Sciatic nerve crush evokes a biphasic TGF-β and Decorin modulation in the rat spinal cord

Kritis A[1], Kapoukranidou D[1], Michailidou B[2], Hatzisotiriou A[1], Albani M[1]

[1] Laboratory of Physiology, Department of Physiology and Pharmacology, Medical School, Aristotle University of Thessaloniki, Greece
[2] 2nd Department of Neurology, AHEPA University Hospital, Aristotle University, Thessaloniki, Greece

Abstract
Background and aim: Inherent property of the motoneurons of the peripheral nervous system is their ability to recover, at least in part, upon injury. To this end different factors are expressed and are thought to play important role in the regeneration processes. These factors are diverse, and range from transcription factors and chemokines, to molecules of the extracellular matrix. Transforming growth factor beta (TGF-β) is a protein with diverse actions controlling cell growth and proliferation. In the extracellular matrix it is found bound to decorin a proteoglycan involved in cell adhesion and cell signaling. In the present study we investigate the expression of TGF-β and decorin at different time points, in the regenerating sciatic nerve of a seven day old rat, having suffered nerve crush injury, over a period of one month.
Materials and methods: To achieve this, we evoked injury to male Wistar rats by exposing and applying pressure to the sciatic nerve using watchmaker's forceps. After that at 12h, 24h, 48h, 72h, one week, and one month intervals we investigated the gene expression of decorin using RT-PCR, and followed the expression of TGF-β molecule by immunohistochemistry in frozen sections of the L4-L5 region of the rat spinal cord.
Results: We report that both decorin mRNA and TGF-β protein exhibit a concerted, biphasic expression after 12 hours and one month having the animal suffered the nerve crush.
Discussion: Our data reveal a biphasic modulation of TGF-β protein and decorin mRNA expression at lumbar segment of the spinal cord of animals having suffered unilateral sciatic nerve crush. We postulate that their concerted expression both at an early and a late phase after the nerve injury is of importance and can be part of a repair or neuroprotective mechanism as yet unclarified. Hippokratia 2010; 14 (1): 37-41

Key words: TGF-beta, decorin, nerve crush, sciatic nerve, neurodegeneration

Corresponding author: Kritis A, Laboratory of Physiology, Medical School, Aristotle University, Thessaloniki, Greece, e-mail: kritis@med.auth.gr

Peripheral nerve injury elicits a complex response pertaining to both nerve degeneration and repair through mechanisms that are not fully understood yet. In neonatal rats the survival and development of motoneurons is dependent upon the functional interaction with their target muscles. Peripheral nerve injury during this period caused degeneration and loss of motoneurons[1,2]. In contrast to the central nervous system, mature neurons of the peripheral nervous system are able to survive and regenerate after injury. To this end different molecules such as cytokines and neurotrophins as well as molecules of the extracellular matrix are expressed and are thought to be part of a repair mechanism or act against nerve repair and axon regeneration[3-7]. The processes seem to be well coordinated since both the receptors for these molecules as well as their corresponding intracellular effectors' expression is modulated[4,8,9]. A central role to regulate the repair process was assigned to members of the TGF-β superfamily including TGF-β, activin and BMP which are all expressed by injured motoneurons[4,10-12]. In mammals TGF-β can be found in three isoforms TGF-β1, TGF-β2,

and TGF-β3 which are highly conserved among species[13]. TGF-β is a pleiotropic cytokine synthesized in a latent form which upon activation exerts its functions as a homodimer through three classes of receptors TβRI, TβRII and TβRIII found in most cell types[14]. Receptor activation is achieved when TGF-β binds to TβRII which then forms a heterodimer with TβRI which in turn is phosphorylated by the constitutively active kinase domain of TβRII. Thus, the molecular switch is set to "ON" and downstream signaling is initiated. The activated TβRI then interacts with the Smad proteins which in turn are classified into three classes depending on their structure and function. R-Smads are directly phosphorylated and activated by the activated TβRI, co-Smad (common partner) is Smad-4 and I-Smads exert inhibitory function competing with R-Smads for the activated TβRI receptor. Smads are then transferred to the nucleus were they interact with other signalling proteins to modulate the transcription of certain target genes[15-17]. In the extracellular matrix, TGF-β can be bound to biglycan, decorin, fibromodulin and betaglycan the later being a synonym for TRβIII. This

association of TGF-β with the proteins of the extracel-
lular matrix and specifically with decorin attenuates its
function by generating an extracellular reservoir for the
factor, or enhances its activity by helping to present TGF-
β to its receptors or even activates alternative TGF-β sig-
nal transduction pathways by TGF-β signalling through
lipoprotein-receptor related protein (LRP-1)[18-21]. Specifi-
cally the interaction of TGF-β with decorin is of interest
as decorin is a proteoglycan involved in cell adhesion and
cell signaling. Decorin belongs to a superfamily of small
leucine-rich proteoglycans (SLRPs) and it carries a single
glucosaminoglycan chain attached to its aminoterminus
and its core protein consists of 12x24-aminoacid leucine-
rich repeats. Furthermore, decorin is expressed in most
tissues and in the brain its expression is developmentally
regulated[22,23]. It binds to the insulin-like growth factor-I,
and interacts with TNF-α[24,25]. Furthermore, by binding to
the EGF receptor decorin activates the mitogen activated
protein kinase (MAP kinase) signaling pathways lead-
ing to p21 induction, and ultimately to cell cycle arrest[26].
Previous studies have decorin implicated in suppression
of tumor cell-mediated angiogenesis[27], attributed to it a
functional role in adult brain injury and repair[28] and dem-
onstrated that it promotes axonal growth across rat spinal
cord injuries[7].

In this study we monitored the expression of TGF-β
protein and decorin mRNA in the lumbar region of the
spinal cord of seven day old rats after unilateral injury of
the sciatic nerve over a period up to one month.

Methods
Nerve crush
Wistar rats were obtained from the Laboratory of
Physiology (Medical School, Aristotle University of
Thessaloniki, Greece). We complied with the guide-
lines for animal use established by the American Physi-
ological Society approved by the local ethical commit-
tee in accordance with EEC Council Directive 86/609.
The day of birth was counted as P0 (zero). Sciatic nerve
crush was performed on the left side at the 7th postnatal
day (P7) and successful axonotomy was certified post-
operatively by clinical tests with the right side serving
as control as described previously[29,30]. Only animals
in which successful axonotomy was verified were in-
cluded in our study. For each time point we divided the
animals into two groups, control and nerve crush. Each
group consisted of at least 7 animals and the TGF-β
and decorin expression was determined at 12 hours, 24
hours, 48 hours, 72 hours, 1 week and 1 month after
axonotomy.

Cardiac Perfusion
Control and operated animals, were deeply anaesthe-
tized and underwent the procedure of cardiac perfusion
with a fixative containing paraformaldehyde 4% in phos-
phate buffered saline (PBS). The spinal cord was then
removed, post-fixed for 4 hours in the same fixative and
finally stored in 30% sucrose at -40˚ C.

Immunochemistry
The L4-L5 region of the rat spinal cord was identi-
fied with the aid of a stereoscope. For identification, the
control dorsal horn (right side) was marked with a fine
micropin. Serial frozen sections of 4μm were cut with a
HM 505 E Microm cryostat, collected on superfrost plus
glass slides (Fisher Scientific, Pittsburgh, PA, USA) and
stored at -70˚ C.

For immuno-staining, the sections were microwave
fixed with 2% paraformaldehyde[31] incubated for 10 min-
utes in dual endogenous enzyme block solution, for 3
hours with the primary antibody (diluted to 1:400) ac-
cording to the vendors protocol. The antibody was a
monoclonal anti-TGF-β1 antibody with possible actions
for the other isoforms of TGF-β (NCL-TGFB, Novocas-
tra Laboratories Ltd Newcastle upon Tyne NE12 8EW,
U.K.). The sections were then processed using a horse-
radish peroxidase (HRP) conjugated secondary antibody
for 30 min at room temperature and developed using di-
amino benzidine (DAB) as a substrate.

mRNA extraction and RT-PCR analysis
Twelve, 24, 48, 72 hours, 1 week and 1 month af-
ter the nerve injury, the animals were sacrificed, and the
region of the spinal cord (L4 – L5) was excised. Total
RNA was extracted with the RNeasy Mini RNA extrac-
tion kit from Qiagen (Qiagen Greece BioAnalytica S.A.
Athens). Two μg of total RNA were subjected to reverse
transcription using 200 units of MMLV-RT (Promega
Greece SB Biotechnology Suppliers S.A. Athens) in 100
μl reaction volume, under standard conditions. Five μl of
the reverse transcription reaction mixture were subjected
to PCR amplification in 25 μl reaction volume, including
primers for the amplification of the beta-actin mRNA as
internal control for gene expression as described by oth-
ers[32,33]. Amplification started with an initial step of 5 min
denaturation at 94°C followed by 30 cycles, each cycle
consisted of denaturation at 94°C for 30 sec annealing at
55°C for 1 min and extension at 72°C for 1 min. Finally,
the PCR products were extended for 10 min at 72°C. Five
μl of each PCR reaction mixture were analyzed in a 1.7%
agarose gel. Visualization of DNA bands was achieved
with UV illumination of ethidium bromide-stained gels.
The intensity of the DNA bands was measured using the
software from Kodak Digital Science 1DTM.

Primers
Primers were designed to be specific for both rat and
human mRNA. For this purpose decorin mRNA sequnc-
es for R. rattus (Z12298), R.norvegicus (X59859) and
Homo sapiens (BC005322) were aligned using the Clust-
al W algorithm and the computing facilities of EBI http://
www.ebi.ac.uk/. The primers then were designed out of
regions of complete homology. For beta-actin sence 5´
ACACTGTGCCCATCTACGAGG 3´ and antisence 5´
AGGGGCCGGACTCGTCATACT 3´ providing a pcr
amplification product of 625 bp and for decorin sence 5´
ATGATTGTCATAGAACTGGGC 3´ and antisence 5´

ATTGTTGTTATGAAGGTAGAC 3´ with an amplicon of 385 bp.

Statistical Analysis

Non parametric data was analyzed using the Kruskal Wallis test and ANOVA. When significant, post hoc analysis Dunett test was used. The data is presented as mean ± SE of n ≥ 6 in each group. Results were considered significant with a probability z (p)<0.01 marked with(*).

Results

Animals successfully axonotomized lost reactive movements concerning the plantar- and the dorsi-flexion reflexes and the ability of normal movement of the left hindlimb when suspended by their tails. Impaired movement was evident up to two weeks after the nerve crush when gradual recovery was observed. By the end of one month the animals adopted an apparent normal walking pattern and reactive movements concerning the plantar- and the dorsi-flexion reflexes were restored. The ability of animals for normal movement of the left hindlimb when suspended by their tails was also restored albeit diminished in strength and velocity. Our data suggests that peripheral nerve injury to a 7 day old newborn rat can be repaired and nerve regeneration and reinervation of the muscles involved is achieved resulting in apparently normal movement.

In our study the expression of TGF-β isoforms was assessed by immuno-staining of lumbar sections of spinal cords of axonotomized and control animals. We detected a strong immuno-reactivity for the TGF-β isoforms specific for the motoneurons of operated animals 12 hours and 1 month after the nerve crush (Figure 1, panel A and B).

No immuno-reactivity for TGF-β was detected in any of the other groups or in the control animals. In the sections where motoneurons were positively stained for TGF-β, staining was observed both ipsilatelral and contralateral to the sciatic nerve crush.

Decorin mRNA was expressed at the L4 - L5 region of the rat spinal cord of the 7 day old rat albeit to a much
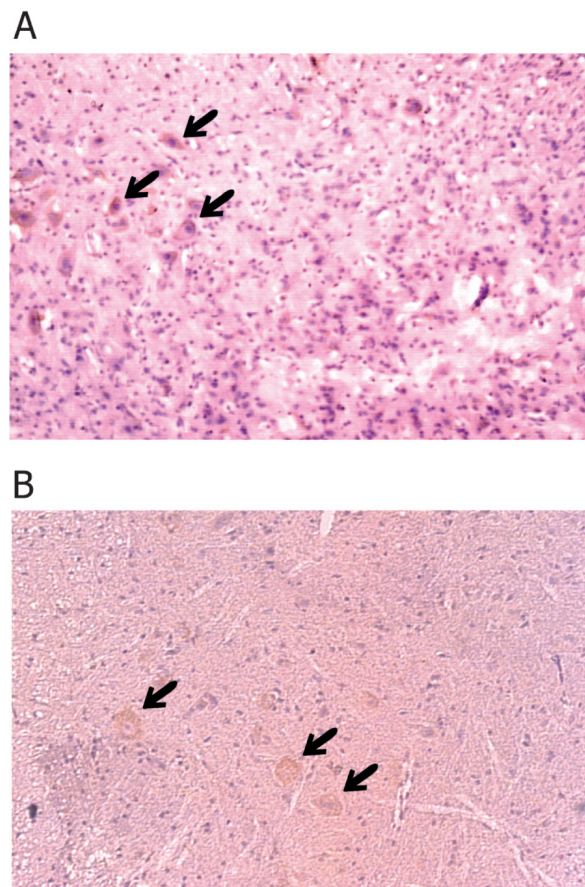

Figure 1: Immunohistochemical staining for TGF-β of spinal cord lumbar sections of animals having undergone sciatic nerve crush. Panel **A** 12 hours after the nerve crush and panel **B** one month after the nerve crush. Arrows indicate motoneurons expressing TGF-beta stained with anti-TGF-β antibody.

lower level compared to the expression of the beta-actin gene (Table 1).

Twelve hours after the unilateral sciatic nerve crush decorin mRNA levels increased significantly about three-

Table 1: Relative intensities of ethidium bromide stained PCR bands.

|	**C**	**12h**	**24h**	**48h**	**72h**	**1W**	**1M**
Rat 1	0.334013	1.027608	0.414493	0.485814	0.509921	0.412569	0.793991
Rat 2	0.37458	1.025733	0.452675	0.512758	0.535783	0.445701	0.806745
Rat 3	0.344025	1.027142	0.423949	0.492418	0.516262	0.420717	0.797141
Rat 4	0.323691	1.028089	0.404722	0.479035	0.503411	0.40419	0.790741
Rat 5	0.38937	1.025057	0.466509	0.522704	0.545324	0.457859	0.811388
Rat 6	0.359056	1.016068	0.433853	0.498485	0.521717	0.429359	0.794485
Mean	0.354934	1.026637	0.434228	0.499649	0.523202	0.429616	0.800572
Fold mRNA compared to control	1	2.892477	1.223406	1.407724	1.474085	1.210412	2.255553
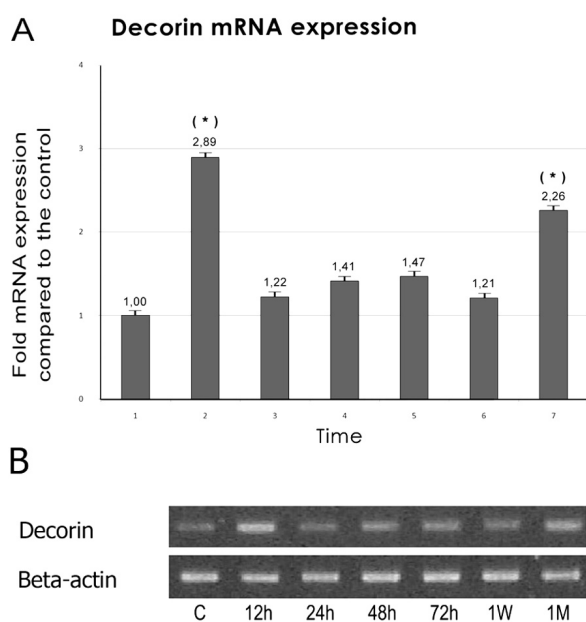
Figure 2: Expression of Decorin mRNA was monitored from 12 hours after the sciatic nerve crush and over a period of one month. (A) The relative mRNA signal intensity at each time point is compared with that of the control. Bars represent means ± SE. (*) Significant difference compared with the control, (p < 0.01) (B). Agerose gel electrophoresis of RT-PCR products for decorin and beta-avtin mRNA from injured animals at different time points after sciatic nerve crush.

fold compared to those of the control animals (p < 0.01). At later time points decorin mRNA expression returned to normal levels with small variations of no statistical significance. However one month after the nerve crush, the levels of decorin mRNA expression were again significantly elevated, (p < 0.01), about 2.5–fold compared to those of the control animals (Figure 2).

Discussion

Our data reveal a biphasic modulation of TGF-β protein and decorin mRNA expression at lumbar segment of the spinal cord of animals having suffered unilateral sciatic nerve crush. This modulation of expression occurs at two stages, an early one after 12 hours and a late one, 1 month after the axonotomy. Bearing in mind that these molecules are functionally related and both alone and together activate different signaling pathways we conclude that their concerted expression both at an early and a late phase after the nerve injury is of importance and can be part of a repair or neuroprotective mechanism not clarified yet.

It is well established that injury to the peripheral nerve triggers a complex, developmental stage specific, gene expression response, which regulates interplay of motor neuron death or survival. It has been reported that in spinal cord injuries inflammation is a key feature of this interplay, causing substantial secondary damage and thus undermin-

ing the processes of neuroregeneration[34,35]. In addition it has been reported that IL-10, a powerful anti-inflammatory cytokine, administered at the site of sciatic nerve crush reduced scar formation and permitted better regeneration of the damaged axons[36]. In this inflammatory response TGF-β is an important factor and it has been shown that inhibition of its expression by neutralizing antibodies at the site of sciatic nerve repair reduced scar formation but did not enhance nerve regeneration[37]. In contrast TGF-β inhibition by decorin over-expression in the rat brain induced severe inflammation and acute neuronal death[37]. Others reported that TGF-β is not a neurotrophic factor by itself rather it potentiated the action of neurotrophins either though interaction with other growth/differentiation factors[38] or by activating other signaling pathways such as the MAP Kinases pathway (MAPK/ERK/p38) that also has been reported to be activated in peripheral nerve injury[39,40].

On the other hand decorin has been shown to help reduce scar formation by inhibiting the expression of other chondroitin sulfate proteoglycans and promote axon growth by direct interaction with myelin in adult spinal cord injuries[7,41]. Our data is consistent with these reports and it would account for the late expression of decorin being involved in processes employed in remyelination. Additional implication of decorin in nerve repair comes from reports stating that the MAPK ERK pathway is activated in peripheral nerve after injury[39,40].

Our findings are in agreement with the above reports and clearly demonstrate that TGF-β and decorin expression is modulated as part of the response to sciatic nerve injury. This modulation of expression is biphasic and includes an early and a late stage consistent with the inflammatory process during which both cellular and exudative components are mobilized. It is of interest that these molecules are expressed in a concerted manner after the sciatic nerve crush. Both of these molecules activate different branches of the same pathway (MAPK) making it an important target for further investigation. The fact that TGF-β and decorin are functionally related both in a negative and a positive way and are expressed simultaneously implies that their function depends on the microenvironment created at the site of the injury or that it is exerted through other unidentified mechanisms as yet. Recent reports revealed that TGF-β required decorin for signaling through the large endocytotic lipoprotein related receptor-1[18]. It is of interest to elucidate the contribution of this pathway to nerve injury and repair and whether it is indeed activated upon of sciatic nerve injury. This would help identify the events following the injury and assign to both TGF-β and decorin specific places in the nerve repair processes.
Conflict of interest: none.

References

1. Lawson SJ, Lowrie MB. The role of apoptosis and excitotoxicity in the death of spinal motoneurons and interneurons after neonatal nerve injury. Neuroscience. 1998; 87: 337-348.
2. Lowrie MB, Vrbova G. Dependence of postnatal motoneurones on their targets: review and hypothesis. Trends Neurosci. 1992; 15: 80-84.
3. Navarro X, Vivo M, Valero-Cabre A. Neural plasticity after peripheral nerve injury and regeneration. Prog Neurobiol. 2007; 82: 163-201.
4. Snider WD, Zhou FQ, Zhong J, et al. Signaling the pathway to regeneration. Neuron. 2002; 35: 13-16.
5. Szpara ML, Vranizan K, Tai YC, et al. Analysis of gene expression during neurite outgrowth and regeneration. BMC Neurosci. 2007; 8: 100.
6. Hossain-Ibrahim MK, Rezajooi K, Stallcup WB, et al. Analysis of axonal regeneration in the central and peripheral nervous systems of the NG2-deficient mouse. BMC Neurosci. 2007; 8: 80.
7. Davies JE, Tang X, Denning JW, et al. Decorin suppresses neurocan, brevican, phosphacan and NG2 expression and promotes axon growth across adult rat spinal cord injuries. Eur J Neurosci. 2004; 19: 1226-1242.
8. Makwana M, Raivich G. Molecular mechanisms in successful peripheral regeneration. FEBS J. 2005; 272: 2628-2638.
9. Okuyama N, Kiryu-Seo S, Kiyama H. Altered expression of Smad family members in injured motor neurons of rat. Brain Res. 2007; 1132: 36-41.
10. Jiang Y, McLennan IS, Koishi K, et al. Transforming growth factor-beta 2 is anterogradely and retrogradely transported in motoneurons and up-regulated after nerve injury. Neuroscience. 2000; 97: 735-742.
11. D'Antonio M, Droggiti A, Feltri ML, et al. TGFbeta type II receptor signaling controls Schwann cell death and proliferation in developing nerves. J Neurosci. 2006; 26: 8417-8427.
12. Buss A, Pech K, Kakulas BA, et al. TGF-beta1 and TGF-beta2 expression after traumatic human spinal cord injury. Spinal Cord. 2008; 46: 364-371.
13. Clark DA, Coker R. Transforming growth factor-beta (TGF-beta). Int J Biochem Cell Biol. 1998; 30: 293-298.
14. Brand T, Schneider MD. Transforming growth factor-beta signal transduction. Circ Res. 1996; 78: 173-179.
15. Massague J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. EMBO J. 2000; 19: 1745-1754.
16. Miyazawa K, Shinozaki M, Hara T, et al. Two major Smad pathways in TGF-beta superfamily signalling. Genes Cells. 2002; 7: 1191-1204.
17. Wrana JL, Attisano L, Wieser R, et al. Mechanism of activation of the TGF-beta receptor. Nature. 1994; 370: 341-347.
18. Cabello-Verrugio C, Brandan E. A novel modulatory mechanism of transforming growth factor-beta signaling through decorin and LRP-1. J Biol Chem. 2007; 282: 18842-18850.
19. Takeuchi Y, Kodama Y, Matsumoto T. Bone matrix decorin binds transforming growth factor-beta and enhances its bioactivity. J Biol Chem. 1994; 269: 32634-32638.
20. Yamaguchi Y, Mann DM, Ruoslahti E. Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Nature. 1990; 346: 281-284.
21. Droguett R, Cabello-Verrugio C, Riquelme C, et al. Extracellular proteoglycans modify TGF-beta bio-availability attenuating its signaling during skeletal muscle differentiation. Matrix Biol. 2006; 25: 332-341.
22. Iozzo RV. Matrix proteoglycans: from molecular design to cellular function. Annu Rev Biochem. 1998; 67: 609-652.
23. Kappler J, Stichel CC, Gleichmann M, et al. Developmental regulation of decorin expression in postnatal rat brain. Brain Res. 1998; 793: 328-332.
24. Schonherr E, Sunderkotter C, Iozzo RV, et al. Decorin, a novel player in the insulin-like growth factor system. J Biol Chem. 2005; 280: 15767-15772.
25. Tufvesson E, Westergren-Thorsson G. Tumour necrosis factor-alpha interacts with biglycan and decorin. FEBS Lett. 2002; 530: 124-128.
26. Moscatello DK, Santra M, Mann DM, et al. Decorin suppresses tumor cell growth by activating the epidermal growth factor receptor. J Clin Invest. 1998; 101: 406-412.
27. Grant DS, Yenisey C, Rose RW, et al. Decorin suppresses tumor cell-mediated angiogenesis. Oncogene. 2002; 21: 4765-4777.
28. Stichel CC, Kappler J, Junghans U, et al. Differential expression of the small chondroitin/dermatan sulfate proteoglycans decorin and biglycan after injury of the adult rat brain. Brain Res. 1995; 704: 263-274.
29. Gougoulias N, Kouvelas D, Albani M. Protective effect of PNQX on motor units and muscle property after sciatic nerve crush in neonatal rats. Pharmacol Res. 2007; 55: 370-377.
30. Gougoulias N, Hatzisotiriou A, Kapoukranidou D, et al. Magnesium administration provokes motor unit survival, after sciatic nerve injury in neonatal rats. BMC Musculoskelet Disord. 2004; 5: 33.
31. Barsony J, Pike JW, DeLuca HF, et al. Immunocytology with microwave-fixed fibroblasts shows 1 alpha, 25-dihydroxyvitamin D3-dependent rapid and estrogen-dependent slow reorganization of vitamin D receptors. J Cell Biol. 1990; 111: 2385-2395.
32. Enomoto T, Fujita M, Cheng C, et al. Loss of expression and loss of heterozygosity in the DCC gene in neoplasms of the human female reproductive tract. Br J Cancer. 1995; 71: 462-467.
33. Jonson T, Mahlamaki EH, Karhu R, et al. Characterization of genomically amplified segments using PCR: optimizing relative-PCR for reliable and simple gene expression and gene copy analyses. Genes Chromosomes Cancer. 2000; 29: 192-199.
34. Fleming JC, Norenberg MD, Ramsay DA, et al. The cellular inflammatory response in human spinal cords after injury. Brain. 2006; 129: 3249-3269.
35. Yang L, Blumbergs PC, Jones NR, et al. Early expression and cellular localization of proinflammatory cytokines interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in human traumatic spinal cord injury. Spine. 2004; 29: 966-971.
36. Atkins S, Loescher AR, Boissonade FM, et al. Interleukin-10 reduces scarring and enhances regeneration at a site of sciatic nerve repair. J Peripher Nerv Syst. 2007; 12: 269-276.
37. Boche D, Cunningham C, Docagne F, et al. TGFbeta1 regulates the inflammatory response during chronic neurodegeneration. Neurobiol Dis. 2006; 22: 638-650.
38. Krieglstein K, Strelau J, Schober A, et al. TGF-beta and the regulation of neuron survival and death. J Physiol Paris. 2002; 96: 25-30.
39. Yongchaitrakul T, Pavasant P. Transforming growth factor-beta1 up-regulates the expression of nerve growth factor through mitogen-activated protein kinase signaling pathways in dental pulp cells. Eur J Oral Sci. 2007; 115: 57-63.
40. Agthong S, Kaewsema A, Tanomsridejchai N, et al. Activation of MAPK ERK in peripheral nerve after injury. BMC Neurosci. 2006; 7: 45.
41. Minor K, Tang X, Kahrilas G, et al. Decorin promotes robust axon growth on inhibitory CSPGs and myelin via a direct effect on neurons. Neurobiol Dis. 2008; 32: 88-95.