NEURAL REGENERATION RESEARCH
Volume 7, Issue 22, August 2012
www.nrronline.org

Cite this article as: Neural Regen Res. 2012;7(22):1703-1708.

Sonic hedgehog elevates N-myc gene expression in neural stem cells★

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Abstract
Proliferation of neural stem cells is regulated by the secreted signaling molecule sonic hedgehog. In this study, neural stem cells were infected with recombinant adeno-associated virus expressing sonic hedgehog-N-enhanced green fluorescent protein. The results showed that overexpression of sonic hedgehog in neural stem cells induced the increased expression of Gli1 and N-myc, a target gene of sonic hedgehog. These findings suggest that N-myc is a direct downstream target of the sonic hedgehog signal pathway in neural stem cells. Sonic hedgehog and N-myc are important mediators of sonic hedgehog-induced proliferation of neural stem cells.

Key Words
stem cells; neural stem cells; sonic hedgehog signal pathway; N-myc gene; proliferation; target gene; neural regeneration

Research Highlights
(1) Neural stem cells were infected with recombinant adeno-associated virus expressing sonic hedgehog-N-enhanced green fluorescent protein.
(2) We verified that N-myc is a direct downstream target of the sonic hedgehog signal pathway in neural stem cells.

Abbreviations
SHH, sonic hedgehog; EGFP, enhanced green fluorescent protein; rAAV, recombinant adeno-associated virus

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Received: 2012-03-21
Accepted: 2012-06-30
(NY20110223004/WLM)

Liu DS, Wang SY, Cui Y, Shen L, Du YP, Li GL, Zhang B, Wang RZ. Sonic hedgehog elevates N-myc gene expression in neural stem cells. Neural Regen Res. 2012;7(22):1703-1708.

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doi:10.3969/j.issn.1673-5374.2012.22.004

INTRODUCTION

Sonic hedgehog (SHH) plays a critical signaling role in the patterning, proliferation, regeneration, and cell fate determination of a broad range of cells and tissues[1]. In the developing nervous system, SHH regulates patterning of the neural tube[2] and modulates the proliferation and differentiation of neural progenitors[3-6]. Neural stem cells are self-renewing, multipotent progenitor cells that reside in the subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus within the nervous system, and are capable of differentiating into all major neural cell types, namely, neurons, astrocytes and oligodendrocytes[7-9]. Recent studies have demonstrated that SHH is required to maintain the progenitor cell niche and the neural regeneration niche in the telencephalon[1, 10-12], but the mechanism by which the SHH signaling pathway regulates the proliferation and regeneration of neural stem cells remains unclear.

A previous study has shown that SHH induces high levels of N-myc expression[13]. Overexpression of N-myc is sufficient to promote proliferation, and N-myc activity is

necessary for SHH-induced proliferation. In this study, we assumed that N-myc is a direct target gene of SHH signal pathway in neural stem cells.

Exogenous SHH enhances the proliferation of neural stem cells[14-16], but the responsiveness of neural stem cells to SHH is poor. Although numerous mitogens, neurotrophins and other factors modulate the proliferation and neural regeneration of neural stem cells, the mechanisms underlying this process are poorly understood.

In the present study, we isolated and cultured neural stem cells from the subventricular zone of the postnatal rat brain and the amino-terminal active fragment of SHH (SHH-N) was cloned. The plasmid pSNAV2.0-CMV-SHH-N-IRES-enhanced green fluorescent protein (EGFP) was established using enzyme cutting and ligation, and then transfected into the packaging cell line 293T to acquire recombinant adeno-associated virus (rAAV) with SHH. Real-time quantitative PCR analysis was performed after cultured neural stem cells had been infected with the rAAV-SHH-N-EGFP vector for 48 hours to detect the levels of mRNA for SHH, N-myc and Gli1.

RESULTS

Observation of neural stem cell morphology

After 3 days of primary culture of neural stem cells, groups of 2–4 cells were gathered and grew in suspension (Figure 1A). By 7 days, lots of adherent cells died and cells in suspension formed neurospheres (Figure 1B). By 14 days, some neurospheres were fused (Figure 1C; supplementary Figure 1 online).

SHH-N-encoding sequence and construction of pSNAV2.0-CMV-SHH-N-IRES-EGFP

RNA was extracted from neural stem cells that had been primarily cultured for 7 days. Reverse transcription was performed to obtain the SHH-N-encoding sequence. This sequence was 594 bp in length and matched the reported sequence in the National Center for Biotechnology Information (NCBI) database, which is displayed in Figure 2. The sequence was cloned into a pSNAV2.0-CMV-SHH-N-IRES-EGFP carrier vector (supplementary Figure 2 online).

SHH-N protein expression in 293T cells and identification

pSNAV2.0-CMV-IRES-EGFP was successfully transfected into 293T cells, and green fluorescent protein was expressed. SHH-N protein was identified by western blot assay. The characteristic fragment was 20 kDa (Figure 3).

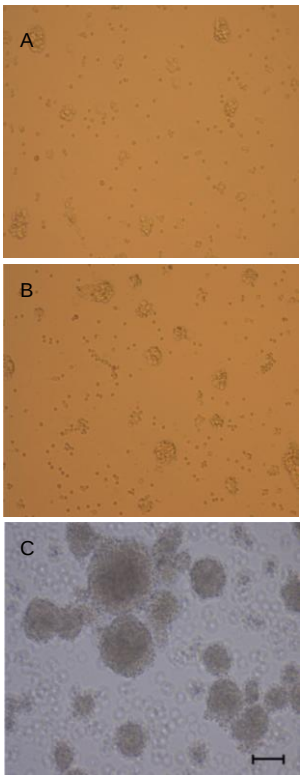

Figure 1 Morphology of primary cultured rat neural stem cells (inverted phase contrast microscope, bar: 100 μm).

(A) A few cell masses can be seen at 3 days in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12; (B) suspended neurospheres at 7 days in serum-free DMEM/F12; (C) partial fusion of neurospheres at 14 days in serum-free DMEM/F12.

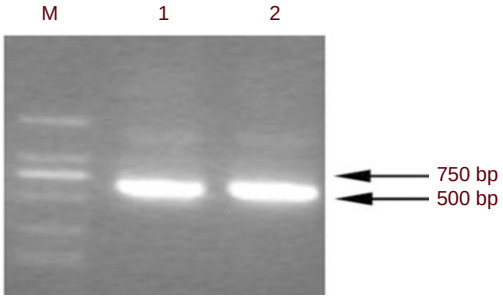

Figure 2 Results of agarose electrophoresis of PCR amplified products of SHH-N.

SHH: Sonic hedgehog; M: marker; 1, 2: SHH-N.

Figure 3 SHH-N expression in 293T cells after pSNAV2.0-CMV-SHH-N–IRES-EGFP plasmid transfection (western blot assay).

EGFP: Enhanced green fluorescent protein; SHH: sonic hedgehog.

Observation of EGFP expression in the rAAV-SHH-N-EGFP-infected group

EGFP, as a reporter protein for SHH-N, was expressed in neural stem cells 14 days after rAAV-SHH-N-EGFP infection (Figure 4, supplementary Figure 3 online).

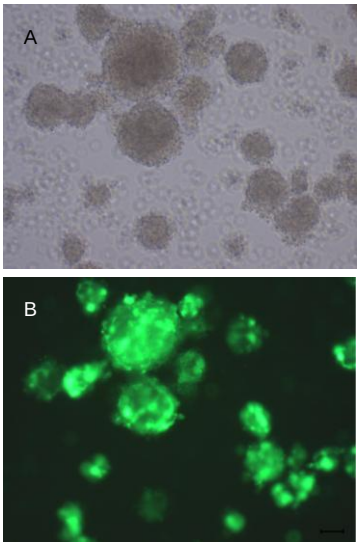

Figure 4 Observation of enhanced green fluorescent protein (EGFP) expression in neural stem cells (NSCs) after rAAV-SHH-EGFP infection (× 100).

(A) Primary culture of NSCs after rAAV-SHH-N-EGFP infection for 14 days.

(B) The same cells shown in A, observed under inverted fluorescence microscope.

rAAV: Recombinant adeno-associated virus; SHH: sonic hedgehog.

Real-time quantitative PCR analysis of SHH-N, N-myc, and Gli1

Compared with the rAAV-EGFP group, real-time PCR showed 3.3-fold induction of SHH-N ($P < 0.01$), 2.3-fold induction of N-myc ($P < 0.05$), and 6.4-fold induction of Gli1 ($P < 0.01$) in the rAAV-SHH-N-EGFP group (Figure 5).

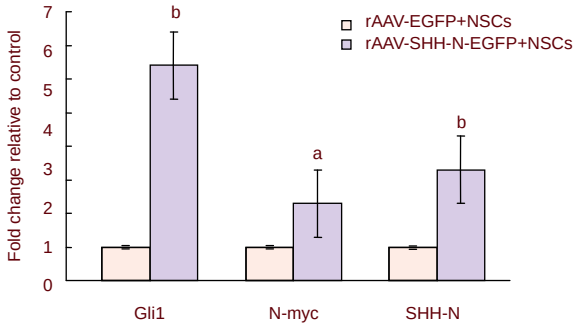

Figure 5 Real-time quantitative PCR analysis of SHH-N, N-myc and Gli1 mRNA expression.

The data are expressed as mean ± SD of three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$, *vs.* rAAV-EGFP+NSCs group (control; *t* test). rAAV: Recombinant adeno-associated virus; SHH-N: the amino-terminal active fragment of sonic hedgehog; EGFP: enhanced green fluorescent protein; NSCs: neural stem cells.

DISCUSSION

The hedgehog signaling plays a pivotal role in organogenesis and differentiation during development and is also involved in the proliferation, cell-fate specification and regeneration of neural stem cells[14, 17-20]. SHH is a potent mitogen for neural progenitor cells of the adult hippocampus[9, 14, 21]. Rat hippocampal progenitors proliferated when cultured in SHH. Furthermore, delivery of SHH to the hippocampus through the use of an adeno-associated viral vector led to significant increase in cell proliferation *in vivo*[8, 14, 22]. Thus, the SHH signal pathway is involved in neural stem cell proliferation and regeneration.

The precise mechanisms by which SHH promotes cell proliferation and tumor formation are unknown[11, 13, 23-25]. In most cells, the transmembrane protein Patched represses transcription of SHH target genes[5, 16, 26-27]. When SHH binds to Patched, the repression is relieved, and a protein called Smoothened becomes activated. Smoothened activation leads, through steps that are poorly understood, to posttranslational modification and nuclear translocation of Gli-family transcription factors. Once in the nucleus, Gli proteins bind to DNA and regulate target gene transcription[28-29].

However, it remains unclear which target genes are responsible for the promotion of neural stem cell proliferation and neural regeneration by SHH signaling in neural stem cells. Previous microarray analysis of genes that are regulated by SHH in granule cells showed that SHH induces expression of the transcription factor N-myc, which is implicated in cell cycle progression[13, 30-31]. This analysis also found that overexpression of N-myc is sufficient to promote cell proliferation, and that N-myc activity is necessary for SHH-induced proliferation[13, 26, 32]. Moreover, members of the Myc family have been reported to be involved in differentiation processes in other cell types, including epithelial, neural crest and hematopoietic stem cells[30-31], although, to our knowledge, previous reports have not directly demonstrated that Myc is involved in the SHH signaling pathway in neural stem cells. The results of this study confirm that (1) the sequence of the SHH-N gene in neural stem cells is coincident with that reported in the NCBI database; (2) the pSNAV2.0-CMV-SHH-N-IRES-EGFP expression vector and

rAAV-SHH-N-EGFP vector were successfully established and packaged; and (3) induction of N-myc and Gli1 was enhanced in the rAAV-SHH-N-EGFP-treated group compared with the control group. N-myc is a direct downstream target of the SHH signaling pathway in neural stem cells. The increase in N-myc transcription stimulated by SHH suggests that N-myc might be an important mediator of SHH-induced proliferation and neural regeneration. Although examination of sequences in and around the N-myc gene has not revealed any consensus Gli-binding sites, it is possible that such sites are present in other parts of the gene, or that SHH regulates N-myc expression through Gli-independent mechanisms. It would be interesting to investigate the possible roles of N-myc in the SHH signaling pathway in future studies.

MATERIALS AND METHODS

Design

A randomized, controlled, cell experiment.

Time and setting

This experiment was performed at the Chinese Academy of Medical Sciences, Department of Neurosurgery, Peking Union Medical College Hospital, China in May 2010.

Materials

A total of 10 male specific pathogen-free Sprague-Dawley rats aged 3 days and weighing 4 g were supplied by the Animal Institute, Chinese Academy of Medical Sciences. Experiments were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[33].

Methods

Isolation, culture and identification of neural stem cells

Rat brain was obtained by craniotomy after anesthesia by 10% chloral hydrate. Tissue from the subventricular zone was isolated under aseptic conditions[34]. Meninges and blood vessels were stripped off under a microscope, mechanically cut into pieces, and filtered through a screen (mesh size 150 μm). The specimens were washed in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12; Gibco, Carlsbad, CA, USA) and centrifuged. DMEM/F12 was a 1:1 mixture of DMEM and F12. Then, 2-mL aliquots with a cell density of 5×10^5/mL were seeded into cell culture flasks, and cultured in serum-free DMEM/F12 medium supplemented with N2, basic fibroblast growth factor 10 μg/L, epidermal growth factor 20 μg/L, heparin 4×10^4 U/L, penicillin 1×10^5 U/L and streptomycin 1×10^6 U/L at 37°C in 5% CO_2 in a saturated humidity incubator for 7 days. The fluid was replaced every 2 days. Obtained cells were identified as neural stem cells by anti-nestin immunocytochemical staining (results not shown).

Cloning and sequencing of SHH-N

(1) Extraction of RNA and reverse transcription. RNA was extracted from primary cultured neural stem cells. Annealing reactions consisting of RNA (4 μL), oligo-dt (1 μL), and diethylpyrocarbonate-treated water (7.4 μL) were then performed at 65°C for 10 minutes. Reverse transcription reactions consisted of the following: buffer 4 μL, RNase inhibitor 0.5 μL, deoxynucleotide (dNTP) mix 2 μL, dithiothreitol 1 μL, reverse transcriptase 1.1 μL. Reactions were performed at 50°C for 30 minutes, 85°C for 5 minutes, and 20°C for 1 minute. cDNA (SuperScript III Preamplification System for First Strand cDNA Synthesis kit; Invitrogen, Carlsbad, CA, USA) was stored at −80°C.

(2) Amplification and recovery of the SHH-N fragment. Reactions consisted of the following: 10 × buffer II 5 μL, cDNA 1 μL, dNTP (10 mM) 1 μL, forward primer (10 μM) 1 μL (5'-CGA ATT CGC ATG CTG CTG CTG GCG AG-3'), reverse primer (10 μM) 1 μL (5'-CGG TCG ACT CAG CCT CCC GAT TTG GCC-3'), pyrobest enzyme 0.5 μL, and water 40.5 μL. Reaction conditions were as follows: 95°C for 5 minutes, thirty cycles of 95°C for 0.5 minutes, 55°C for 0.5 minutes, and 72°C for 1 minute, then 72°C for 10 minutes and 4°C for 1 minute. The SHH-N fragment was recovered using a DNA recovery kit (Anxygen, Union City, CA, USA).

pSNAV2.0-CMV-SHH-N-IRES-EGFP construction

After double enzyme digestion of the pSNAV2.0-CMV-Laz-IRES-EGFP vector and the SHH-N fragment, we obtained a pSNAV2.0-CMV-SHH-N-IRES-EGFP vector using T4 ligase. After transformation and bacterial challenge, the plasmid was extracted using a plasmid extraction kit.

SHH-N protein identified by western blot assay

293T cells (gifted by the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences) were seeded in medium at a cell density of $10^6/cm^2$ overnight. DMEM was used 1 hour before transfection. The pSNAV2.0-CMV-SHH-N-IRES-EGFP plasmid was transfected into cells and the DMEM was replaced with 10% fetal bovine serum DMEM. Cells were cultured for 36 hours, observed under an inverted phase contrast fluorescence microscope, and then collected. Cells were

lysed in 4°C radioimmunoprecipitation assay buffer and centrifuged. Supernatants were retained and western blot assays were conducted using SHH antibody (N-19) to identify SHH-N protein.

rAAV-SHH-N-EGFP packaging and purification

rAAV-SHH-N-EGFP and rAAV-EGFP were packaged, purified, and concentrated by Gene Technology Company (Beijing, China). Virus titer was detected using a digoxin-labeled H1 probe by dot blot analysis (Gene Technology Company). The level was 2×10^{11} v.g/mL.

Infection of neural stem cells with rAAV-SHH-N-EGFP in vitro

neural stem cells were cultured for 14 days. Then, 1×10^5 cells were seeded onto cell culture plates. rAAV-SHH-N-EGFP viral vector (multiplicity of infection 1×10^5) and rAAV-EGFP were added. Cells were infected with rAAV-SHH-N-EGFP or rAAV-EGFP, with a multiplicity of infection (v.g/cell) of 1×10^5. Cells were cultured at 37°C in 5% CO_2 in a saturated humidity incubator for 14 days, and observed under a fluorescence microscope. The fluid was replaced every 2 days.

Real-time quantitative PCR analysis

The primers used for real-time quantitative PCR are listed in Table 1. RNA was extracted 48 hours after infection.

Table 1 Real-time PCR primer sequence

Primer	Sequence	Length (bp)
SHH-N	U: 5'-TTG CTT CCT CGC TGC TGG T-3'	519
	D: 5'-ATG ATG GCC GTC CTC ATC C-3'	
Gli1	U: 5'-ATC ACC TGT TGG GGA TGC TGG AT-3'	3 501
	D: 5'-GGC GTG AAT AGG ACT TCC GAC AG-3'	
N-myc	U: 5'-GCG GTA ACC ACT TTC ACG AT-3'	1 099
	D: 5'-ATG ATG GCC GTC CTC ATC C-3'	
GAPDH	U: 5'-GAG GCC GGT GCT GAG TAT GTC-3'	1 307
	D: 5'-CCA GGC GGC ACG TCA GA-3'	

U: Upstream; D: downstream; SHH-N: the amino-terminal active fragment of sonic hedgehog.

Real-time PCR reactions consisted of the following: TransStart Green qPCR SuperMix 12.5 μL, forward primer (10 μM) 0.5 μL, reverse primer (10 μM) 0.5 μL, cDNA 2 μL, ddH_2O 12.5 μL, dye 0.5 μL. Reaction conditions were as follows: pre-denaturation at 94°C for 2 minutes, 45 cycles of denaturation at 94°C for 20 seconds and annealing at 58°C for 1 minute. The gain value was 2.0. The mean values for three wells were recorded. Results were analyzed using iQ5 real-time PCR analysis software (Bio-Rad, Hercules, CA, USA), and average values were obtained.

Statistical analysis

Values are presented as mean ± SD. Differences between groups were analyzed by *t* test. A value of $P < 0.05$ was considered statistically significant, and all statistical tests were two-sided.

Acknowledgments: We thank Professor Yongsheng Chang from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences for providing cells and medium.

Funding: This project was funded by the National Natural Science Foundation of China, No.81171401; Science and Technology Development Program of Dalian City, No. 2008J99JH268; the Scientific Research Program of Higher Learning School of Department of Education of Liaoning Province, No. L20100108.

Author contributions: Dongsheng Liu, Shouyu Wang, Yanping Du, Yan Cui and Lun Shen participated in molecular genetic studies, sequence alignment, and drafted the manuscript. Guilin Li participated in study design and performed statistical analysis. Renzhi Wang and Bo Zhang conceived the study and participated in study design and coordination. All authors read and approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: This experimental protocol was approved by the Animal Ethics Committee of Dalian Medical University of China.

Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org.

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(Edited by Yang XF, Zhu WJ/Qiu Y/Song LP)