SOX2 promotes tumorigenicity and inhibits the differentiation of I-type neuroblastoma cells

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Abstract. SOX2 is a transcription factor associated with the pluripotency, proliferative potential, and self-renewing properties observed with embryonic stem cells and germ cells. SOX2 expression has been reported in several cancers and is implicated in tumorigenesis. We previously found that SOX2 expression was correlated to the clinical stage of neuroblastoma. Recently, we found that SOX2 overexpression occurs in I-type neuroblastoma cells (BE(2)-C cells). To elucidate the tumorigenic function of SOX2, we established a SOX2 overexpressed BE(2)-C cell line. SOX2 overexpressed cells showed higher tumorigenicity than control cells and exhibited decreased expression levels of marker proteins of N- or S-type cells after agent-induced differetiation. By contrast, in cells where SOX2 mRNA expression was knocked down by gene-specific siRNA, tumorigenicty was significantly decreased and the expression levels of marker proteins of N- or S-type cells were upregulated. In conclusion, our findings indicate an important function for SOX2 in promoting tumorigenicity of I-type neuroblastoma cells and in inhibiting their differentiation, suggesting that SOX2 might be a potential therapeutic target in neuroblastoma.

Introduction

Neuroblastoma (NB) is the most common extracranial solid pediatric tumor and accounts for 10% of childhood cancers (1). NB arises from neural-derived crest cells, and this tumor is remarkable for its biological heterogeneity, displaying a broad spectrum of clinical behavior, which ranges from spontaneous regression or maturation into a benign form that is referred to as ganglioneuroma, to rapid tumor progression and even death (2).

Although major advances have been made in the surgical and chemotherapeutic treatment of NB, the morbidity and mortality still remain high. Thus far, the molecular mechanisms responsible for the pathogenesis of NB remain elusive. In recent years, emerging evidence has suggested that tumorigenesis is dependent on a small subset of cells within the tumor itself. These are termed as cancer stem cells (CSCs). Additionally, CSCs have been identified and isolated from hematopoietic malignant and solid tumors, including glioblastoma, breast and colon cancer (3-7). Moreover, NB stem cells (NBSCs) have been isolated from NB cell lines (8,9).

Cell lines that have been established from human NB also show the same cellular heterogeneity. Based on morphological appearance, biochemical properties and growth patterns, three major cell types have been identified in NB cell lines. These have been designated as N-(neuroblastic), S-(substrate-adherent and non-neuronal) and I-type (intermediate) NB cells (10). I-type cells exhibit a morphology that is intermediate to those of N- and S-type cells. These cells have small but flattened cell bodies, with or without neurite-like processes, attach modestly to the substrate, and express low levels of both N- and S-type cell marker proteins (11).

Several lines of evidence suggest that I-type cells might represent a population of NBSCs or malignant neural crest stem cells. I-type cells are multipotent and can differentiate into either N- or S-type cells when induced by specific agents (12). I-type cells express stem cell markers such as CD133 and c-kit (13). In contrast with N- and S-type cells, I-type cells exhibit significantly higher clonogenic activity in soft agar culture and exhibit tumorigenic potential in immunodeficient mice. Moreover, the numbers of I-type cells in primary NB tumors correlate with disease progression (10,13).

SOX2 is a member of the SOX (SRY-related high mobility group box) gene family, which contains a high mobility group (HMG) domain that is very similar to that found in the sex-determining gene SRY (14). The SOX family of transcription factors is expressed during various phases of embryonic development, which affects cell fate and differentiation (15). SOX2 plays an important role in the maintenance of self-renewal

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and the potential for differentiation. Further studies revealed that SOX2 is required for the self-renewing proliferation of many normal and cancer stem cells (16-20). Moreover, SOX2 over-expressing mice displayed extensive hyperplasia, and about half of the mice expressing the highest levels of SOX2 also developed carcinoma over a 12-34-week period (21). However, little is known about the function of SOX2 in NB tumorigenesis.

In our previous studies, we found that SOX2 was overexpressed in human NB tissues, and its expression correlated to the clinical stage of NB, but not with other clinicopathological parameters including patient gender and age, tumor size, location and histological classification (22). These findings suggest that the expression of SOX2 might correlate with the genesis and progression of NB.

In the present study, we examined the expression of SOX2 in I-type neuroblastoma cells using the human neuroblastoma cell line BE(2)-C as a model. BE(2)-C cells have a typical I-type phenotype, and show consistent morphological and biochemical responses to differentiation-inducing agents (12,23,24). We further established stable cell lines that overexpressed and had downregulated expression of SOX2 by infecting BE(2)-C cells via lentiviral transduction vectors, following which we explored the functions of SOX2 in cell proliferation, clonogenicity, tumorigenicity and differentiation.

Materials and methods

Cell lines and animals. The NB cell line BE(2)-C and the retrovirus packaging cell line 293T were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). BE(2)-C was cultured in DMEM/F12 (Gibco-BRL, Grand Island, NY, USA) and supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biochrom AG, Berlin, Germany) at 37˚C in a humidified 5% CO_2 atmosphere. 293T cells were maintained in DMEM (Gibco-BRL) and supplemented with 10% FBS, 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 μg/ml streptomycin (Sigma-Aldrich). The 4-week-old male nude mice (BALB/c-nu/nu) were obtained from the Shanghai Slac Laboratory Animal Co., Ltd., (Shanghai, China) and were housed in laminar-flow cabinets under specific pathogen-free conditions. Animal care and experimental protocols were performed in accordance with the procedures and guidelines established by the Shanghai Medical Experimental Animal Care Commission, and the study was approved by the Ethics Committee of Children's Hospital of Fudan University.

Reverse transcriptase-PCR analysis. Total RNA was isolated from cultured cells by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed as previously described (22). The PCR mixture was initially incubated at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 59˚C for 30 sec and extension at 72˚C for 40 sec. Primer pairs used for RT-PCR analysis of SOX2 were 5'-AACTCCATGACCAGCTCGCAGAC-3' and 5'-TGG GAGGAAGAGGTAACCACAG-3' with an expected PCR product size of 158 bp, and 5'-TAGTTGCGTTACACCC TTTCTTG-3' and 5'-TGCTGTCACCTTCACCGTTC-3' for β-actin with an expected product size of 156 bp. β-actin was used as an internal control.

Immunofluorescence. Cells were washed with PBS, fixed with cold methanol for 30 min at 4˚C, rehydrated in PBS, permeabilized with 0.25% Triton X-100 for 10 min, and then blocked with 0.5% BSA. They were incubated overnight with the primary antibody (Sox2, 1:100, cst2748; Cell Signaling Technology, Beverly, MA, USA) at 4˚C, followed by washes with PBS, and incubation with the secondary antibody for 1 h at 37˚C. Cells were stained with Hoechst 33342 to visualize nuclei and examined with a fluorescence microscope (Olympus, Tokyo, Japan).

Lentivirus vector construction and transduction. The entire coding sequence of SOX2 cDNA was amplified and cloned into the pLenti-CMV-RFP vector obtained from Addgene. The sequence of SOX2 shRNA was amplified and cloned into the pGCSIL-GFP vector, which was also obtained from Addgene. The expression of SOX2 was confirmed by quantitative real-time PCR and western blot analysis. Lentivirus production and transduction were performed according to instructions supplied by Addgene http://www.addgene.org.

Quantitative real-time PCR analysis. Quantitative real-time PCR was performed in a reaction mixture containing 10 μl SYBR Premix (Takara Bio, Shiga, Japan), 0.8 μl each of the primer (10 μM), 0.4 μl of the ROX reference dye II, 2 μl of the cDNA and 6 μl of dH_2O. Primer pairs used for real-time PCR analysis of SOX2 were 5'-ATCCCATCACCCACAGCAA-3' and 5'-TCGGCATCGCGGTTTTT-3' with an expected PCR product size of 80 bp. All real-time PCR reactions were performed using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: initiation at 95˚C for 30 sec, amplification of 40 cycles at 95˚C for 5 sec and 60˚C for 34 sec. Each experiment was done in triplicate and normalized to the β-actin gene as an internal control.

Western blot analysis. Experiments were performed as previously described (22). Briefly, cells were lysed in RIPA buffer for protein extraction. The supernatant was collected, and protein concentrations measured by an enhanced BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). Protein samples (20 μg) were separated in a 10% SDS-PAGE gel and then transferred onto a PVDF membrane. Membranes were blocked in 5% non-fat milk for 1 h and then probed with primary antibodies followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies. Primary monoclonal antibodies used were as follows: rabbit polyclonal anti-Sox2 (1:500, cst2748; Cell Signaling Technology), mouse monoclonal anti-peripherin (1:200, P5117; Sigma-Aldrich), mouse monoclonal anti-S-100 (1:200, sc-52204; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-NF-68 (1:5,000, ab78159l; Abcam, Cambridge, UK), and rabbit polyclonal anti-Vimentin (1:1000, cst3932; Cell Signaling Technology). A mouse monoclonal anti-β-actin antibody (1:1,000, sc-69879; Santa Cruz Biotechnology) was used for the internal control. Membranes were developed using an ECL

detection system (Thermo Fisher Scientific, Waltham, MA, USA). Band intensities were determined using the Image Lab 3.0 software.

Cell proliferation assays. In the cell proliferation experiments, the neuroblastoma cells were seeded into 96-well plates at $1x10^4$ cells/well for absorbance value assay or into 6-well plates at $1x10^6$/well for flow cytometric analysis. Cells divided into groups according to the different treatment: BE(2)-C group, pCMV-SOX2 group, pCMV group, SOX2-shRNA group and control shRNA group. The number of viable cells was measured indirectly as the absorbance value (AV) using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay as previously described (at 0, 24, 48 and 72 h) (25). Briefly, 10 μl of CCK-8 dye was added to each well, and the plate was incubated for 2 h at 37˚C. The absorbance values were then measured at 450 nm using a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA). Each measurement was performed in triplicate and the experiments were repeated 3 times.

To evaluate the cell growth, flow cytometric analysis was performed using a FACScan flow cytometer (FACSCalibur; Becton-Dickinson) at 72 h. The proliferation index (PI) was then calculated with the following formula: PI (%)=[(S + G_2M)/(G_0G_1 + S + G_2M)] x 100%.

Colony formation assays. Cells were seeded into 6-well plates at a density of 500 cells/well and cultured at 37˚C for 2 weeks. At the end of the incubation, the cells were fixed with 100% methanol and stained with 0.1% crystal violet. Megascopic cell colonies were counted by Image-Pro Plus 5.0 (Media Cybernetics, Silver Spring, MD, USA). Each measurement was performed in triplicate and the experiments were each carried out at least three times.

Cell differentiation assays. Cells were seeded into 6-well plates at a density of $2x10^5$ cells/well. RA (2 μl) or BrdU was added to each well, and the cells were cultured at 37˚C for 1 week. Cell morphology was observed everyday under phase contrast microscope. At the end of the incubation, the cells were harvested and lysed in ice-cold RIPA buffer containing proteinase inhibitors for protein extraction. The proteins extracted were used for further western blot analysis. Each measurement was performed in triplicate.

Xenograft experiments. Cells ($2x10^6$ per mouse) were injected sub-cutaneously into the right upper flank of three groups of 4-week-old male nude mice. Six animals per group were used in each experiment. Mice were then monitored weekly for tumor size and evidence of morbidity. Tumor size was measured in two dimensions with Vernier calipers, and volume was calculated according to the formula: V = (length x width^2)/2. The xenograft in each mouse was excised at the time of sacrifice after inoculation for 5 weeks.

Statistical analysis. Statistical analyses were performed using the SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). The data were expressed as the mean ± SEM from at least three separate experiments. Differences in mean values were analyzed by the Student's t-test and one-way analysis of variance. An α value of $P<0.05$ was considered to be statistically significant.

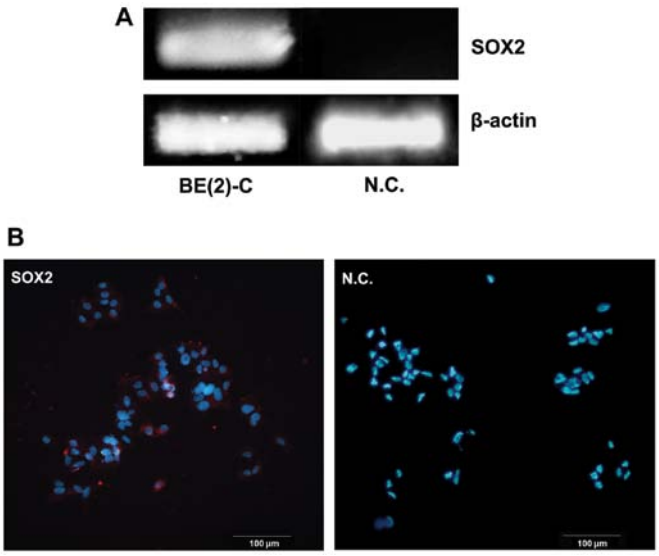

Figure 1. SOX2 expression in BE(2)-C cells. (A) SOX2 mRNA level detected by RT-PCR. β-actin was used as an internal control. (B) Representative immunofluorescence staining of SOX2. SOX2 was mainly localized in the nuclei of BE(2)-C cells. Scale bar, 100 μm. N.C., negative control.

Results

SOX2 expression in BE(2)-C cells. We examined the mRNA expression levels of Sox2 gene in BE(2)-C cells by regular RT-PCR. There was detectable expression of Sox2 gene in this cell line while not in the negtive control (Fig. 1A). At the same time, the expression was further confirmed at protein level by immunofluorescence staining. It was primarily located in the nuclei of BE(2)-C cells (Fig. 1B).

SOX2 promotes proliferation of BE(2)-C cells in vitro. To explore the function of SOX2 in BE(2)-C cells, we established SOX2-overexpressing and downregulated sublines of SOX2 (pCMV-SOX2 and SOX2-shRNA), with empty plasmid and non-functional shRNA as control. The expression of SOX2 at the RNA level of pCMV-SOX2 cells was ~2.75 times that of the wild-type. However, the Sox2 mRNA level of the Sox2-shRNA cells was less than half of the wild-type group (Fig. 2A). The protein expression level of SOX2 was confirmed by western blot analysis (Fig. 2B).

The proliferation of the cells was measured by the CCK-8 assay. At 72 h, AV was 1.210±0.148 for the BE(2)-C group, 1.124±0.131 for the pCMV group, 1.626±0.175 for the pCMV-SOX2 group, 0.812±0.083 for the Sox2-shRNA group, and 1.156±0.097 for the control shRNA group (Fig. 3A). AV was increased by 39.3% in the pCMV-SOX2 group, when compared with that of BE(2)-C group and pCMV group ($P<0.05$). AV was decreased by 45.7% in the SOX2-shRNA group, when compared with that of control shRNA group and BE(2)-C group ($P<0.05$).

Furthermore, to determine whether SOX2 increases the cell number by increasing proliferation, cellular DNA content was measured by flow cytometry (Fig. 3B). The proliferation

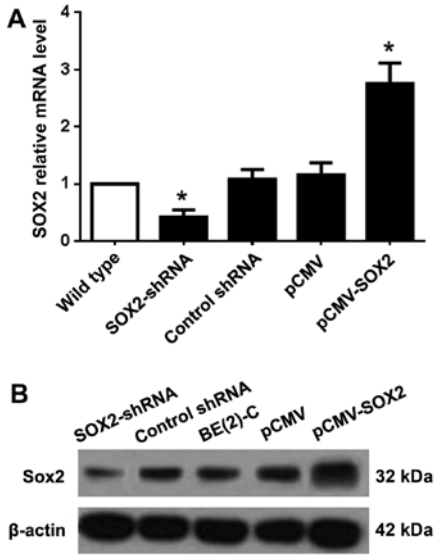

Figure 2. Effect of SOX2 transfection on SOX2 mRNA and protein expression in BE(2)-C cells. (A) Quantitative real-time PCR analysis of SOX2 mRNA expression after gene transfection. *P<0.05 vs. wild-type (BE(2)-C cell). (B) Expression of SOX2 protein confirmed by western blot analysis.

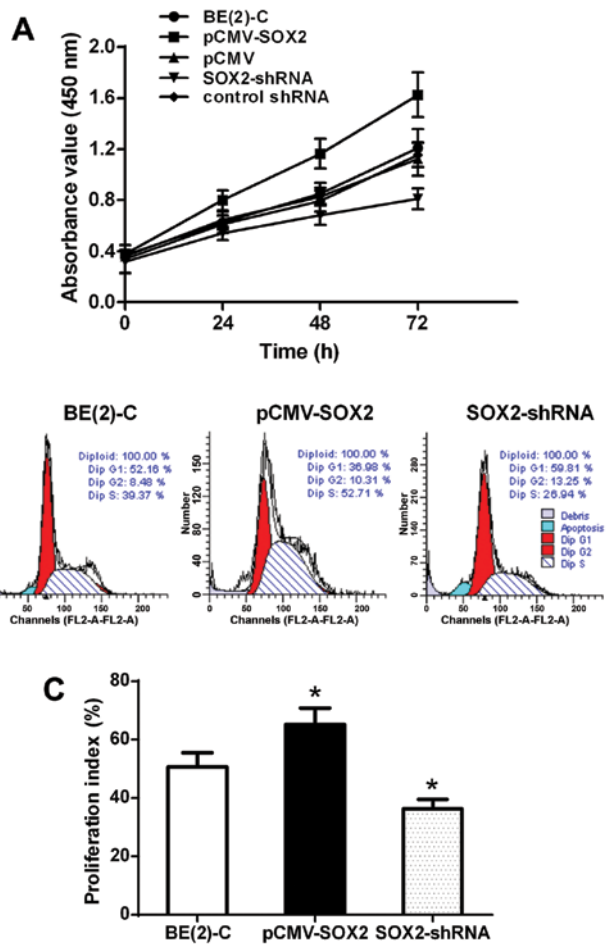

Figure 3. SOX2 promotes proliferation of BE(2)-C cells. (A) Growth curves of cells from day 0 to day 3. The Cell Counting Kit-8 assay was performed at the indicated time-points to quantify the number of viable cells. Differences were analyzed by ANOVA. (B) Representative cell cycle analyses by FACS measurement. SOX2 arrested the cell cycle in the G2/M phase of the cell cycle. (C) Proliferation index of the three groups as determined on day 3. *P<0.05 vs. BE(2)-C.

Table I. Statistical analyses of the cell cycle determinations.

Group	Cell cycle		
	G1 (%)	S (%)	G2 (%)
BE(2)-C	48.3±5.2	38.6±3.5	13.1±4.6
pCMV-SOX2	34.8±3.8	55.2±4.3	10.1±2.9
SOX2-shRNA	63.7±5.9	21.8±5.7	14.5±3.2

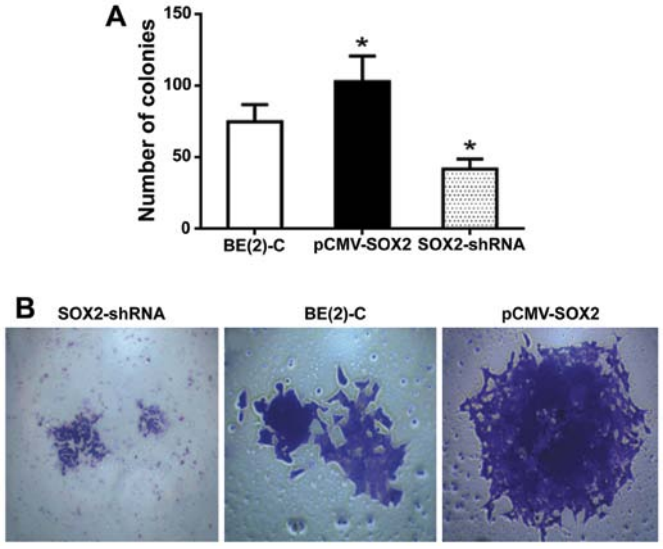

Figure 4. SOX2 promoted colony formation of BE(2)-C cells. (A) Quantification of clone numbers. *P<0.05 vs. BE(2)-C. (B) Clone formation by the three groups under light microscopy (original magnification, x400).

index (PI) was 65.2±5.6% in the pCMV-SOX2 group, and was significantly higher than the BE(2)-C group (50.7±4.8%) and SOX2-shRNA group (36.3±3.2%) (P<0.05; Fig. 3C and Table I).

SOX2 promotes colony formation of BE(2)-C cells in vitro. The colony formation assay showed that the colony forming numbers were significantly increased in pCMV-SOX2 cells (103±18) with enhanced SOX2 and were decreased in SOX2-shRNA cells (42±7) with downregulated SOX2 expression when compared with wild-type cells (75±12, Fig. 4A; both P<0.05). By light microscopy, the colony spheres of pCMV-SOX2 cells displayed large bodies with more cells than that found in SOX2-shRNA treated cells (Fig. 4B).

SOX2 promoted tumorigenicity in vivo. Subcutaneous nodules were observed 12 days after initiating the tumor in the pCMV-SOX2 group, 15 days in the BE(2)-C group, and 17 days in the SOX2-shRNA group. Tumor size was measured weekly after inoculation for 21 days, following which, a tumor growth curve was constructed according to the tumor volume (Fig. 5A and Table II). The tumor volume was found to be significantly different between the three groups (P<0.05). Typical appearances of tumor nodules and views of the dissected tumor in nude mice are shown in Fig. 5B.

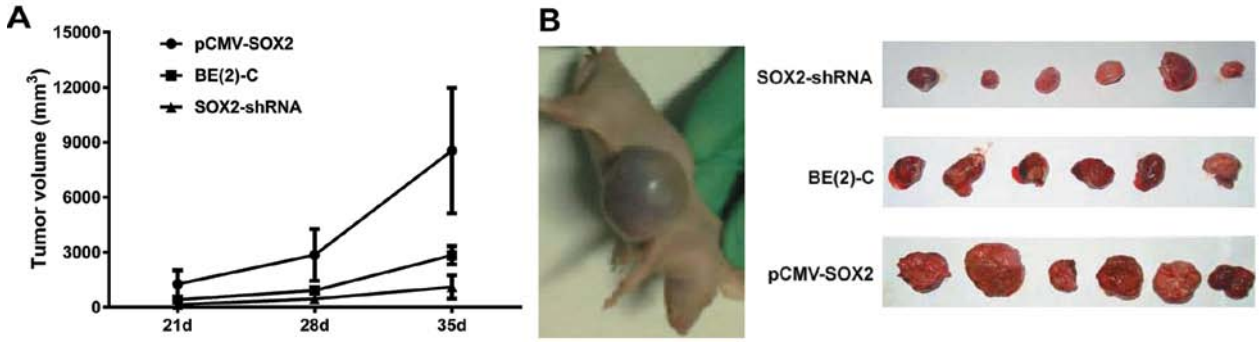

Figure 5. SOX2 promotes tumorigenicity *in vivo*. (A) Tumor growth curves of the three groups. (B) Typical appearance of tumor nodules (left) and view of the dissected tumors (right).

Table II. The volume of tumors in nude mice inoculated with different transfected BE(2)-C cells.

		Volume (mm^3)		
Group	Number	21 days	28 days	35 days
pCMV-SOX2	6	1264.0±754.8	2855.8±1412.7[a]	8546.8±3416.2[b]
BE(2)-C	6	405.4±133.1	914±254.5[a]	2837.2±497.1[b]
SOX2-shRNA	6	143.9±67.1	452.8±232.9[a]	1101.8±639.8[b]

[a]P<0.05, F=13.842; [b]P<0.05, F=10.441.

Table III. Relative expression levels of marker proteins after RA induced differentiation.

Group	Peripherin	P-value	NF-68	P-value
BE(2)-C	0.465±0.030		0.618±0.026	
pCMV-SOX2	0.182±0.016	0.012	0.287±0.013	0.001
pCMV	0.487±0.021	0.363	0.665±0.022	0.077
SOX2-shRNA	0.698±0.029	0.003	0.924±0.067	0.008
Control shRNA	0.486±0.038	0.496	0.647±0.071	0.562

Table IV. Relative expression levels of marker proteins after BrdU induced differentiation.

Group	Vimentin	P-value	S-100	P-value
BE(2)-C	0.583±0.056		0.441±0.023	
pCMV-SOX2	0.192±0.020	0.003	0.138±0.016	0.000
pCMV	0.576±0.058	0.848	0.443±0.062	0.962
SOX2-shRNA	0.850±0.031	0.005	0.719±0.097	0.033
Control shRNA	0.597±0.028	0.725	0.475±0.027	0.174

Downregulation of SOX2 in BE(2)-C cells promotes agent-induced differentiation. As shown in Fig. 6A, BE(2)-C cells were induced to differentiate towards N-type cells after RA treatment, and S-type cells after BrdU treatment, respectively. Peripherin and NF-68 were N-type cell marker proteins. Vimentin and S100 were S-type cell marker proteins. To investigate the effect of SOX2 on differentiation properties of BE(2)-C cells, we performed western blot analysis to detect the marker proteins after agent treatment. We found that SOX2-shRNA cells exhibited increased expression levels of marker proteins of N- or S-type cells when compared with BE(2)-C and pCMV-SOX2 cells after agent-induced differentiation (Fig. 6B and C, Tables III and IV; both P<0.05).

Discussion

Cancer stem cells have been described as small populations of cells that have higher tumorigenicity, differentiation ability and self-renewal ability (26). As CSCs have these characteristics, they are thought to be associated with cancer recurrence after treatment and distant metastasis. Additionally, CSCs have been shown to be related to resistance to various treatments. Therefore, elimination of CSCs is essential for cancer treatment (26). Thus, CSCs are likely to be the most relevant targets in the treatment of neuroblastoma and further studies on the characterization of these cells will help in the design of more successful neuroblastoma therapies.

Several lines of evidence have shown that neuroblastoma could arise from its own cancer stem cells (9,12,13,27,28). *In vitro*, neuroblastoma cancer stem cells may correspond with a population having an intermediate phenotype (I-type). These cells express features of both N-type and S-type cells, possess multipotent differentiation properties, and can be induced to differentiate into neuroblastic or glial cell phenotypes. Of interest, I-type cells are more malignant than N-type and S-type cells in athymic mice. This further confirms that I-type

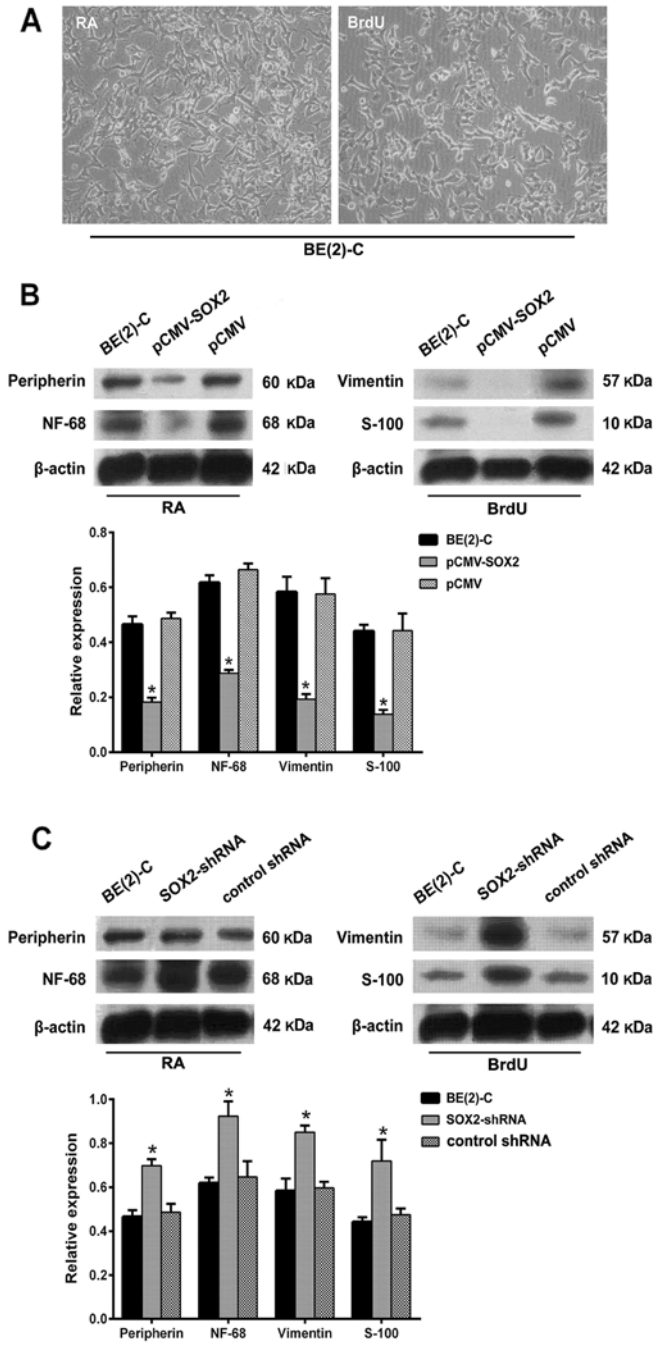

Figure 6. Effects of SOX2 on the differentiation properties of BE(2)-C cells. (A) Cell morphology after agent-induced differentiation (original magnification, x100). BE(2)-C cells were induced to exhibit S-type cells after BrdU treatment, and N-type cells after RA treatment. (B) Upregulation of SOX2 inhibited agent-induced differentiation of BE(2)-C cells. Differentiation-associated marker proteins decreased in pCMV-SOX2 cells. (C) Downregulation of SOX2 promoted agent-induced differentiation. Differentiation-associated marker proteins increased in SOX2-shRNA cells. Peripherin and NF-68 were N-type cell marker proteins, and vimentin, S100 were S-type cell marker proteins. *P<0.05 vs. BE(2)-C.

cells may also be considered the so-called tumor initiating cells (TICs) or cancer stem cells (9,12,13,27,28). Based on the above, we chose to evaluate the function of SOX2 in the BE(2)-C neuroblastoma cell line that is composed of I-type cells (12,29).

In the present study, we examined the expression of SOX2 in BE(2)-C cells, and tests were carried out with model systems displaying overexpressed and downregulated SOX2 in BE(2)-C cells. BE(2)-C is a I-type neuroblastoma cell line, wherein SOX2 is highly expressed. We found that overexpression of SOX2 in BE(2)-C cells could promote both cell proliferation and growth of the tumor by cloning formation assay. At the cellular level, knocking down SOX2 expression resulted in an accumulation of cells in the G0/G1 phases of the cell cycle and decreased the proportion of cells in the S and G2/M phases. In addition, SOX2 expression also enhanced tumor formation in nude mice. However, suppression of SOX2 had the opposite effect. Taken together, these data suggested that exogenous SOX2 expression might promote neuroblastoma tumorigenesis both *in vitro* and *in vivo*. These results were consistent with previous studies in which SOX2 was found to be overexpressed and could promote cell proliferation and tumorigenesis (16-20).

An examination of changes in cell morphology showed that the BE(2)-C cells were induced to differentiate towards N-type or S-type cells after RA or BrdU treatment, respectively. Furthermore, pCMV-SOX2 cells exhibited decreased expression levels of marker proteins of N- or S-type cells when compared with BE(2)-C and SOX2-shRNA cells. Conversely, downregulation of SOX2 showed an inverse series of results. The data suggested that SOX2 overexpression inhibited BE(2)-C cells from differentiating towards less malignant cell type, indicating that SOX2 plays an important role in the maintenance of the differentiation potential of BE(2)-C cells. These results also suggested that deregulated SOX2 expression distorted the balance toward a transformed phenotype, leading to the development of neuroblastoma.

Our results are consistent with the growing body of evidence that cancer is caused by deregulation of transcription factors that affect cell fate and proliferation. Additionally, recent studies have suggested that dysregulation of self-renewal plays a key role in the generation of CSCs. Thus, it is reasonable to suggest that the high-expression levels of SOX2 were related to the generation of neuroblastoma CSCs. However, the mechanism remains unknown and requires further analysis. In our future work, we propose to use microarray analysis to identify genes regulated by SOX2 by comparing expression profiles of BE(2)-C cells and SOX2-shRNA cells.

In conclusion, we have demonstrated SOX2 expression in the human neuroblastoma I-type cell line BE(2)-C. SOX2 promoted BE(2)-C cell proliferation, colony formation and tumorigenesis. Additionally, SOX2 maintained BE(2)-C cells in an undifferentiated state. Our results may provide further evidence for the cancer stem cell theory. By contrast, it may also imply the existence of undifferentiated cells in these tumors, in which SOX2 contributes to the character of these cells. These results demonstrate that SOX2 might play an important role in NB tumorigenesis and suggests a possible therapeutic target in NB.

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