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# Sox2 promotes survival of satellite glial cells *in vitro*

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ABSTRACT

Sox2 is a transcriptional factor expressed in neural stem cells. It is known that Sox2 regulates cell differentiation, proliferation and survival of the neural stem cells. Our previous study showed that Sox2 is expressed in all satellite glial cells of the adult rat dorsal root ganglion. In this study, to examine the role of Sox2 in satellite glial cells, we establish a satellite glial cell-enriched culture system. Our culture method succeeded in harvesting satellite glial cells with the somata of neurons in the dorsal root ganglion. Using this culture system, Sox2 was downregulated by siRNA against Sox2. The knockdown of Sox2 downregulated ErbB2 and ErbB3 mRNA at 2 and 4 days after siRNA treatment. MAPK phosphorylation, downstream of ErbB, was also inhibited by Sox2 knockdown. Because ErbB2 and ErbB3 are receptors that support the survival of glial cells in the peripheral nervous system, apoptotic cells were also counted. TUNEL-positive cells increased at 5 days after siRNA treatment. These results suggest that Sox2 promotes satellite glial cell survival through the MAPK pathway via ErbB receptors.



## 1. Introduction

The peripheral somatic sensory nervous system is composed of dorsal root ganglion cells (DRG neurons) and two types of glial cells. One type is satellite glial cells (SGCs), which envelop the cell body of all DRG neurons with their thin cytoplasm [1]. The other is Schwann cells, which cover the axon. Myelinating Schwann cells ensheath a large-caliber axon by forming a myelin sheath, and non-myelinating Schwann cells ensheath fine axons without myelin sheaths.

Sox protein is encoded by the *Sox* (SRY-related-HMG-box) gene. *Sox2* is classified in the SoxB1 family according to the similarity of the amino acid sequence of the HMG domain [2]. Sox2 is thought to be expressed in stem cells, e.g., inner cell mass, developmental and adult neural stem cells, to promote cell proliferation and to inhibit differentiation [3–5]. Moreover, it is also reported that Sox2 promotes the cell survival of neural stem cells, several epithelial cells and cancer cells [6–8].

During the developmental stage of peripheral nervous system (PNS), Sox2 is expressed in neural crest stem cells and regulates the differentiation to DRG neurons [9,10]. Sox2 is also expressed in immature Schwann cells and inhibits myelination [11]. In adult PNS, Sox2 is re-expressed in undifferentiated myelinating Schwann cells after nerve injury and regulates their sorting after nerve injury [12]. Recently, our work revealed that certain types of PNS glial cells, including SGCs, express Sox2 in normal adult rats [13]. However, the roles of Sox2 in these glial cells of the normal animal were not known. In this study, we examined the role of Sox2 in SGCs *in vitro*. For *in vitro* experiments, SGCs need to be discriminated from Schwann cells. Some researchers use partially dissociated DRGs [14]. In this method, SGCs are morphologically discriminated from Schwann cells, and the experiment was finished before the migration of SGCs from the soma of the DRG neurons because migrated SGCs and migrated Schwann cells cannot be distinguished morphologically. Other researchers discriminate SGCs from Schwann cell using cell markers. In mouse sensory ganglions, it is known that SGCs express glutamine synthetase, but Schwann cells do not [15]. This cell marker permit discrimination of SGCs from Schwann cells when the cells are completely dissociated [16]. However, there was previously no culture method to discriminate SGCs from Schwann cells or other cells in rats. In this study, we established the method of SGC-enriched cultures, and then Sox2 expression was downregulated with siRNA. Sox2 knockdown caused the downregulation of ErbB receptors, which are necessary to support glial cell survival. Moreover, a decrease in activated MAPK, downstream of ErbB, was also confirmed in Sox2 knockdown. In addition, TUNEL-positive cells significantly increased in Sox2 knockdown experiments.

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These results suggest that Sox2 regulates ErbB2 and ErbB3 signaling-related cell survival of SGCs.

## 2. Materials and methods

### 2.1. Animals

Eight-to nine-week-old male Wistar rats were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). The Animal Committee of Kansai Medical University approved all experimental protocols, and all studies were performed in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985).

### 2.2. SGC culture

Wistar rats were deeply anesthetized by sodium pentobarbital (50 mg/kg i.p.). Lumber DRGs (L2-L6) were harvested in ice-cold Hank's balanced salt solution (HBSS; Nacalai Tesque Inc., Kyoto, Japan), desheathed and cut into sections of approximately 1 $mm^3$. Then, the tissue was incubated in Dulbecco's modified Eagle medium-F12 (1:1) (DMEM/F12; GIBCO, Tokyo) supplemented by 0.2% collagenase, DNase I and penicillin/streptomycin (P/S) for 1 h at 37 °C with shaking, using a bioshaker. The partially dissociated ganglions were spun down, and HBSS was added, followed by trituration with a Pasteur pipette. The suspension was passed through a 70 μm cell strainer and then centrifuged at 1000 rpm for 5 min through 30% Percoll (GE Healthcare Bio-Science, PA, USA) in HBSS to collect SGCs with neuronal somata. Collected cells were washed three times with DMEM/F12 (1:1) containing 10% fetal calf serum (FCS) and P/S followed by seeding ($1.6 \times 10^3$ DRG neurons/ml) on 0.002% poly-L-lysine-coated culture dishes. The total volume of medium was changed to DMEM/F12 (1:1) containing 10% FCS at 12 h after seeding.

### 2.3. RNA interference

Cells were transfected with a mixture of 3 forms of double-stranded siRNAs against Sox2 mRNA at 2 days in *vitro* (DIV) using lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA). The sequences of sense chains were as follows: 5′-CCAC-GACGCUCAUGAAGAAdTdT-3′; 5′-CGGCACAGAUGCAGCCGAUdTdT-3′; 5′-GUGCAAAAGAGGAGAGUAAdTdT-3′. Non-coding double-stranded RNA (5′-UACUAUUCGACACGCGAAGdTdT-3′) was used as a control (Bonac Corporation, Fukuoka, Japan). The final concentration of each RNA was 33 nM. Half the volume of the medium was replaced 3 days after siRNA treatment.

### 2.4. Immunocytochemistry

Cultured cells were fixed with 4% formaldehyde (FA) in 0.1 M phosphate buffer (PB, pH 7.4) for 10 min, then rinsed with 0.3% Triton X-100 in 0.1 M phosphate buffered saline (PBST). Cells were incubated in primary antibody diluted with PBST for 12 h. Primary antibodies were as follows: goat anti-Sox2 (1:2000, Santa Cruz,

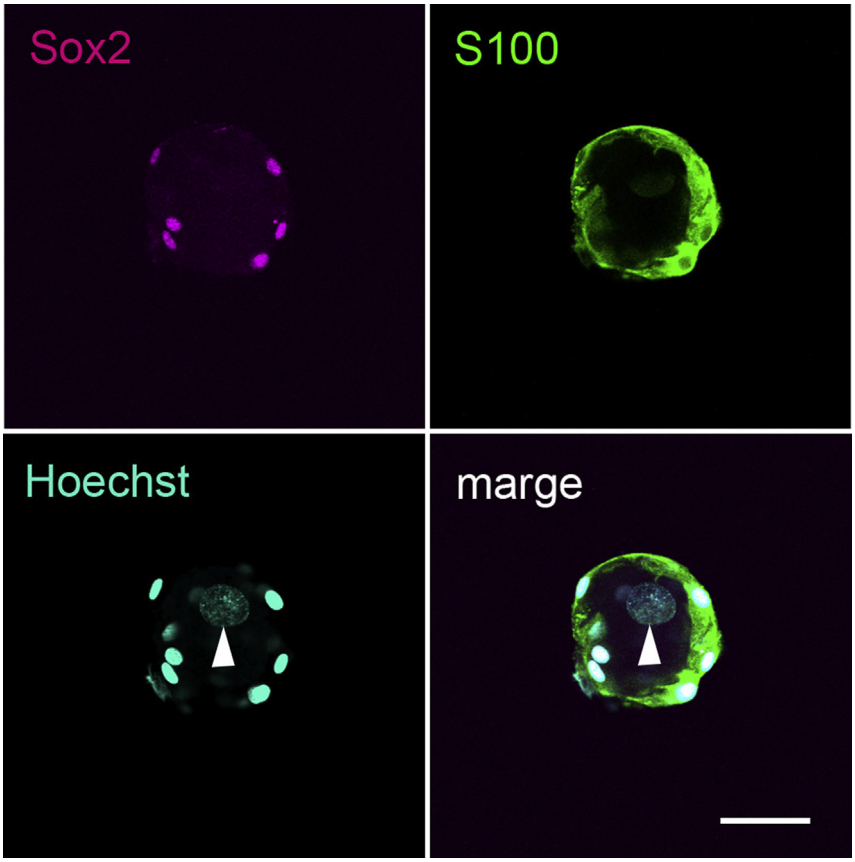


**Fig. 1.** Harvested glial cells showed SGC morphology. Micrographs were captured with a confocal laser microscope. Cells were stained with anti-Sox2 (magenta) and -S100 (green) antibodies at 2 h after seeding. Almost all S100 positive glial cells are Sox2 positive. The nucleus of the DRG neuron is indicated by an arrowhead. The glial cells cover the cell body of the DRG neuron, indicating these cells are SGCs. DNA (cyan). Bar: 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Biotechnology, Santa Cruz, CA, USA) and rabbit anti-S100 (1:10000, Dako Japan, Tokyo, Japan) antibodies. After rinsing with PBST, the cells were incubated in Cy2-or Cy3-labeled secondary antibodies against appropriate animals for 1 h (1:200, Jackson ImmunoResearch, West Grove, PA, USA). Then, the cells were rinsed and mounted with mounting medium containing Hoechst dye 33,258 (Nacalai). Micrographs were obtained with a confocal laser microscope (LSM 510-META, Carl Zeiss, Oberkochen, Germany).

### 2.5. Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End labeling (TUNEL)

Cultured SGCs were fixed with FA in PB for 10 min and rinsed with PBS. For the TUNEL assay, the in situ Cell Death Detection Kit was used (Roche Diagnostics, Mannheim, Germany). Briefly, after washing, cells were permeabilized with 0.1% sodium citrate containing 0.1% Triton X-100 and incubated with TUNEL reaction mixture. Cells were rinsed with PB and mounted in mounting medium. Photographs were captured using a fluorescent microscope (Axiolpan2, Carl Zeiss). The ratios of TUNEL-positive cells to all cells were calculated.

### 2.6. Quantitative reverse transcription PCR

RNA collection and reverse transcription was conducted based on previous reports (Koike et al., 2014). Sox2, ErbB2, ErbB3 and HPRT mRNA was measured on Mini Opticon (Bio-Rad laboratories, CA, USA) with MESA GREEN qPCR MasterMix Plus (Eurogentec, Seraing, Belgium). Hypoxanthine phosphoribosyltransferase (HPRT) was used as the internal standard. Primer sets were as follows: Sox2 forward 5′-ACTAGGGCTGGGAGAAAGAA-3′, Sox2 reverse 5′-AAAACAGTCGCTCTCCTCCT-3′; ErbB2 forward 5′-GAGCCTCTCTCCACATGACC-3′, ErbB2 reverse 5′-TAAAGGAGGCTGAGGCTGAA-3′; ErbB3 forward 5′-CGTCATGCCAGATACACACC-3′, ErbB3 reverse 5′-AGGGCTACCCCTTCTCTTCC-3′; HPRT forward 5′-ATTGACACTGGTAAAACAATG-3′, HPRT reverse 5′-GAGGTCCTTTTCACCAGCAAG-3′. The PCR conditions were as follows: 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each cDNA amount was calculated using a standard curve, and Sox2, ErbB2 and ErbB3 were

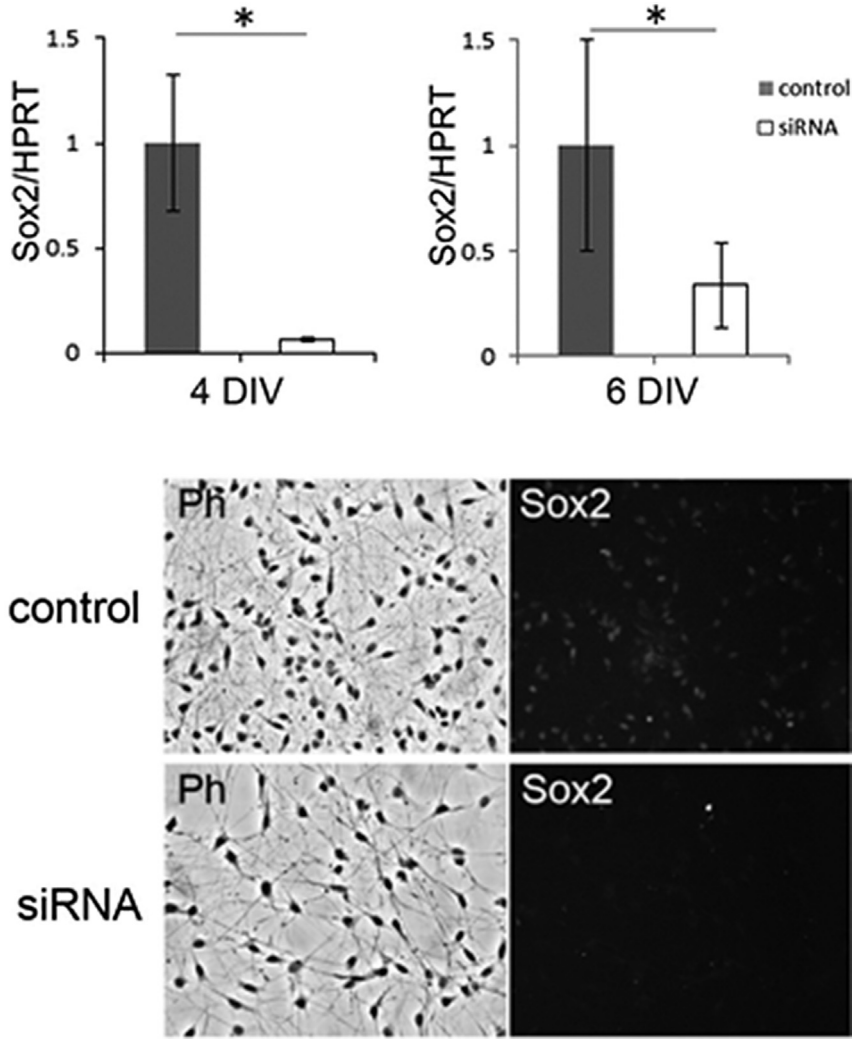


**Fig. 2.** Expression level of Sox2 was decreased by siRNA. Upper panels show Sox2 mRNA expression levels at 4 and 6 DIV. The expression level was compared using quantitative RT-PCR. The relative Sox2 level was significantly decreased in Sox2 knockdown. The lower panels show that Sox2 immunoreactivity was not observed in Sox2 knockdown. Ph: phase difference. At 4 DIV, cultivation was repeated 4 times in each group, and Student's *t*-test was performed. At 6 DIV, cultivation was repeated 3 times in each group, and Welch's *t*-test was performed. Data represent the mean ± S.D. $^{*}P < 0.05$. Gray bar: Sox2 knockdown; white bar: control.

normalized by HPRT. The specificities of the products were confirmed by sequencing.

### 2.7. Protein isolation and western blotting

Cells were dissolved in RIPA buffer containing 50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 0.1% SDS and 0.5% 7-deoxycholate. Then, the samples were sonicated, combined with 1/6 volume of 6-fold sample buffer and incubated for 5 min at 95 °C. The protein was electrophoresed with acrylamide gel and transferred to a PVDF membrane. Blocking was performed with PBS containing 0.1% Tween-20 (PBSTw) and 1% bovine serum albumin, and the samples were then incubated in primary antibody diluted with PBSTw. Primary antibodies were as follows: rabbit anti-MAPK, anti-phospho-MAPK, anti-Akt and anti-phospho-Akt antibody (1:1000, Cell Signaling Technology, Tokyo, Japan). Then, the membrane was washed and incubated in HRP-conjugated secondary antibody (1:10,000, Jackson). After washing, the membrane was reacted with reagent for chemiluminescence, Chemi-Lumi One Super (Nacalai), and pictures were obtained using a chemiluminescence-detecting device, ImageQuant LAS-4000mini (Fuji Film, Tokyo, Japan). The ratios of phospho-MAPK to MAPK and phospho-Akt to Akt were measured with the software Multi Gauge (Fuji Film).

### 2.8. Statistical analysis

The data were initially subjected to the F-test to determine whether the data showed homoscedasticity. Student's *t*-test was used for data that showed homoscedasticity. Welch's *t*-test was used for data that showed unequal variance. The significance level of the static test was set at $p < 0.05$.

## 3. Results

### 3.1. SGC culture

Two hours after seeding, nearly all glial cells exhibited the characteristic morphology of SGCs: they adhered to the somata of DRG neurons and exhibited a flat shape and Sox2 immunoreactivity (Fig. 1). In this culture, there were very few glial cells that did not adhere to neuronal soma. Schwann cells with axons were not observed. Thus, these harvested cells were neuronal somata and the SGCs covering them.

The SGCs began to migrate from DRG neurons and radially expanded by proliferation at 2 DIV.

### 3.2. Sox2 knockdown induced increasing of TUNEL positive cells

Sox2 was downregulated by lipofection of siRNA at 2 DIV. The efficiency of downregulation was assessed using quantitative RT-PCR and immunocytochemistry. Sox2 mRNA was significantly reduced in knockdown against the control at 4 and 6 DIV (Fig. 2). In immunocytochemical assessment at 4 and 6 DIV, Sox2 immunoreactivity completely disappeared in Sox2 knockdown but not in a mock transfected control (Fig. 2).

We examined apoptosis of the cells because decreasing numbers of cells and floating corps of cells were observed in Sox2 knockdown. To label the apoptotic cells, TUNEL was performed, and the ratio of TUNEL-positive cells to SGC nuclei was calculated at 7 DIV. At 7 DIV, a significant increase in the TUNEL-positive ratio was found in knockdown (Fig. 3, $p = 0.044$).

### 3.3. ErbB2 and ErbB3 mRNA were decreased in Sox2 knockdown

Previous reports elucidated that ErbB2/3 signaling is sufficient for the cell survival [17]. To examine whether downregulation of ErbB2 and ErbB3 occurs, quantitative RT-PCR was performed for ErbB2 and ErbB3 mRNA. In the knockdown group, significant decreases in *ErbB2* and *ErbB3* mRNA were observed at 4 and 6 DIV (Fig. 4A). At 4 DIV, *ErbB2* has decreased by 61% and *ErbB3* by 68% ($p = 0.024$ in ErbB2, $p = 0.008$ in ErbB3). At 6 DIV, *ErbB2* has decreased by 83% and *ErbB3* by 48% ($p = 0.001$ in ErbB2, $p = 0.033$ in ErbB3). To confirm the effect of signaling via ErbB receptors, the activation of MAPK and Akt, downstream of ErbB receptors, was examined by western blotting. A decline of approximately 50% in phosphorylated MAPK was observed in the Sox2 knockdown experiment at 6 DIV (Fig. 4B, $p = 0.037$); however, the activity of Akt was unchanged (Fig. 4B, $p = 0.473$).

## 4. Discussion

In this study, we examined the role of Sox2 in cultured SGCs using siRNA against Sox2. Sox2 knockdown elicited increased apoptosis of SGCs. Our previous histochemical study elucidated that Sox2 is expressed in all SGCs of the normal adult rat. In addition, only a small number of SGCs underwent apoptosis [13]. These results suggested that Sox2 also maintains SGC survival *in vivo*.

Some researchers have used the cultivation of SGCs in their study; however, each method includes problems in harvesting rat

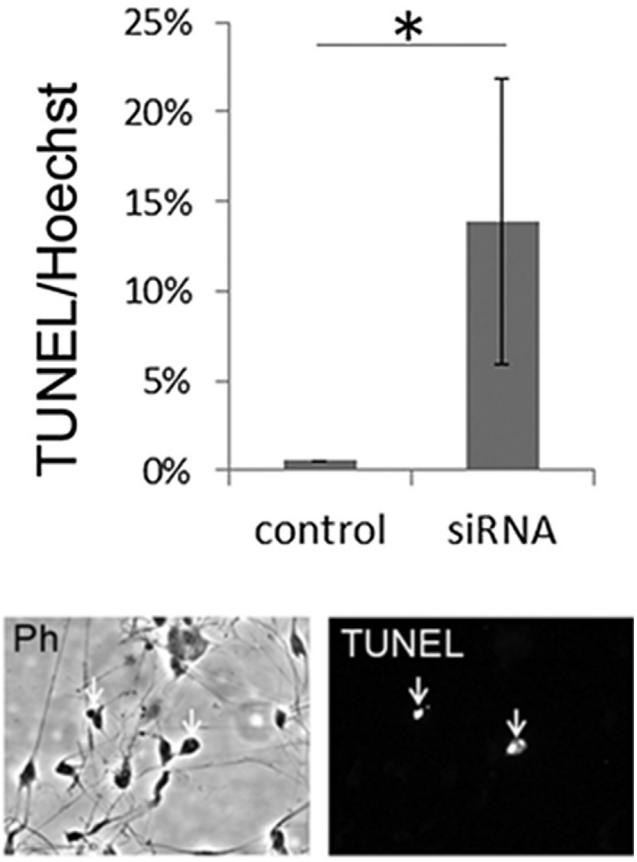


**Fig. 3.** TUNEL-positive cells were increased by Sox2 knockdown. Apoptotic cells were labeled with TUNEL staining, and the ratio of TUNEL-positive cells to all cells was calculated at 7 DIV. TUNEL-positive ratio is significantly increased in Sox2 knockdown. Cultivation was repeated 4 times in control and 3 times in knockdown. Welch's *t*-test was performed. Data represent the mean ± S.D. $^*P < 0.05$.

SGCs. Poulsen et al. completely dissociated the trigeminal ganglion of the rat, and all harvested cells were seeded [18]. In this report, SGCs were segregated from Schwann cells using the cell marker glutamine synthetase, which is expressed in mouse SGCs [15]. However, in rats, glutamine synthetase is expressed in both SGCs and Schwann cells [19]. Therefore, glutamine synthetase is not an SGC-specific marker in rats. Fex Svenningsen et al. harvested NG2, one of proteoglycan, positive glial cells as SGCs from embryonic day 17 to postnatal day 8 rat DRGs by using culture plates coated with anti-NG2 antibody to catch NG2 positive cells [20]. However, in adult PNS, it is reported that there are other NG2-positive cells in addition to SGCs [21]. Therefore, NG2 is not an SGC-specific marker in adult animals. In this study, we established an SGC-enriched culture method to harvest SGCs without Schwann cells. This method consists of 3 major procedures. First, DRGs were partially dissociated using collagenase followed by pipetting to separate SGCs adhering to the somata of DRG neurons from Schwann cells sheathing axons. Second, dissociated cells were passed through a cell strainer to remove the mass of DRG tissues, including Schwann cells. Third, SGCs adhering to neuronal soma were segregated from other cells by density gradient of Percoll. Through this method, cells adhering to DRG neuronal soma were harvested. These glial cells definitely exhibited SGC-specific features: the harvested cells cover the somata of DRG neurons with their thin cytoplasm and show immunoreactivity for S100 protein, a pan-glial cell marker in PNS. In addition to these observations, hardly any contamination of Schwann cells was found. These results suggest that almost all harvested cells were SGCs and neurons.

It is known that Sox2 is involved in cell survival. In cancer cells, it has been reported that Sox2 knockdown elicited apoptosis in human lung cancer cells by causing downregulation of the anti-apoptotic molecule survivin [22]. Another research group has reported that Sox2 promotes the survival of lung cancer cells by inducing BCL2L1 expression [23]. It is known that in the nervous system, Sox2 directly promotes survivin expression to prevent apoptosis in the neural stem cells of the E14.5 mouse brain [8]. Sox2 knockdown increased activated caspase3-positive cells in human neural crest stem cells [9]. In the same manner, our study showed that Sox2 knockdown led to increased TUNEL-positive SGC, but the survivin expression level was not altered at 4 DIV (data not shown). These results suggest that the prevention of apoptosis is among the fundamental functions of Sox2, although the signaling pathway for cell survival is different among cell types.

In this study, Sox2 knockdown caused decreases in the ErbB2/3 mRNA expression level and phosphorylated MAPK, downstream of the ErbB receptor, before increasing the TUNEL-positive cell ratio. Significant alteration of activated Akt, downstream of the ErbB receptor, was not observed. Many studies have elucidated that ErbB is

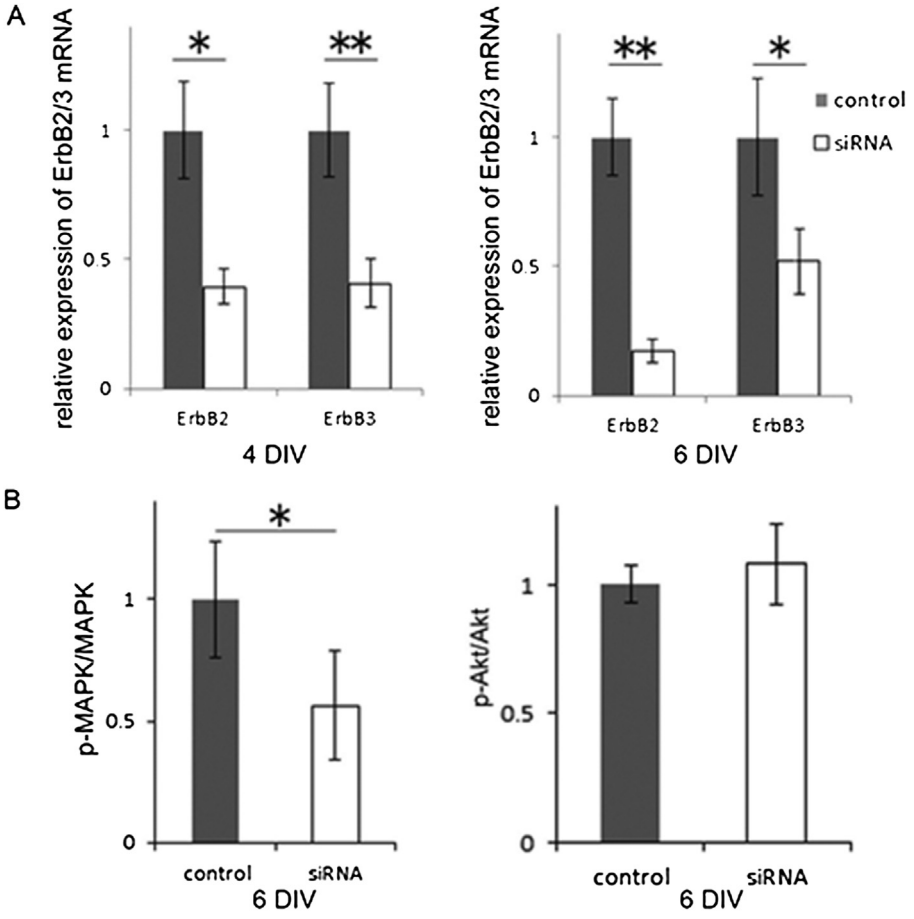


**Fig. 4.** (A) Expression levels of ErbB2 and ErbB3 mRNA were decreased by Sox2 knockdown. ErbB2 and ErbB3 mRNA expression levels were compared using quantitative RT-PCR at 4 and 6 DIV. Relative levels of ErbB2 and ErbB3 significantly decreased in Sox2 knockdown. At 4 DIV, cultivation was repeated 5 times in ErbB2 and 4 times in ErbB3. At 6 DIV, cultivation was repeated 3 times in each group. Student's *t*-test was performed. (B) Sox2 knockdown caused decline in phosphorylated MAPK but not Akt. Ratios of phospho-MAPK to MAPK and phospho-Akt to Akt were measured by western blot at 6 DIV. Significant decrease in MAPK activity was observed in Sox2 knockdown experiment at 6 DIV. No significant change in Akt activity was observed. Cultivation was repeated 4 times. Student's *t*-test was performed. Data represent the mean ± S.D. $^{*}P < 0.05$. $^{**}P < 0.01$. Gray bar: Sox2 knockdown; white bar: control.

sufficient for cell survival [24]. In PNS, it is known that Schwann cell precursor survival depends on neuregulin-ErbB signaling through the Akt and MAP-kinase pathways, which are downstream of ErbB. The signaling cascade finally activates the anti-apoptotic factor Bcl and the inhibitory apoptotic factors Bad and Bax [25–28]. Furthermore, a recent study showed that Sox2 has the ability to bind the promoter region of ErbB2 [29]. These previous results support our findings that Sox2 promotes SGC survival through ErbB signaling.

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.141.

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