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Gene expression profile of Sox1, Sox2, p53, Bax and Nestin in neural stem cells and adult mouse brain tissues

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Abstract: Histone deacetylation is a key modulator involved in cell proliferation, apoptosis, and mRNA transcription. However, the effects of histone deacetylation on C17.2 neural stem cells (NSCs) remain unclear. Here, the histone deacetylase inhibitors nicotinamide and trichostatin A (TSA) were used to determine the role of histone deacetylation on gene transcription in NSCs. The results showed that the mRNA expression of p53, Sox1, Sox2, and Bax were significantly higher in E14.5 NSCs than in C17.2 NSCs. Nestin, a marker gene of neuronal differentiation, did not differ significantly between E14.5 NSCs and C17.2 NSCs. The transcription levels of p53 and Nestin were significantly higher in C17.2 NSCs than in differentiated brain tissues, and the expression of Bax, Sox1, and Sox2 was higher in the olfactory bulb than in other brain tissues. Nicotinamide and TSA treatment decreased the transcription of Sox2, p53, Nestin, and Bax in C17.2 NSCs, although the difference was statistically significant only for Sox2 and Nestin, Sox1 transcription was not detected. These results demonstrated that mRNA expression profiles differ between C17.2 NSCs, E14.5 NSCs, and adult mouse brain tissues, and HDAC inhibitors regulate gene expression by modulating histone acetylation.

Introduction

Neural stem cells (NSCs) are involved in the formation of neurons by differentiating into astrocytes and oligodendrocytes during brain development (Snyder *et al.,* 1992). Many cell factors are involved in the differentiation and maintenance of NSCs. Overexpression of Sox1 and Sox2 increases the number of neural progenitor cells but prevents further neural differentiation (Archer *et al.,* 2011). Sox1 is a key cell factor that contributes to the differentiation of stem cells towards terminal cells and promotes tumorigenesis (Ahmad *et al.,* 2017).

p53 is a widely studied protein that plays a role in apoptosis, senescence, genomic stability, proliferation, stem cell maintenance, and angiogenesis inhibition (Surget *et al.,* 2014). Mutations of p53 lead to inactivation of the protein, resulting in increased proliferation of neural progenitor cells (NPCs) and the development of hyperplasia of the SVZ subventricular zone (SVZ) in p53-deficient mice *in vivo* (Kumar *et al.,* 2015).

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Nestin is a type VI intermediate filament protein. It is a protein marker for NSCs, and is expressed mostly in neural cells associated with radial growth of axons (Guérette *et al.,* 2007; Michalczyk and Ziman 2005). Nestin positively regulates the proliferation, survival, and invasiveness of breast cancer stem cells (CSCs) by promoting Wnt/β-catenin activation (Zhao *et al.,* 2014). The apoptosis regulator Bax, also known as bcl-2-like protein 4, is encoded by the *bax* gene in humans. Bax is upregulated by the tumor suppressor p53 and involved in p53-mediated apoptosis (Westphal *et al.,* 2014). Bax inactivation by curcumin inhibits ischemia-induced mitochondrial apoptosis, underlying the neuroprotective function of curcumin (Xie *et al.,* 2018). Histone deacetylases (HDACs) are involved in the regulation of gene transcription in various cells (Cunliffe and Casaccia-Bonnefil 2006). Trichostatin A (TSA), an inhibitor of type I and type II HDACs, and nicotinamide, which inhibits type III HDACs, are commonly used to investigate the regulation of gene transcription (Surjana *et al.,* 2010). Despite extensive research, the regulatory function of nicotinamide and TSA in NSCs remains unclear.

The aim of the present study was to understand the impact of HDACs on C17.2 NSCs. A large body of epigenetics research indicates that nicotinamide inhibits cell proliferation by disrupting normal cell cycle progression and inducing neuronal differentiation (Griffin *et al.,* 2013).

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We showed that the levels of Sox1, Sox2, p53, Nestin, and Bax differed between two types of NSCs (C17.2 and E14.5 NSCs) and between different brain regions of adult mice, and nicotinamide induced significantly differential transcription of Sox2 and Nestin in C17.2 NSCs.

Materials and Methods

Animals and tissues

C57BL/6J mice were mated, and primary NSCs were obtained from the brains of embryonic day 14.5 (E14.5) mice. Under the dissecting microscope, the meninges and choroid plexus were removed, and the hippocampus was cut with tissue scissors and incubated in pre-cooled D-Hank solution. The tissue was dissociated into individual cells using a 1 ml pipette and centrifuged, and the supernatant was discarded. The cells were suspended in D-Hank solution and spread onto 6-well culture plates. Neurosphere formation and immunofluorescence detection of nestin were used to characterize NSCs. Four brain regions (cerebellar cortex, cerebral cortex, hippocampus, and olfactory bulb) were extracted from the brains of mature male inbred C57BL/6J mice aged 2.5-3 months. Generally accepted ethical guidelines for the care and use of experimental animals were followed, and the experimental procedures were approved by the Ethics Committee on Animal Experimentation of Shanghai University.

NSC culture

Mouse C17.2 NSCs were cultured as described by Snyder et al. in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (FCS, Gibco) and 5% horse serum (HS, Gibco) in a 5% CO_2 atmosphere at 37°C (Snyder *et al*., 1992). Primary neurospheres (E14.5 NSCs) were obtained from the fetal mouse hippocampus and cultured in DMEM/F12 (Gibco) with N2 (Gibco) and B27 (Gibco) supplements containing penicillin (100 U/ml, Gibco), streptomycin (100 μg/ml, Gibco), basic fibroblast growth factor (20 μg /ml, Gibco), and epidermal growth factor (20 ng/ml, Gibco).

Treatment of C17.2 NSCs with HDAC inhibitors

TSA and nicotinamide (Beyotime) were used to analyze the effects of HDACs on the transcription levels of p53, nestin, sox1, sox2, and bax. First, C17.2 NSCs were precultured for 24 h before the addition of nicotinamide (20 mM) and TSA (250 nM) to the culture medium and an additional 24 h of culture. The control group was treated with the equivalent volume of vehicle.

Extraction of RNA and cDNA synthesis

RNA was extracted from the cerebral cortex, hippocampus, cerebellar cortex, and olfactory bulb. C17.2 NSCs and E14.5 NSCs were exposed to nicotinamide (20 mM) and TSA (250 nM) for 48 h, and RNA was extracted using the RNAiso^{TM} Plus kit (TaKaRa) following the manufacturer's protocol. RNA concentration was determined using a spectrophotometer (BioPhotometer/Plus, Eppendorf) at 260/280 nm. cDNA was synthesized from 5 μg RNA from different tissues using DNase I (Sigma) at 37°C for 30 min. First-strand cDNA was synthesized using the ReverTra Ace kit (TOYOBO) and oligo(dT) following the instruction manual. cDNA samples were stored at -80°C until use.

Quantitative PCR and universal PCR

The expression levels of Sox1, Sox2, p53, Bax, and Nestin in the four brain tissues were analyzed by real-time PCR in a BioRad mini (CFX Connect, BioRad) with SYBR Green Real-time PCR Mix (TOYOBO) according to the product manual. The Nestin primer was provided by Dr. Jiao Wang (Shanghai University). The primers are listed in Tab. 1 (Elkouris *et al.,* 2011; Masahito *et al.,* 2010; Yeo *et al.,* 2006). The PCR program parameters were as follows: 95°C for 5 min, followed by 38 cycles of 30 s at 95°C for DNA denaturation, 30 s at 58°C for primer annealing, and 35 s at 72°C for extension. The program was terminated with a 7 min extension at 72°C. BioRad CFX Manager software was used for relative quantification of PCR data. The expression levels of Sox1, Sox2, p53, Bax, and Nestin were also evaluated in E14.5 and C17.2 NSCs treated with nicotinamide and TSA. β-actin was used as the internal reference to normalize the transcription levels of target mRNAs. A melting curve was used as an evaluation index to determine the specificity of PCR products. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative transcription levels of target mRNAs.

TABLE 1

Primer sequences and temperature of genes in Real-time PCR

Gene Symbol	Sequence(5'-3')	Annealing(°C)
Sox1	AGCCTCCAGAGCCCGACTTTG	62
	AAGAGCTGGCGGGAAGTAAACC	
Sox2	GCGGAGTGGAAACTTTTGTCC	58
	CGGGAAGCGTGTACTTATCCTT	
p53	ACGCTTCTCCGAAGACTGGAT	62
	AGCAACAGATCGTCCATGCAG	
Bax	CTAAAGTGCCCGAGCTGATCA	58
	TGAGGACTCCAGCCACAAAGA	
Nestin	CCTCAACCCTCACCACTCTATTT	49
	TGTCCAGACCACTTTCTTGTATT	
β-actin	TTGCAGCTCCTTCGTTGC	60
	CACGATGGAGGGGAATACAG	

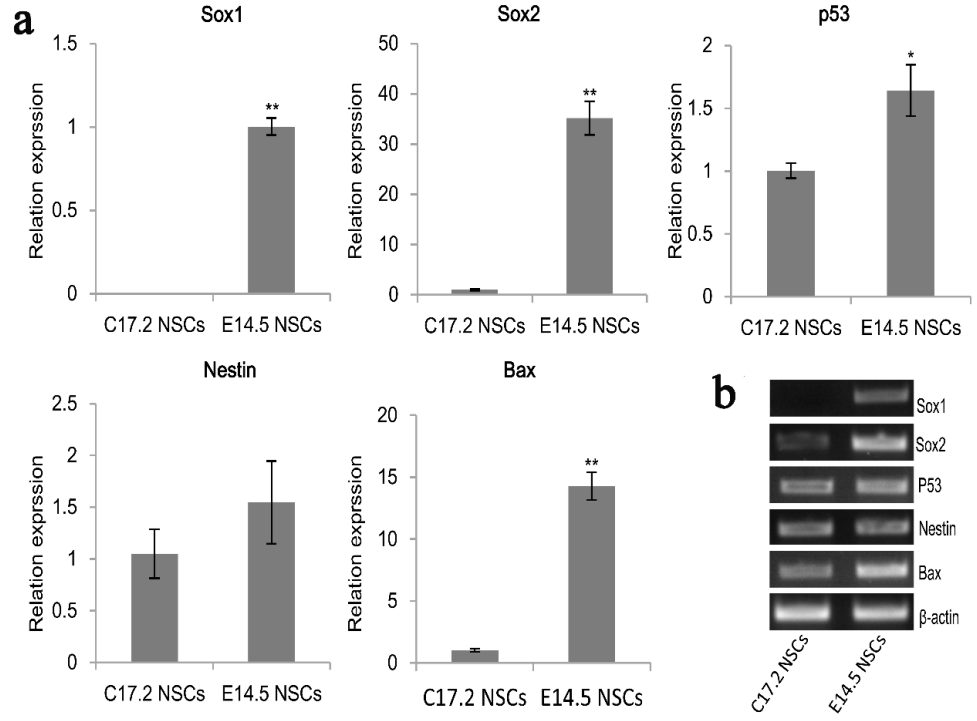

FIGURE 1. Expression of Sox1, Sox2, p53, Nestin, and Bax in C17.2 and E14.5 NSCs. (a) RNA extracted from C17.2 NSCs and E14.5 NSCs was subjected to quantitative PCR analysis. Quantitative data (mean ± SEM) were based on three independent experiments and normalized to β-actin. $^{*}p < 0.05$; $^{**}p < 0.01$. (b) PCR products were detected by electrophoresis in an ethidium bromide agarose gel.

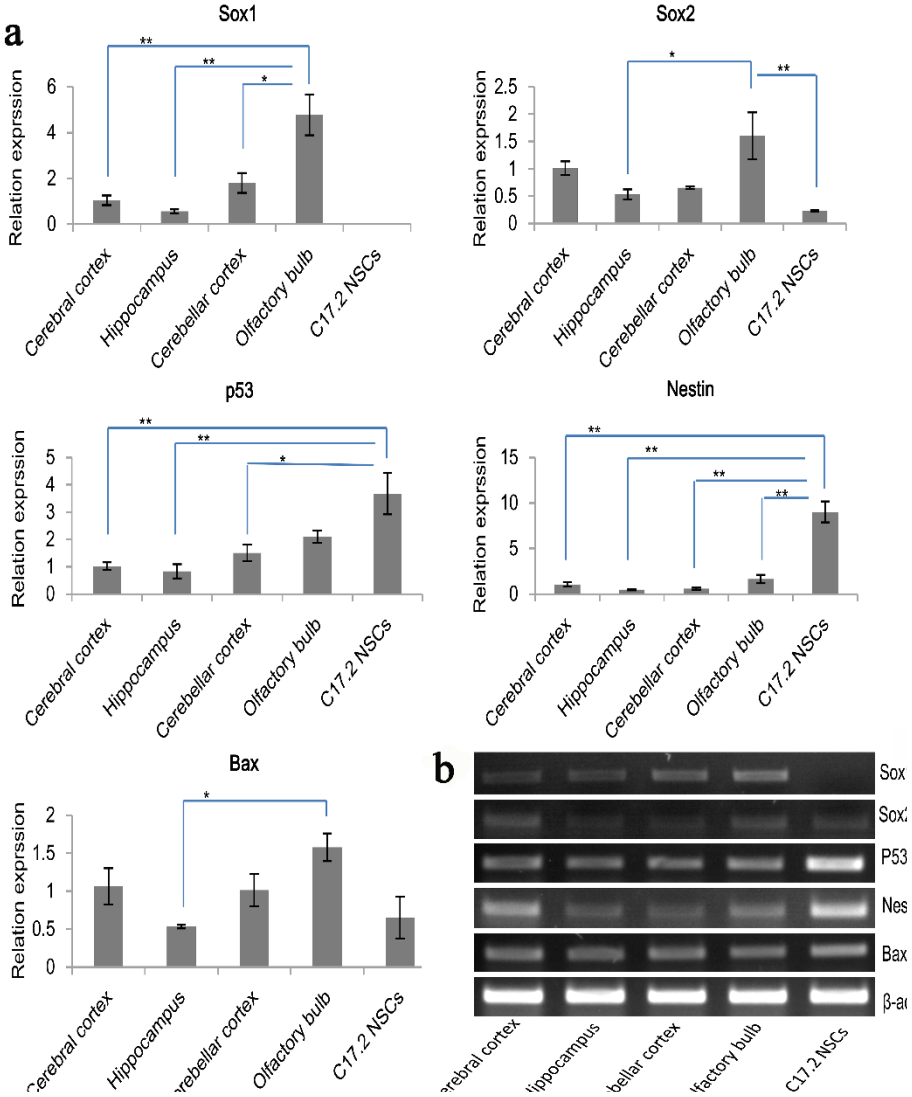

FIGURE 2. Sox1, Sox2, p53, Nestin, and Bax expression in different mouse brain tissues. (a) RNA from tissues and cells was isolated and subjected to quantitative PCR analysis. Quantitative data (mean ± SEM) were based on three independent experiments and normalized to β-actin. $^{*}p < 0.05$; $^{**}p < 0.01$. (b) PCR products were detected by electrophoresis in an ethidium bromide agarose gel.

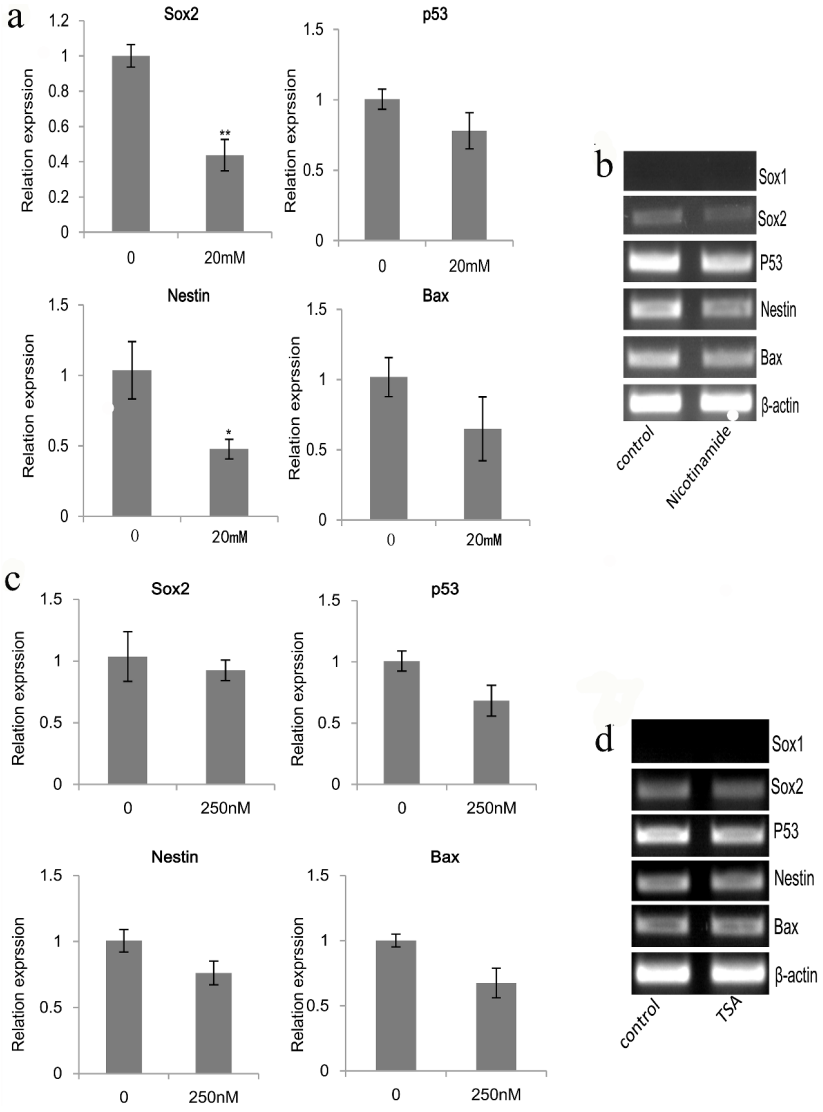

FIGURE 3. Effects of nicotinamide and TSA on the expression of Sox1, Sox2, p53, Nestin, and Bax in C17.2 NSCs. (a) C17.2 NSCs were cultured and treated with nicotinamide (20 mM), and RNA from these cells was isolated and subjected to quantitative PCR analysis. Quantitative data (mean ± SEM) were normalized to β-actin, and data were based on three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$. (b) PCR products of Sox1, Sox2, p53, Nestin, and Bax were detected by electrophoresis after exposure to nicotinamide. (c) C17.2 NSCs were treated with TSA (250 nM), and RNA from these cells was isolated and subjected to quantitative PCR analysis. Quantitative data (mean ± SEM) were based on three independent experiments and normalized to β-actin. $^{*}p < 0.05$; $^{**}p < 0.01$. (d) PCR products of Sox1, Sox2, p53, Nestin, and Bax were detected by electrophoresis after exposure to TSA.

Statistical analysis

Data were expressed as the mean ± standard error of the mean. Student's t-test and one-way analysis of variance (ANOVA) were used to assess the differences between two groups and more than two groups, respectively. The P-value was corrected by Bonferroni correction. Statistically significant differences were considered at *P*-values of < 0.05.

Results

Transcription levels of genes in NSCs

The transcription patterns of Sox1, p53, Nestin, Sox2, and Bax genes were assessed in E14.5 and C17.2 NSCs cultured under standard conditions. The results showed that the transcription levels of Sox2, p53, Sox1, and Bax were significantly higher in E14.5 NSCs than in C17.2 NSCs. The expression of Nestin did not differ significantly between E14.5 NSCs and C17.2 NSCs (Fig. 1).

Gene expression in brain tissues of adult mice

p53 and Nestin expression was significantly higher in C17.2 NSCs than in four brain tissue types (cerebral cortex, hippocampus, cerebellar cortex, and olfactory bulb) (Fig. 2). The olfactory bulb is the key neural structure affecting the sense of smell. The expression of Sox1, Sox2, and Bax was significantly higher in the olfactory bulb than in the cerebral cortex, hippocampus, and cerebellar cortex. Sox1 and p53 were markedly higher in the cerebellar cortex than in the cerebral cortex. Sox2 and nestin expression was higher in the cerebral cortex than in the cerebellar cortex. Bax gene expression did not differ between the cerebral cortex and the cerebellar cortex. The hippocampus is mainly responsible for long-term memory and orientation conversion showed generally low levels of Sox1, Sox2, p53, Nestin, and Bax gene expression.

Nicotinamide modulates gene transcription

To gain further insight into the transcriptional regulation of Sox2, p53, Sox1, Nestin, and Bax, and to determine whether it is under epigenetic control, the effects of nicotinamide and TSA on the mRNA transcription of these genes were evaluated in C17.2 NSCs. The results showed that nicotinamide significantly inhibited the transcription of Sox2 and Nestin; p53 and Bax were also downregulated but the difference did not reach statistical significance (Figs. 3(a) and 3(b)). TSA downregulated Sox2, p53, Nestin, and Bax, but the difference did not reach statistical significance (Figs. 3(c) and 3(d)). Nicotinamide and TSA had no effect on Sox1 mRNA levels.

Discussion

NSC research is a topic of interest that has received wide attention. Sox1 and Sox2 are members of the SoxB1 transcription factor family and are two pivotal transcription factors involved in early neurogenesis (Sottile *et al.,* 2006). Sox1 is the earliest marker in NSCs at the embryonic stage, and Sox1 upregulation prevents cell differentiation and maintains the embryo in the neuroectodermal stage (Suter *et al.,* 2009). In the present study, however, Sox1 transcription was not detected in C17.2 NSCs. The HDAC inhibitors nicotinamide and TSA had no effect on Sox1 transcription, indicating that histone acetylation was not involved in the transcriptional regulation of Sox1. Sox1 and Sox2 are crucial for the maintenance of the NSC stage. The tumor suppressor p53 is important for cell proliferation, development, and aging (Harris and Levine 2005). p53 is involved in the regulation of proliferation and survival of NPCs in association with neuroinflammation, suggesting that some neurological conditions could be improved and promote neurogenesis (Guadagno *et al.,* 2015). Interaction of p53 with apoptosis stimulating protein of p53 regulates the conversion of rhesus monkey embryonic stem cells to a neural fate concomitant with apoptosis, the expression of p53 and Bax were approximately consistent (Wang *et al.,* 2018). The present results indicated that p53 levels were higher in NSCs than in four brain tissues, suggesting that NSCs need more p53 than adult brain tissues during nervous system development. Nestin is an important marker protein to distinguish NSCs from other cell types. Nestin is present in transformed cells in human malignancies and is expressed in many tissues under pathological conditions (Neradil and Veselska, 2015). Nestin is upregulated in stromal cells of human colorectal cancer tissues, and knockdown of nestin inhibits the proliferation and migration of colorectal cancer cells (Li *et al.,* 2015).

Nestin upregulation promotes the expression of epithelial-mesenchymal transition-related proteins in gastric adenocarcinoma (Liu *et al.,* 2015). Bax belongs to the Bcl-2 gene family. The mitochondrial fission protein dynamin-regulated protein 1 protects hippocampal NSCs against lipotoxicity-associated oxidative stress by preserving mitochondrial integrity and inhibiting mitochondrial apoptotic cascades caused by an increased Bcl-2/Bax ratio, preventing cytochrome c release, and inhibiting caspase-3 activation (Kim *et al.,* 2017). HDAC inhibitors such as valproate/valproic acid and TSA affect postnatal neurogenesis, especially in individuals whose brains are actively undergoing key postnatal time windows of development (Foti *et al.,* 2013). We previously showed that Bcl-2, p38, cyt c, and p-4EBP1 suppressed proliferation and promoted apoptosis of C17.2 NSCs in response to nicotinamide and TSA through different molecular mechanisms (Wang *et al.,* 2012)</style>2012. The transcription of Bax was inhibited by nicotinamide and TSA, although the difference was not statistically significantly, implying that histone acetylation does not modulate the transcription of Bax.

To the best of our knowledge, the present study is the first to report the transcription levels of Sox1, Sox2, p53, Nestin, and Bax in C17.2 NSCs, E14.5NSCs, and different adult mouse brain tissues. The results showed that the HDAC inhibitor nicotinamide significantly downregulated Sox2 and Nestin in C17.2 NSCs. Taken together, these findings provide insight into the relationship between HDACs and the expression of Sox2 and Nestin in NSCs.

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Conflicts of interest

The authors declare no conflict of interest.

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