The *noggin2* gene of *Gekko japonicus* (Gekkonidae) is down-regulated in the spinal cord after tail amputation

Y. Liu, Y.L. Zhou, Y.Y. Qian, Y.J. Wang, F. Ding, X.S. Gu and M. Liu

Jiangsu Key Laboratory of Neuroregeneration, Nantong University, Nantong, Jiangsu Province, PR China

Corresponding autor: M. Liu
E-mail: liumei@ntu.edu.cn

ABSTRACT. The cDNA encoding noggin2 protein was obtained from the brain and spinal cord cDNA library of *Gekko japonicus*. The size of the *noggin2* transcript and its expression in different tissues were analyzed by Northern blot analysis. *In situ* hybridization revealed positive hybridization signals in both gray and white matter of the spinal cord. Changes in *noggin2* expression in the spinal cord after tail amputation were examined by real-time PCR. The *noggin2* was expressed in the normal spinal cord and down-regulated three days after tail amputation, reaching the lowest level at two weeks, during the time course when we followed the expression levels. We concluded that the expression of *noggin2* is affected by the process of spinal cord injury and regeneration.

Key words: *Gekko japonicus*; Molecular cloning; *Noggin2*; Regeneration; Spinal cord

INTRODUCTION

It is known that adult central nervous system (CNS) fails to regenerate following injury in most species, but there are examples in which spinal cord regeneration can occur following specific types of lesions, such as in urodele amphibians (newts and salamanders) and lizards (Simpson Jr., 1968; Egar et al., 1970; Echeverri and Tanaka, 2002; Chernoff et al., 2003). It is still poorly understood why this potential is lost with evolution and development and becomes very limited in adult mammals. It is of great interest to examine those cases in which vertebrate spinal cord demonstrates intrinsic regeneration capacity (Brockes, 1997). Understanding the mechanisms of spontaneous spinal cord regeneration will generate an additional set of tools applicable to human spinal cord injury.

Gekko japonicus, a member of the family Gekkonidae, is well known for its remarkable ability to regenerate its tail and recover full movement and function in a certain time after amputation (Alibardi, 1995; Echeverri and Tanaka, 2002). The gecko tail has major axial structure including the spinal cord. Thus, the understanding of tail regeneration in gecko could contribute to the molecular mechanism of spinal cord regeneration. With the aim toward the investigation of the comprehensive gene expression profile of the reptilian CNS, we constructed a cDNA library of the brain and the spinal cord from *G. japonicus*, and the cDNA clones were randomly selected and sequenced for expressed sequence tag (EST) analysis (Liu et al., 2006). The sequence of a cDNA clone from the library shared significant homology with *noggin2* of *Gallus*, and we further cloned the full-length *noggin2* cDNA of gecko.

The *noggin* gene was first identified from *Xenopus* embryos in 1992 by Smith and Harland. Since *noggin* can mimic the actions of the Spemann organizer of the amphibian gastrula, it was originally characterized as a component of the Spemann organizer, and it can induce dorsalization and the formation of neural tissue from ectoderm. *Noggin* genes are highly conserved among species, while the different species have different members of the *noggin* gene family. Generally, in lower animals, there are two or more members of the *noggin* family, such as in *Xenopus tropicalis*, *X. laevis*, and zebrafish (Fürthauer et al., 1999; Fletcher et al., 2004; Eroshkin et al., 2006). However, for higher mammals, only a single *noggin* gene has been found in their genome, such as in *Homo sapiens*, *Rattus norvegicus*, *Mus musculus*. Comparison of the three zebrafish noggin proteins reveals that *noggin1* and *noggin3* are highly related, and *noggin2* appears to be the most divergent member of the family, while *noggin2* can also antagonize ventralizing bone morphogenetic proteins (BMPs), as other members. Noggin is a secreted protein with a hydrophobic amino-terminus and cysteine-rich carboxy-terminus, and it binds to BMP-2, -4, -5, -6, and -7 with various degrees of affinity and prevents them from forming a complex with their receptors (Valenzuela et al., 1995; Zimmerman et al., 1996; Aspenberg et al., 2001). Noggin has been shown to play important roles in growth and patterning of the neural tube and somite (McMalon et al., 1998), bone and cartilage development (Reddi, 2001), and growth and differentiation in the anagen hair follicle (Kulessa et al., 2000). Noggin was also found to be involved in the development of embryos, and was regarded to be endogenous neural induced signals at a certain stage of development (Lamb et al., 1993). Therefore, it is of interest to investigate whether noggin plays a role in the spinal cord regeneration of gecko. In the present study, we carried out the molecular characterization of *noggin2* gene of gecko and described its expression change in the spinal cord after tail amputation.

MATERIAL AND METHODS

Animals

Adult *G. japonicus* were from the laboratory animal center of Nantong University. They were freely fed mealworms and given water during the whole experiment, and they were housed in an air-conditioned room with a controlled temperature of 26°C and saturated humidity. All experimental protocols pertinent to animals were given prior approval by the Laboratory Animal Care and Use Committee of the medical school. We minimized the number of animals used, and used cooling anesthesia to reduce their suffering. Adult geckos were used for tail amputation at the sixth caudal vertebra. The caudotomy was performed at the site of the sixth tail segment, by inserting a nylon slipknot and pulling gently, to mimic the conditions of autotomy in the natural environment of the animals.

Molecular cloning of full-length cDNA of *noggin2*

The EST (GenBank accession No. EB171660) of gecko *noggin2* gene was obtained from the cDNA library of gecko brain and spinal cord, and the fragment did not contain 5' and 3' regions of the cDNA. To obtain the full-length transcript of *noggin2* gene, we conducted the 5' and 3' rapid amplification of cDNA ends (RACE), using a commercially available kit (BD Biosciences Clontech). Total RNA was extracted and purified from the brain and the spinal cord of gecko using TRIZOL reagent (Invitrogen, Co., Ltd.) according to the manufacturer protocol. The primers designed for RACE are as follows: 5'-CTTGATGTAACGAGGCCAGAACCG-3' (5' gene-specific primer), 5'-GGTGTACG CCCACAGCCACTGC-3' (5' nested gene-specific primer); 5'-GGAGCAATTTCGACCCTGCGT TC-3' (3' gene-specific primer), 5'-AATCTCTCCAGCCCTGACCCGC-3' (3' nested gene-specific primer). We then used CDS primer and BD SMART II A oligo provided with the kit to synthesis the RACE-Ready first-strand cDNA. 5'- and 3'-RACE polymerase chain reaction (PCR) was performed with the Universal Primer A Mix under the following conditions: one cycle of 94°C for 3 min; five cycles of 94°C for 40 s and 72°C for 3 min; five cycles of 94°C for 30 s, 70°C for 40 s and 72°C for 3 min; 15 cycles of 94°C for 30 s, 68°C for 40 s, and 72°C for 3 min; 15 cycles of 94°C for 30 s, 66°C for 40 s, and 72°C for 3 min, and one cycle of 72°C for 10 min. The PCR-amplified DNA was purified and cloned into pGEM-T Easy vector (Promega) for sequencing. The full-length cDNA sequence was obtained by combining the fragment with the overlapped region.

Bioinformatics analysis of *noggin2*

The sequence of *noggin2* was analyzed for coding probability with DNATools programs. Comparison against the GenBank protein database was performed using the BLAST network server at the National Center for Biotechnology Information. The theoretical molecular weight and pI of the noggin2 protein were analyzed by ProtParam tool in ExPASy (http://www.expasy.ch/tools/protparam.html).

Northern blot analysis

Digoxigenin (DIG)-labeled *noggin2* riboprobes of 664 bp were synthesized *in*

vitro from linearized plasmid, following DIG-UTP supplier instructions (Roche). Total RNAs were prepared with Trizol (Invitrogen) from various tissues including brain, spinal cord, heart, lung, liver, kidney, and ovary. Twenty micrograms RNAs of each tissue was electrophoresed and blotted onto nylon membranes (Schleicher & Schuell Bioscience Inc.). RNAs were cross-linked to nylon membranes by UV-light cross-linking using a cross-linker (CX-2000, UVP, USA). Prehybridization was performed at 60°C in standard hybridization buffer containing 50% (v/v) formamide, 5X SSC, 0.2% (w/v) SDS, and 2% (w/v) blocking reagent (Roche) for 2 h. The membranes were then hybridized in the same buffer containing DIG-labeled RNA riboprobes (100 ng/mL in DIG Easy Hyb) for 20 h at 68°C, and washed twice in 2X SSC with 0.1% SDS at 25°C for 15 min each and twice in 0.1X SSC with 0.1% SDS at 60°C for 15 min each. They were then incubated in blocking solution (100 mM maleic acid, 150 mM NaCl and 1% blocking reagent) for 30 min at 37°C, and in the blocking solution with anti-DIG-AP (Roche, 1:5000) overnight at 4°C. After washing twice with 100 mM maleic acid buffer, pH 7.5, and 0.3% Tween-20 each for 15 min at 25°C, the membrane was incubated in detection buffer including 100 mM Tris-HCl, pH 9.5, with 100 mM NaCl for 5 min. The hybridized bands were visualized by CDP-Star (Roche) and recorded with X-ray film.

In situ hybridization

Spinal cord was dissected from perfused gecko and fixed overnight on 4% paraformaldehyde, pH 7.0. The 14-μm thick sections were cut at the site of the caudal vertebra and thawed onto RNase-free silicane-coated slides. Sections were prehybridized for 2 h and hybridized with 5 ng DIG-labeled probe (see above) overnight at 42°C in a humid chamber, and washed twice in 2X SSC with 0.1% SDS at 50°C for 10 min each, twice in 1X SSC with 0.1% SDS at 50°C for 15 min each, and twice in 0.1X SSC with 0.1% SDS at 50°C for 30 min each. They were then incubated in blocking solution (100 mM maleic acid, 150 mM NaCl and 1% blocking reagent) for 30 min at 37°C, and in blocking solution with anti-DIG-AP (Roche, 1:1000) overnight at 4°C. After washing twice with 100 mM maleic acid buffer, pH 7.5, each for 15 min at 25°C, the signals were detected using NBT/BCIP (Roche).

Real-time PCR analysis

The primers used were designed for real-time PCR using the Primer 5.0 software and are described as follows: *noggin2*-F: 5'-CCTGCTGGAAACCACTCG-3' and *noggin2*-R: 5'-CACCTGCCATATCCATCACC-3', for gecko *noggin2* cDNA (GenBank accession No. HM002546), and elongation factor 1 alpha (EF-1α)-F: 5'-GATGGAAAGTGACCCGCA-3' and EF-1α-R: 5'-GAGGAAGACGCAGAGGTTTG-3' for EST00347 (GenBank accession No. C0818260), the sequence of gecko eukaryotic EF-1α. The primers of the *noggin2* and EF-1α were synthesized by Shanghai Invitrogen Biotechnology Co., Ltd. Geckos for the tail amputation model had their tails broken at the sixth caudal vertebra. Total RNAs of spinal cord from the third to the fifth caudal vertebrae were prepared respectively (N = 5) at 1, 3, 7, and 14 days post-amputation. The first-strand cDNA was synthesized using Omniscript Reverse Transcription Kit (QIAGEN) in a 20-μL reaction system containing 2 μg total RNA, 0.2 U/

µL M-MLV reverse transcriptase, 0.5 mM dNTP mix, 1 µM Oligo-dT primer. The first-strand cDNA products (1 µL) were amplified with gene-specific primers to investigate the expression of *noggin2*. The amplification of EF-1α was used for quantitative normalization of the target gene. The PCR amplification was performed in 12.5 µL Evagreen Mix (Biotium Inc.), with 20 pmol of each primer and 10.5 µL ddH$_2$O in a final volume of 25 µL. After cDNA/primer denaturation at 95°C for 15 min, the PCR amplification was carried out in 40 cycles at 95°C for 15 s, and 60°C for 45 s (fluorescence collection). Normalization was carried out simultaneously by amplification of EF-1α under the same conditions described above. At the same time, a negative control without the first-strand cDNA was also performed. We set the threshold at the level of the middle steady portion of the reaction cycles-versus-fluorescence curve, and we calculated the cycle threshold values of target genes using customized software (Rotor Gene Analysis Software 6.0, Corbett Research Pty Ltd.). Finally, the PCR products were separated by 1.5% agarose gel electrophoresis in the presence of ethidium bromide and visualized using a GeneGenius imaging system (SynGene). The identity of the real-time PCR products was confirmed by DNA sequencing.

RESULTS AND DISCUSSION

Molecular cloning and bioinformatic analysis of *noggin2*

Based on the EST analysis from the brain and the spinal cord cDNA library of *G. japonicus*, we obtained the full-length cDNA sequence of *noggin2* gene (shown in Figure 1) using 5'- and 3'-RACE, which were submitted to GenBank (accession No. HM002546). The full-length cDNA of *noggin2* was 1397 bp, and the longest open reading frame, covering 226 to 873 bp, encoded a polypeptide of 215 amino acids. The nucleotide sequence of *noggin2* and its deduced amino acid sequence were analyzed by ProtParam tool in ExPASy (http://cn.expasy.org/), the predicted molecular weight was 25 kDa, and the theoretical pI was 9.02. The analysis of the predicted gecko noggin2 protein revealed that it contains a signal peptide, which is similar to the known noggin2 protein of other species. The sequence comparison was conducted through a database search using the BLAST program (http://www.ncbi.nlm.nih.gov). The noggin2 amino acid sequences of different species including *Danio rerio* (NM_130992), *Trachinotus blochii* (GU001783), *Takifugu rubripes* (AY779056), *X. laevis* (AY913768), *G. japonicus* (HM002546), *Gallus gallus* (AY779058) were aligned using the MegAlign program of DNASTAR by the CLUSTAL method. The results showed that at the amino acid level, similarity was 60-72% with the previously reported sequences of *noggin2* in NCBI (Figure 2).

Northern blot analysis and *in situ* hybridization analysis of *noggin2* in normal geckos

We detected *noggin2* expression by Northern hybridization in brain, spinal cord, lung, heart, kidney, ovary, and liver from adult gecko. It was expressed in all tissues examined, with relatively higher expression levels in spinal cord, liver and ovary. The positive bands of *noggin2* mRNA of approximately 1.4 kb was detected in the assay (Figure 3), which was con-

sistent with the full-length cDNA of *noggin2* described above. In addition, a larger transcript was detected in the spinal cord and ovary as well, indicating that another type of *noggin* may be transcribed. We intend to confirm its existence in further studies.


Figure 1. Sequence of *noggin2* cDNA (GenBank accession No. HM002546) and its deduced amino acid sequence. The full-length cDNA of *noggin2* was 1397 bp, and the open reading frame encoded a polypeptide of 215 amino acids. The primers of 5'-RACE and 3'-RACE are boxed. The shaded amino acids at the amino-terminus of noggin2 protein are the predicted signal peptide. Numbering of nucleotide and amino acid sequences is shown on the left.


Figure 2. Alignment of *noggin2* using the MegAlign program (DNASTAR) by the CLUSTAL method. Shaded (with solid black) residues are the amino acids that match the consensus. The dashed blue box shows the predicted signal peptides for secretion among different species, and the red box highlights the nine conserved cysteine residues of the mature peptide. Noggin2 amino acid sequences were obtained from previously reported sequences in GenBank. *Danio rerio* (NM_130992), *Trachinotus blochii* (GU001783), *Takifugu rubripes* (AY779056), *Xenopus laevis* (AY913768), *Xenopus tropicalis* (AY672092), *Gekko japonicus* (HM002546), *Gallus gallus* (AY779058).
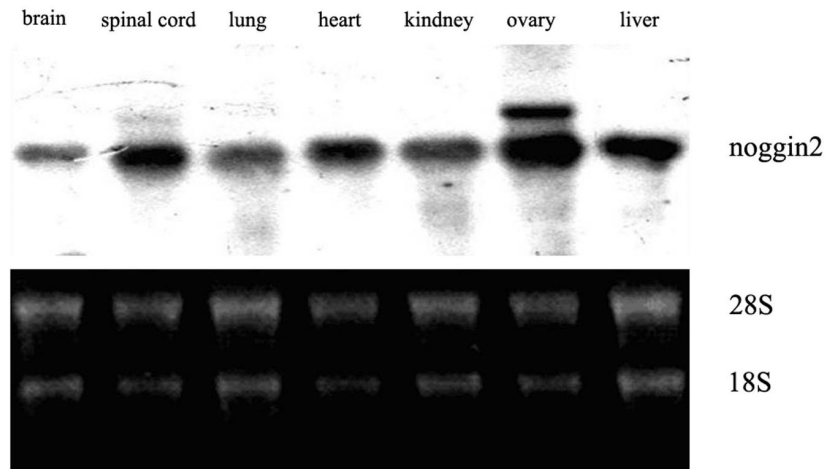
Figure 3. Representative results of Northern blot analyses of *noggin2* mRNA in various tissues of adult gecko. The length of *noggin2* mRNA was about 1.4 kb, and the 18S and 28S rRNAs of corresponding tissues are shown in the lower panel.

To investigate the expression pattern of *noggin2* in the spinal cord of tail in adult gecko, the tissue sections from the sixth caudal vertebra were detected by *in situ* hybridization. The spinal cord in the tail showed similar structure to the spinal cord in the body. The results revealed that the majority of positive hybridization signals were present in the gray matter of spinal cord, while the minority were in the white matter (Figure 4). However, the cell lineage of the *noggin2*-positive cells was not determined, because cell markers were not stained simultaneously in the present study.
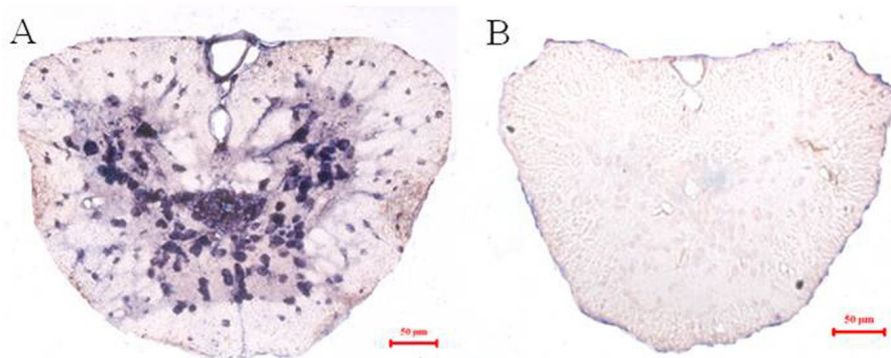
Figure 4. Representative results of *in situ* hybridization analyses of *noggin2* mRNA in the spinal cord of adult gecko. **A.** The majority of positive hybridization signals were present in the gray matter of spinal cord, while the minority were in the white matter. **B.** No signals were observed in the negative control with the sense probe. Scale bars represent 50 µm in A and B.

Real-time PCR analysis of *noggin2* expression in the spinal cord after tail amputation

Total RNA of the gecko spinal cord segments from the third to the fifth caudal vertebrae was used for real-time PCR assay to investigate the expression change of *noggin2* after tail amputation (1, 3, 7, 14 days). The expression level in the spinal cord of *noggin2* after tail amputation gradually decreased from the 3rd day up to 2 weeks (Figure 5).
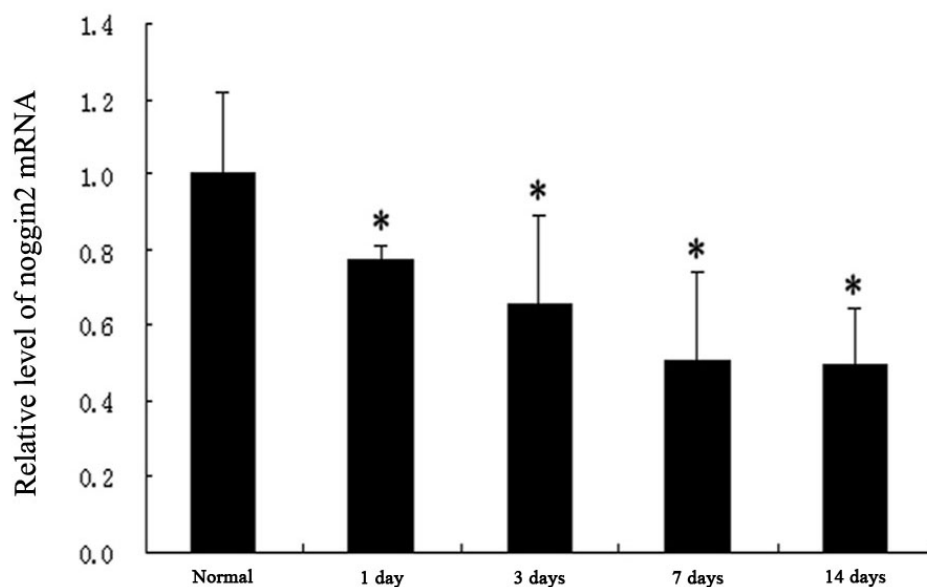
Figure 5. Expression level of *noggin2* in the spinal cord after tail amputation determined by real-time PCR. Time points selected for the experiment were normal control, 1, 3, 7, and 14 days, respectively (N = 5 for each group), after tail amputation. EF-1α was used for quantitative normalization. Data were from three independent experiments. Statistical analysis for significance was performed using one-way analysis of variance. *P < 0.05 compared to normal control.

Noggin protein has been reported to play an important role in neural induction and neural development (Lamb et al., 1993). The function of noggin, which mediates antagonism of BMP signaling, is required for growth and patterning of the neural tube and somite (McMahon et al., 1998). Therefore, noggin is considered to be a more promising neural inducer (Bachiller et al., 2000). The spinal cord regeneration of gecko is a complicated process including neural tissue genesis, and whether noggin2 plays a role in this process is an attractive topic. We cloned the cDNA of *noggin2* with complete open reading frame, and further investigated the expression of *noggin2* in spinal cord by real-time PCR after tail amputation. We found that its expression level was down-regulated during the first 2 weeks after amputation. This leads us to propose that *noggin2* may have certain functions in spinal cord regeneration. However, it is difficult to clarify the significance of the expression change of *noggin2* in our preliminary study now. First, we did not determine the type of cells

expressing noggin2 protein under normal conditions and after injury due to the absence of cell lineage markers in gecko, and this is an important issue to identify its function. Another concern of our study is that we are not sure of the time point at which neurogenesis begins, and the determination of expression level of *noggin2* at this stage may reveal its association with neural regeneration more definitely. Therefore, we will further investigate the temporal and spatial expression pattern of *noggin2* together with cell lineage marker after tail amputation, and this should provide valuable insight into understanding the function of *noggin2* during spinal cord regeneration in gecko.

ACKNOWLEDGMENTS

REFERENCES

Alibardi L (1995). Muscle differentiation and morphogenesis in the regenerating tail of lizards. *J. Anat.* 186: 143-151.

Aspenberg P, Jeppsson C and Economides AN (2001). The bone morphogenetic proteins antagonist noggin inhibits membranous ossification. *J. Bone Miner. Res.* 16: 497-500.

Bachiller D, Klingensmith J, Kemp C, Belo JA, et al. (2000). The organizer factors chordin and noggin are required for mouse forebrain development. *Nature* 403: 658-661.

Brockes JP (1997). Amphibian limb regeneration: rebuilding a complex structure. *Science* 276: 81-87.

Chernoff EA, Stocum DL, Nye HL and Cameron JA (2003). Urodele spinal cord regeneration and related processes. *Dev. Dyn.* 226: 295-307.

Echeverri K and Tanaka EM (2002). Ectoderm to mesoderm lineage switching during axolotl tail regeneration. *Science* 298: 1993-1996.

Egar M, Simpson SB and Singer M (1970). The growth and differentiation of the regenerating spinal cord of the lizard, *Anolis carolinensis*. *J. Morphol.* 131: 131-151.

Eroshkin FM, Ermakova GV, Bayramov AV and Zaraisky AG (2006). Multiple noggins in vertebrate genome: cloning and expression of noggin2 and noggin4 in *Xenopus laevis*. *Gene Expr. Patterns* 6: 180-186.

Fletcher RB, Watson AL and Harland RM (2004). Expression of *Xenopus tropicalis* noggin1 and noggin2 in early development: two noggin genes in a tetrapod. *Gene Expr. Patterns* 5: 225-230.

Fürthauer M, Thisse B and Thisse C (1999). Three different noggin genes antagonize the activity of bone morphogenetic proteins in the zebrafish embryo. *Dev. Biol.* 214: 181-196.

Kulessa H, Turk G and Hogan BL (2000). Inhibition of Bmp signaling affects growth and differentiation in the anagen hair follicle. *EMBO J.* 19: 6664-6674.

Lamb TM, Knecht AK, Smith WC, Stachel SE, et al. (1993). Neural induction by the secreted polypeptide noggin. *Science* 262: 713-718.

Liu Y, Ding F, Liu M, Jiang M, et al. (2006). EST-based identification of genes expressed in brain and spinal cord of *Gekko japonicus*, a species demonstrating intrinsic capacity of spinal cord regeneration. *J. Mol. Neurosci.* 29: 21-28.

McMahon JA, Takada S, Zimmerman LB, Fan CM, et al. (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* 12: 1438-1452.

Reddi AH (2001). Interplay between bone morphogenetic proteins and cognate binding proteins in bone and cartilage development: noggin, chordin and DAN. *Arthritis Res.* 3: 1-5.

Simpson SB Jr (1968). Morphology of the regenerated spinal cord in the lizard, *Anolis carolinensis*. *J. Comp. Neurol.* 134: 193-210.

Smith WC and Harland RM (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell* 70: 829-840.

Valenzuela DM, Economides AN, Rojas E, Lamb TM, et al. (1995). Identification of mammalian noggin and its expression in the adult nervous system. *J. Neurosci.* 15: 6077-6084.

Zimmerman LB, De Jesus-Escobar JM and Harland RM (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 86: 599-606.