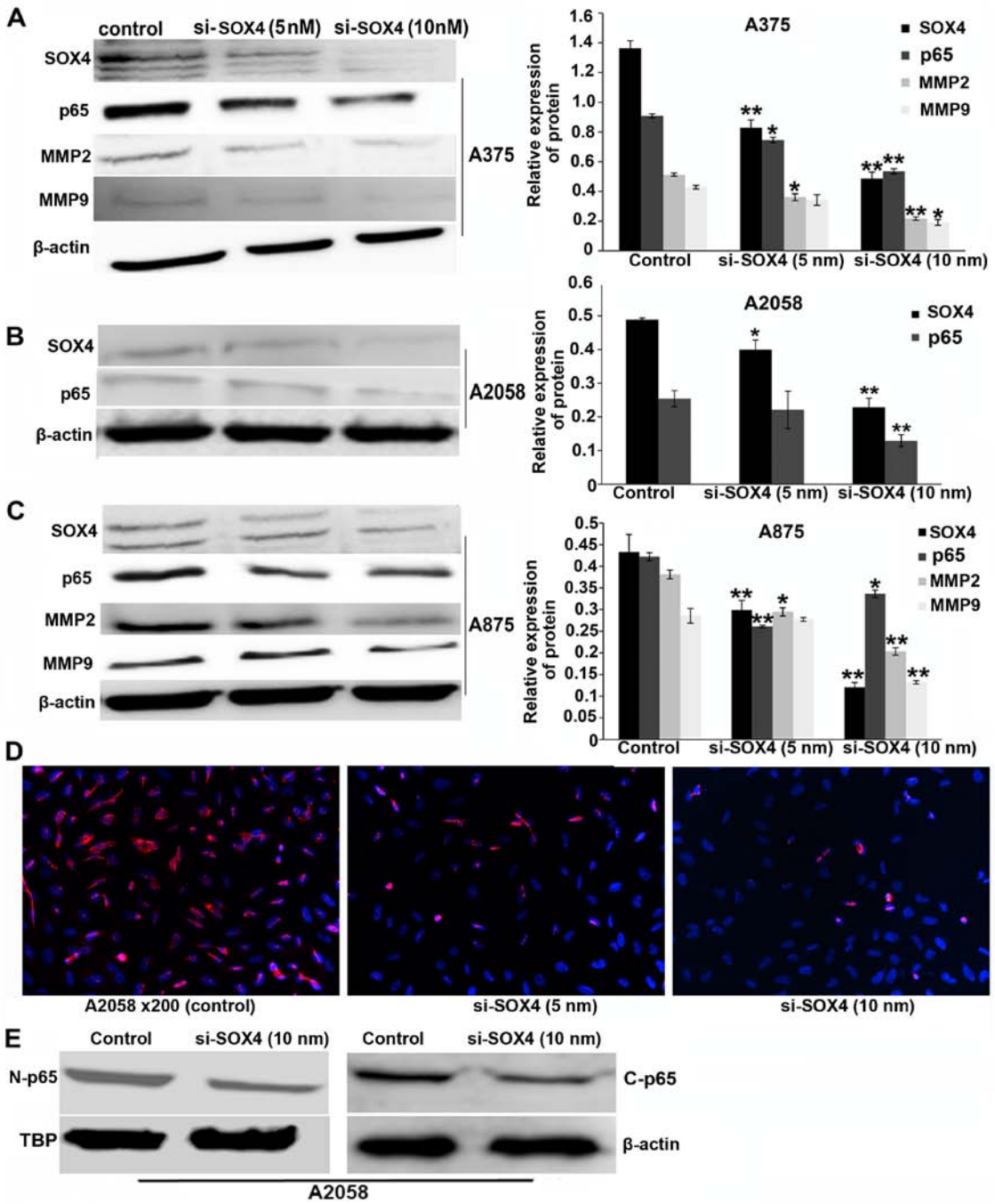

Figure 2. SOX4, p65, MMP2 and MMP9 protein expression levels were downregulated in SOX4-siRNA-transfected cells. A375, A2058, and A875 cells were transfected with si-SOX4 (5 and 10 nM) or scrambled siRNA. (A) Western blot analysis for SOX4, p65, MMP2 and MMP9 protein expression following transfection of A375 cells with SOX4 siRNA for 48 h. (B) Western blot analysis for SOX4 and p65 protein expression following transfection of A2058 cells with SOX4 siRNA transfection for 48 h. (C) Western blot analysis for SOX4, p65, MMP2 and MMP9 protein expression following transfection of A875 cells with SOX4 siRNA for 48 h. (D) Immunofluorescence staining for nuclear factor-κB (NF-κB) p65 expression level: the expression of p65 was analyzed using a specific monoclonal antibody and an Alexa Fluor-594-conjugated secondary antibody. The nuclei were stained with DAPI. Cells were visualized using an Olympus fluorescence microscope. *P<0.05 and **P<0.01 compared to the control. (E) Western blot analysis of p65 expression in the nuclear and cytoplasmic fraction of SOX4 siRNA-transfected A2058 cells. N-p65 represent p65 in nuclear protein, C-p65 represent p65 in cytoplasmic protein.

of SOX4 markedly suppressed the migration of the melanoma cells (P<0.01; Fig. 1B).

NF-κB p65 is downregulated in SOX4-siRNA-transfected cells. NF-κB is one of the key modulators of tumor cell migration and invasion (9,10). In this study, we observed a marked decrease in the level of NF-κB p65 mRNA (P<0.01 and P<0.05) (Fig. 1C) and protein (P<0.01 and P<0.05; Fig. 2A-C) in the SOX4 siRNA-transfected cells compared to the controls. NF-κB is a transcription factor. The NF-κB p65 expression level was analyzed by immunological staining. As demonstrated by immunological staining assay, the expression of nuclear NF-κB was decreased in the SOX4 siRNA-transfected melanoma cells, as compared with that in the scrambled siRNA-transfected cells (Fig. 2D). A similar tendency was observed by western blot analysis (Fig. 2E).

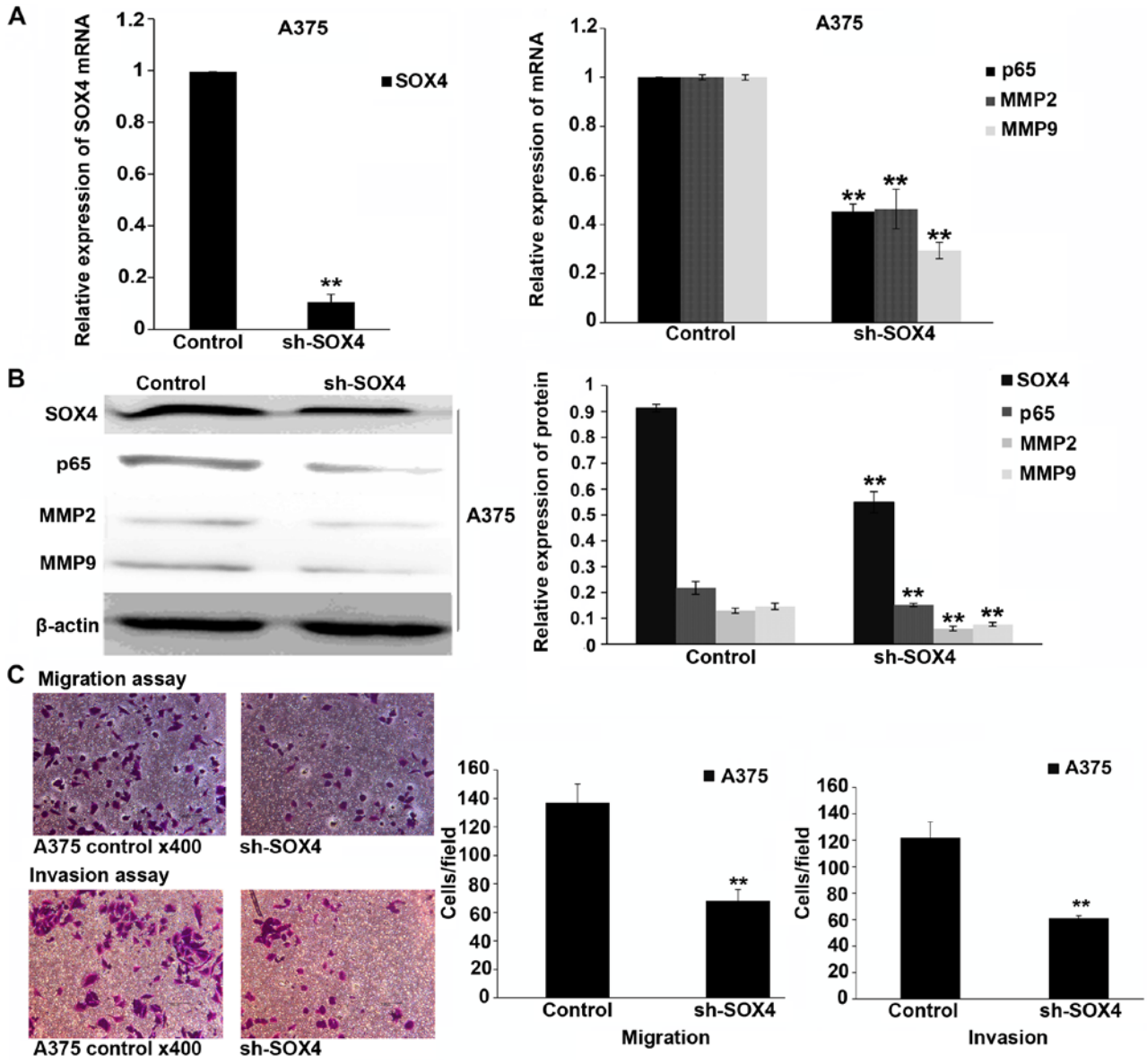

Figure 3. SOX4 shRNA viral transfection decreases melanoma cell migration and invasion, and inhibits the expression of MMP2, MMP9 and nuclear factor-κB (NF-κB) p65. Melanoma A375 cells were transfected with SOX4 shRNA for 72 h. (A) Real-time PCR assay for SOX4, p65, MMP2 and MMP9 mRNA expression. (B) Western blot analysis for SOX4, p65, MMP2 and MMP9 protein expression. (C) Migration and invasion assay. The invading cells were stained with 0.5% crystal violet, and counted in 5 random squares using a microscope. Each experiment was performed in triplicate. **P<0.01 compared to the control. sh-SOX4 represents SOX4 shRNA.

Expression levels of MMP2 and MMP9 are decreased in SOX4 siRNA-transfected melanoma cells. MMP2 and MMP9 are two of the NF-κB target genes. Thus, we performed real-time PCR to determine the mRNA expression of MMP2 and MMP9 following transfection of the melanoma cells with SOX4 siRNA for 24 h. Our results revealed a marked downregulation in the mRNA levels of MMP2 and MMP9 in the A375, A2058 and A875 cells transfected with SOX4 siRNA (P<0.01 and P<0.05; Fig. 1C). Similar results were observed for MMP2 and MMP9 protein expression after 48 h of SOX4 siRNA transfection (P<0.01 and P<0.05; Fig. 2A and C).

SOX4 shRNA viral transfection decreases melanoma cell migration and invasion, and inhibits the expression of MMP2, MMP9 and NF-κB p65. To verify the results observed with SOX4 siRNA transfection, we performed the experiments using shRNA viral transfection. After 72 h, SOX4 expression was suppressed by SOX4 shRNA viral transfection. The mRNA and protein levels of p65, MMP2 and MMP9 were also inhibited in the SOX4 shRNA-transfected cells (P<0.01; Fig. 3A and B). The downregulation of induced by shRNA viral transfection significantly suppressed melanoma cell migration and invasion (P<0.01; Fig. 3C).

Overexpression of SOX4 promotes melanoma cell migration and invasion, and upregulates the expression of MMP2, MMP9 and NF-κB p65. SOX4 was overexpressed by lentiviral transfection. The mRNA and protein levels of p65, MMP2 and MMP9 were upregulated in the melanoma cells after 72 h of transfection (P<0.01; Fig. 4A and B). Migration and invasion assays demonstrated that the overexpression of SOX4 promoted melanoma cell migration and invasion (P<0.01; Fig. 4C).

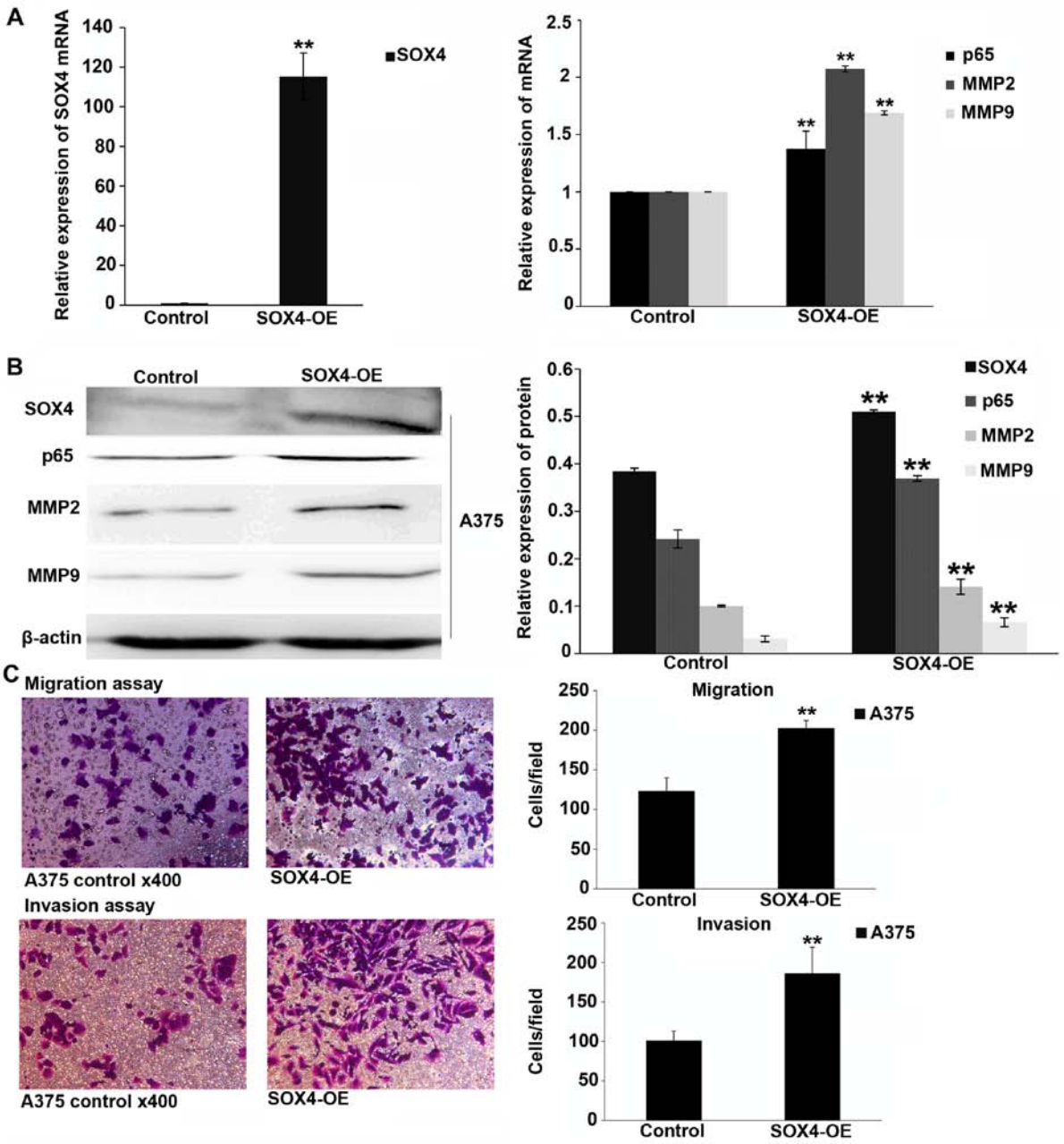

Figure 4. Overexpression of SOX4 promotes melanoma cell migration and invasion and upregulates the expression of MMP2, MMP9 and nuclear factor-κB (NF-κB) p65. Melanoma A375 cells were transfected with SOX4 overexpression lentivirus transfection for 72 h. (A) Real-time PCR assay for SOX4, p65, MMP2 and MMP9 mRNA expression. (B) Western blot analysis for SOX4, p65, MMP2 and MMP9 protein expression. (C) Migration and invasion assay. The invading cells were stained with 0.5% crystal violet, and counted in 5 random squares by microscopic. Each experiment was performed in triplicate. **P<0.01 compared to the control. SOX4-OE represents SOX4 overexpression.

Discussion

SOX4 is highly expressed in many tumors and plays an important role in the occurrence and progression of tumors (20,21). It has been proposed to act as either an oncogene or a tumor suppressor (16). Aaboe *et al* found that SOX4 was overexpressed in bladder cancer tissues compared to normal tissues, but a strong SOX4 expression was found to correlate with increased patient survival (16). Similar results were observed by Zhang *et al* (22). Jafarnejad *et al* reported that the knockdown of SOX4 enhanced melanoma cell invasion and migration (17). In this study, we found that SOX4 siRNA transfection decreased melanoma (A375, A2058 and A875) cell invasion and migration. Similar anti-invasive and anti-migratory effects were observed in the SOX4 shRNA transfected melanoma cells. Different melanoma cell lines were used in the study by Jafarnejad *et al* (17) and our study. SOX4 expressed in different cell lines may bind to different proteins, which regulates different target genes and lead to different biological functions.

NF-κB is a major transcription factor which is present in the cytoplasm in an inactive complex and can be activated by various stimuli, and is linked to various cellular processes in cancer, including inflammation, invasion and metastasis (23). There are 5 members of the NF-κB family in mammals. The most abundant activated form of NF-κB is a heterodimer composed of a p50 and p65 (24). Aggarwal and Sung demon-

strated that the inhibition of NF-κB in melanoma cells can sensitize tumors to chemotherapeutic agents (25). NF-κB has been shown to be upregulated in melanoma and has been specifically identified as being a potential 'master regulator' of melanoma invasion (26,27). In this study, we found that the NF-κB p65 expression level was significantly decreased in the melanoma cells in which SOX4 was knocked down. In addition, the NF-κB p65 expression level was elevated in the melanoma cells overexpressing SOX4.

Activated NF-κB translocates to the nucleus and can induce the expression of MMPs (23). MMPs are the most important metastasis-promoting genes (28) and are responsible for breaking down the extracellular matrix (ECM) microstructure. The excess degradation of the surrounding ECM is one of the hallmarks of tumor invasion and metastasis. MMP2 and MMP9 can degrade several ECM proteins and correlate with tumor invasion and metastasis; they are known to be regulated by several intracellular signaling pathways (29). Highly aggressive melanoma cells express high levels of MMP2 and MMP9 (30). We observed that the knockdown of SOX4 significantly downregulated MMP2 and MMP9 expression, whereas the overexpression of SOX4 upregulated MMP2 and MMP9 expression.

In conclusion, this study demonstrates that SOX4 promotes melanoma cell migration and invasion. These effects were partly mediated through the activation of the NF-κB/p65 signaling pathway. Further *in vivo* studies are required in order to verify the function of SOX4 in melanoma cell migration and invasion.

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