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

Dongsheng Liu¹, Shouyu Wang¹, Yan Cui¹, Lun Shen¹, Yanping Du¹, Guilin Li², Bo Zhang¹, Renzhi Wang²

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

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 tissues¹. In the developing nervous system, SHH regulates patterning of the neural tube² and modulates the proliferation and differentiation of neural progenitors³⁻⁶. 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 oligodendrocytes⁷⁻⁹. Recent studies have demonstrated that SHH is required to maintain the progenitor cell niche and the neural regeneration niche in the telencephalon¹⁰⁻¹², 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 expression¹³. 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).
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).

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).

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. SHH is a potent mitogen for neural progenitor cells of the adult hippocampus. 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. 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. In most cells, the transmembrane protein Patched represses transcription of SHH target genes. 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.

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. 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. 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, 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

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.

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. *P < 0.05, **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.
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 x 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 x 10^4 U/L, penicillin 1 x 10^3 U/L and streptomycin 1 x 10^6 U/L at 37°C in 5% CO2 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, deoxyribonucleotide (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 x 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 10^5/cm² 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.

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.

Table 1 Real-time PCR primer sequence

| Primer | Sequence | Length (bp) |
|--------|----------|-------------|
| SHH-N | U: 5’-TTG CTT CCT GGC TGC TGG T-3’  |
|        | D: 5’-ATG ATG GGC GTC CTC ATG C-3’ | 519 |
| Gli1   | U: 5’-ATC ACC TGT TGG GGA TGG TGG AT-3’ |
|        | D: 5’-GCG GTG AAT AGG ACT TGG GAC AG-3’ | 3501 |
| N-myc  | U: 5’-GCG GTA ACC ACT TTC ACG AT-3’  |
|        | D: 5’-AGT ATG GCC GTC CTC ATG C-3’ | 1099 |
| GAPDH  | U: 5’-GAG GCC GGT GCT GAG TAT GTC-3’  |
|        | D: 5’-CCA GGC GGC ACG TCA GA-3’ | 1307 |

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

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.

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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.

REFERENCES

[1] McMahon AP, Ingham PW, Tabin CJ. Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol. 2003;53:1-114.

[2] Lupo G, Harris WA, Lewis KE. Mechanisms of ventral patterning in the vertebrate nervous system. Nat Rev Neurosci. 2006;7(2):103-114.

[3] Dahmane N, Sánchez P, Gitton Y, et al. The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. Development. 2001;128(24):5201-5212.

[4] Hynes M, Porter JA, Chiang C, et al. Induction of midbrain dopaminergic neurons by Sonic hedgehog. Neuron. 1995; 15(1):35-44.

[5] Kenney AM, Rowitch DH. Sonic hedgehog promotes G1 cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. Mol Cell Biol. 2000; 20(23):9055-9067.

[6] Wechsler-Reya RJ, Scott MP. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. Neuron. 1999;22(1):103-114.
[7] Traiffort E, Angot E, Ruat M. Sonic Hedgehog signaling in the mammalian brain. J Neurochem. 2010;113(3):576-590.

[8] Xu Q, Guo L, Moore H, et al. Sonic hedgehog signaling confers ventral telencephalic progenitors with distinct cortical interneuron fates. Neuron. 2010;65(3):328-340.

[9] Sousa VH, Fishell G. Sonic hedgehog functions through dynamic changes in temporal competence in the developing forebrain. Curr Opin Genet Dev. 2010;20(4):391-399.

[10] Vaillant C, Monard D. SHH pathway and cerebellar development. Cerebellum. 2009;8(3):291-301.

[11] Morikawa Y, Maska E, Brody H, et al. Sonic hedgehog signaling is required for sympathetic nervous system development. Neuroreport. 2009;20(7):684-688.

[12] Huang X, Liu J, Ketova T, et al. Transventricular delivery of Sonic hedgehog is essential to cerebellar ventricular zone development. Proc Natl Acad Sci U S A. 2010;107(18):8422-8427.

[13] Oliver TG, Grasfeder LL, Carroll AL, et al. Transcriptional profiling of the Sonic hedgehog response: a critical role for N-myc in proliferation of neuronal precursors. Proc Natl Acad Sci U S A. 2003;100(12):7331-7336.

[14] Lai K, Kaspar BK, Gage FH, et al. Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. Nat Neurosci. 2003;6(1):21-27.

[15] Palma V, Lim D A, Dahmane N, et al. Sonic hedgehog controls stem cell behavior in the postnatal and adult brain. Development. 2005;132(2):335-344.

[16] Palma V, Ruiz IA. Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex. Development. 2004;131(2):337-345.

[17] Li FH, Xin SJ, Zhang SY, et al. The sonic hedgehog induce vascular adventitial fibroblasts phenotypic modulation, proliferation and migration. Zhonghua Yi Xue Za Zhi. 2009;89(43):3079-3082.

[18] Hor CH, Tang BL. Sonic hedgehog as a chemoattractant for adult NPCs. Cell Adh Migr. 2010;4(1):1-3.

[19] Chen G, Goto Y, Sakamoto R, et al. GLI1, a crucial mediator of sonic hedgehog signaling in prostate cancer, functions as a negative modulator for androgen receptor. Biochem Biophys Res Commun. 2011;404(3):809-815.

[20] Ribes V, Briscoe J. Establishing and interpreting graded Sonic Hedgehog signaling during vertebrate neural tube patterning: the role of negative feedback. Cold Spring Harb Perspect Biol. 2009;1(2):a2014.

[21] Bai LY, Chiu CF, Lin CW, et al. Differential expression of Sonic hedgehog and Gli1 in hematological malignancies. Leukemia. 2008;22(1):226-228.

[22] Sims JR, Lee SW, Topalkara K, et al. Sonic hedgehog regulates ischemia/hypoxia-induced neural progenitor proliferation. Stroke. 2009;40(11):3618-3626.

[23] Fernandez C, Tatard VM, Bertrand N, et al. Differential modulation of Sonic-hedgehog-induced cerebellar granule cell precursor proliferation by the IGF signaling network. Dev Neurosci. 2010;32(1):59-70.

[24] Domantksayaa E, Wacker A, Mauti O, et al. Sonic hedgehog guides post-crossing commissural axons both directly and indirectly by regulating Wnt activity. J Neurosci. 2010;30(33):11167-11176.

[25] Ribes V, Balaskas N, Sasai N, et al. Distinct Sonic Hedgehog signaling dynamics specify floor plate and ventral neuronal progenitors in the vertebrate neural tube. Genes Dev. 2010;24(11):1186-1200.

[26] Pan Y, Wang C, Wang B. Phosphorylation of Gli2 by protein kinase A is required for Gli2 processing and degradation and the Sonic Hedgehog-regulated mouse development. Dev Biol. 2009;326(1):177-189.

[27] Galvin KE, Ye H, Wetmore C. Differential gene induction by genetic and ligand-mediated activation of the Sonic hedgehog pathway in neural stem cells. Dev Biol. 2007;308(2):331-342.

[28] Ingham PW, Mcmahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 2001;15(23):3059-3087.

[29] Yang L, Shen JH, Liu XD. Sonic hedgehog and prostate growth regulation. Zhonghua Nan Ke Xue. 2007;13(8):730-733.

[30] Watt FM, Frye M, Benitah SA. MYC in mammalian epidermis: how can an oncogene stimulate differentiation? Nat Rev Cancer. 2008;8(3):234-242.

[31] Eilers M, Eisenman RN. Myc's broad reach. Genes Dev. 2008;22(20):2755-2766.

[32] Thomas WD, Chen J, Gao YR, et al. Patched1 deletion increases N-Myc protein stability as a mechanism of medulloblastoma initiation and progression. Oncogene. 2009;28(13):1605-1615.

[33] The Ministry of Science and Technology of the People’s Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.

[34] Paxions G, Watson C. The Rat Brain in Stereotaxic Coordinates. Beijing: People’s Medical Publishing House. 2005

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