Temporospatial expression of fibulin-1 after acute spinal cord injury in rats

Guanhua Xu^{1†}, Ying Cui^{2†}, Lingling Wang¹, Jinlong Zhang¹, Aiguo Shen³, Weidong Li¹, Guofeng Bao¹, Yuyu Sun¹, Zhiming Cui¹

¹Department of Spine Surgery, The Second Affiliated Hospital of Nantong University, Nantong University, Nantong, China, ²Department of Orthopedics, General Hospital of Nanjing Military Region, Nanjing, China, ³Department of Immunology, Medical College, Nantong University, Nantong, China

Objective: Fibulin-1 is a matricellular protein that plays important roles in motility inhibition in a variety of cells and blocks the proliferation of cultured neural stem cells. The biological function of fibulin-1 in the spinal cord has not been fully elucidated.

Methods: To clarify the expressions and possible functions of fibulin-1 in spinal cord injury (SCI), we performed an acute spinal cord contusion injury model in adult rats. Our work studied the temporospatial expression patterns of fibulin-1.

Results: Western blot analysis revealed that fibulin-1 levels significantly increased 5 days after spinal cord contusion. Immunohistochemistry confirmed an increased number of fibulin-1 immunopositive cells about 2 mm from the lesion site. Moreover, double immunofluorescence labeling suggested that these changes were especially prominent in neurons and microglia.

Conclusion: These findings suggest that fibulin-1 may be involved in neuronal apoptosis and microglial activation after SCI.

Keywords: Fibulin-1, Glial proliferation, Neuron apoptosis, Rat, Spinal cord injury

Introduction

It is generally accepted that acute spinal cord injury (SCI) is a two-step process involving primary and secondary injury mechanisms.¹ Secondary injury that occurs in injured tissue in the hours and days after the initial insult affects both glia and neurons.^{2–4} Various theories have been proposed concerning the biochemical and cellular mechanisms of secondary SCI. It has been demonstrated that SCI can cause cell cycle activation in mitotic cells, such as astrocytes and microglia,⁵ which results in gliosis, microglial activation, and the formation of a glial scar at the site of injury. Microglia that are excessively activated will release large amounts of toxic substances and inflammatory cytokines. These factors aggravate the nerve damage and are thought to cause a pathological state of irreversible functional damage. Modern research suggests that

apoptosis, calcium signaling, and lipid peroxidation mechanisms also play important roles in the mediation of secondary SCI.^{6,7} There is widespread neuronal apoptosis after SCI, and it can occur hours after an injury to the central nervous system.⁸ However, the mechanisms of delayed post-traumatic neuronal apoptosis are not yet fully understood. Given that various components of the extracellular matrix (ECM) are known to regulate survival in the nervous system,^{9–12} some of the mechanisms may be related to changes in the composition of and signaling by the ECM.

Fibulin-1 belongs to a family of extracellular glycoproteins comprised of seven members named from 1 to 7. All fibulins contain a series of calcium-binding epidermal growth factor-like repeats module and a unique fibulin-type globular domain at its C-terminus.^{13,14} Moreover, fibulin-1 contains an additional protein domain at the N-terminus, which consists of three anaphylatoxin-like motifs and is related to inflammatory peptides released during complement system activation,¹⁵ and its C-terminus domain is variable

[†]These authors contributed equally to this work and should be considered co-first authors.

Correspondence to: Zhiming Cui, Department of Spine Surgery, The Second Affiliated Hospital of Nantong University, Nantong University, Nantong 226001, China. Email: cuizhimingmedsci@163.com

because of alternative splicing. Fibulin-1 is located in basement membranes, elastic fibers, and other connective tissue structures.^{16–19} It participates in diverse biological processes such as embryonic development, motility, tumor growth, and wound repair^{13,20,21} due to the ability of fibulin-1 to influence cell proliferation, migration, and differentiation through binding to receptors, growth factors, and various ECM proteins including fibronectin (FN), laminin, nidogen-1, endostatin, proteoglycans, aggrecan, and versican.^{13,22} It has been reported about that fibulin-1 is expressed in the brain and that it blocks the proliferation of primary cultured rat neural stem cells.²³ However, it is not known whether fibulin-1 expression is induced following acute SCI.

In the present study, we first investigated temporospatial patterns of fibulin-1 at the protein level and assessed its colocalization with active caspase-3 (a marker of apoptosis) and F4/80 (a marker of macrophages and monocytes) using an acute SCI model on adult rats. These experiments were conducted to gain greater insight into the functions of fibulin-1 and its roles in the cellular and molecular mechanisms underlying central nerve injury and repair.

Materials and methods

Animal experiments

Male Sprague Dawley rats weighting 200–225 g were used in the experiments. Male rats were used as a model of SCI to avoid complications due to estrogen production. The rats were anesthetized with pentobarbital at a dose of 50 mg/kg by intraperitoneal injection. Dorsal laminectomies were made at the level of the ninth thoracic vertebra (T9). Contusion injuries ($n = 60$) were performed using the NYU impactor. A 10 g weight and 2.0 mm diameter rod was released from a 25 mm height onto the exposed spinal cord. Sham-operated controls were laminectomized but were not injured. After SCI or sham surgery, sterile 3-0 silk sutures were used to close the overlying muscle and skin. Animals were allowed to recover on a 30°C heating pad. Post-operative treatments included saline (2.0 cc, subcutaneous (s.c.)) for rehydration and Baytril (0.3 cc, 22.7 mg/ml, s.c., twice daily) to prevent urinary tract infection. Bladders were manually expressed twice daily until reflex bladder emptying. All animal experiments were carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was approved by the institutional review board of the the Second Affiliated Hospital of Nantong University (Nantong, China).

Western blot analysis

Western blotting material was prepared from normal or from injured spinal cords at 6 hours, 1 day, 3 days, 5 days, 7 days, and 2 weeks after SCI ($n = 6$ for each time point). To obtain samples, the normal or injured spinal cords were excised and snap frozen at -70°C until use. The portions of spinal cord extending 5 mm rostral and 5 mm caudal to the lesion site were dissected. To prepare lysates, frozen spinal cord samples were minced with ophthalmic scissors in ice. The samples were then homogenized in lysis buffer (1% NP-40, 50 mM/l Tris, pH7.5, 5 mM/l ethylenediaminetetraacetic acid, 1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1% Triton-X100, 1 mM/l phenylmethylsulfonyl fluoride (PMSF), 10 $\mu\text{g}/\text{ml}$ aprotinin, and 1 $\mu\text{g}/\text{ml}$ leupeptin) and clarified by centrifuging 12 000 g for 20 minutes at 4°C . After determination of its protein concentration with the Bradford assay (Bio-Rad, Hercules, CA, USA), the resulting supernatant (50 mg/protein) was subjected to SDS–polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) using a transfer apparatus at 350 mA for 2.5 hours. The membrane was then blocked with 5% non-fat milk and incubated with primary antibody against fibulin-1 antibody (anti-mouse, 1:500; Santa Cruz Biotechnology (SCBT), Santa Cruz, CA, USA) or β -actin (anti-rabbit, 1:2000; Sigma, St Louis, MO, USA) at 4°C . After incubating with horseradish peroxidase-conjugated secondary antibody, protein bands were visualized using an enhanced chemiluminescence system.

Immunohistochemistry

At 1 day, 5 days, and 2 weeks after spinal cord contusion, animals were anesthetized with pentobarbital and perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Naive and injured spinal cords were removed and post-fixed overnight in 4% paraformaldehyde and then replaced with 20% sucrose for 2–3 days, followed by 30% sucrose for 2–3 days. The tissues were then embedded in optimal cutting temperature (OCT) compound, and 8 mm frozen cross sections, 2 mm rostral and caudal to the injury epicenter were prepared. Slide-mounted sections were removed from the freezer, incubated in an oven at 37°C for 30 minutes, and rinsed twice in 0.01 M PBS for 5 minutes. Endogenous peroxidase was quenched by incubating slides in 3% H_2O_2 /methanol for 5 minutes. The sections were rinsed in 0.01 M PBS for 3 minutes and then blocked with 10% goat serum with 0.3% Triton X-100 and 1% (w/v) bovine serum albumin (BSA) for

2 hours at room temperature (RT). Without further rinsing, sections were incubated overnight at 4°C with anti-fibulin-1 antibody (anti-mouse, 1:100; SCBT). After several rinses in 0.01 M PBS, the sections were reacted with a biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA) for 2 hours. Immunohistochemical staining was visualized with diaminobenzidine (Vector Laboratories) after rinses in 0.01 M PBS. Cells with strong or moderate brown staining were counted as positive, cells with no staining were counted as negative, and cells with weak staining were scored separately.

For double immunofluorescent labeling, sections were first blocked with 10% normal serum blocking solution containing 3% BSA and 0.1% Triton X-100 and 0.05% Tween-20 2 hours at RT. Then, the sections were incubated overnight with mouse monoclonal antibody for fibulin-1 (SCBT; 1:200), and different markers as follows: NF200 (neuronal marker, 1:500; Sigma), GFAP (astrocyte marker, 1:200; Sigma), CD11b (microglia marker, 1:500; AbD Serotec, Kidlington, UK), active caspase-3 (1:200; Sigma), and F4/80 (SCBT, 1:500) at 4°C. On the following day, tetramethylrhodamine isothiocyanate conjugated goat-anti-rabbit (1:50, Jackson ImmunoResearch, West Grove, PA, USA) and fluorescein isothiocyanate-conjugated goat-anti-mouse IgG (1:150; Southern Biotechnology Associates, SBA, Birmingham, AL, USA) were added in a dark room and incubated for 2–3 hours at 4°C. After three washes in PBS, the sections were immediately coverslipped with glycerol containing 1,4-diazabicyclo(2, 2, 2)octane (Sigma) and then observed on a Leica DM5000B fluorescence microscope (Wetzlar, Germany).

Quantitative analysis

The number of fibulin-1 positive cells in the spinal cord was counted in a $500 \times 500 \mu\text{m}^2$ area. For each animal, an area was defined in the dorsal horn, the lateral funiculus, and the ventral horn. We counted every fifth section ($50 \mu\text{m}$ apart) to avoid counting the same cell in more than one section. The cell counts were then used to determine the total number of fibulin-1 positive cells per square millimeter. We simultaneously quantified the percentage of cells that expressed fibulin-1. Cells double-labeled for fibulin-1 and the cell-type-specific markers NF200, GFAP, and CD11b were also quantified. To determine the proportion of cells that expressed each marker together with fibulin-1, we counted a minimum of 200 positive cells in both the white matter and gray matter in each section, with the exception NF200, for which we only counted cells in the gray matter. We then recorded the number of cells

that were double-labeled with fibulin-1. Two or three adjacent sections per animal were sampled 2 mm from the epicenter of the injury. All measurements were made by using the Leica QWin software (Leica Microsystems Inc., Buffalo Grove, IL, USA).

Statistical analysis

All statistical analyses were carried out using Stata 7.0 statistical software (StataCorp LP, College Station, TX, USA). One-way analysis of variance followed by the Tukey's *post hoc* multiple comparison tests was used for statistical analysis. The optical densities of immunoreactivities are represented as mean $\pm$ standard error of mean (SEM). P values < 0.05 were considered statistically significant. Each experiment consisted of at least three replicates per condition.

Results

Western blot analysis of fibulin-1 expression levels after SCI

We performed western blot analysis in controls and at various time points after SCI. The results of this analysis are shown in Fig. 1. Fibulin-1 protein levels were low in non-injured (naive) spinal cord, increased at 6 hours after SCI, and reached the highest level at 5 days ($P < 0.01$). Then, fibulin-1 expression gradually decreased for 2 weeks, but the level was still higher in the experimental groups than controls at 2 weeks after injury.

Temporospatial distribution of fibulin-1 immunoreactivity following SCI

To determine the distribution and temporal changes of fibulin-1 expression in contused spinal cord at different

Figure 1 Fibulin-1 expression following SCI. (A) Sample immunoblots probed for fibulin-1 and GAPDH. (B) The ratio of fibulin-1 to GAPDH expression at each time point. The data are means $\pm$ SEM ($n = 3$, * $P < 0.05$, significantly different from naive).

Figure 2 Immunohistochemistry for fibulin-1 2 mm rostral to the SCI epicenter. Low-power views of cross sections immunolabeled with a fibulin-1 antibody in non-injured (naive) spinal cord (A) and 5 days (D) and 2 weeks after injury (G). Higher power views in gray matter (B) and white matter (C) of naive spinal cord showing fibulin-1-positive nuclei. At 5 days after SCI, fibulin-1 reactivity was increased, and nuclear staining was decreased in gray matter (E) and white matter (F). Compared to 5 days, the numbers of fibulin-1-positive cells were decreased, and nuclear staining was decreased in gray matter (H) and white matter (I). Arrows indicate cells with weakly positive fibulin-1 nuclei but positive cytoplasm (E, F, H, and I). Scale bars: left column, 200 μm, middle and right columns, 40 μm. (J) Quantitative analysis of fibulin-1-positive cells in naive animals and 5 days and 2 weeks after SCI. Comparison of mean values of positive cells/mm² between naive and SCI animals. Fibulin-1 expression levels at 5 days and 2 weeks post-SCI were significantly different compared with the naive group. * $P < 0.05$ indicates significant difference compared with naive animals. Error bars represent SEM.

survival times, we performed immunohistochemistry studies. In controls, fibulin-1 was observed at low levels in both gray and white matter (Fig. 2). On day 1 after contusion, fibulin-1 expression was slightly increased in the rostral spinal cord 2 mm from the injury epicenter. At 5 days post-SCI, strong fibulin-1 immunoreactivity was present in cells with intense staining in both gray and white matter. At day 14 after contusion, fibulin-1 staining was decreased compared with 5

days but remained elevated relative to the control group (Fig. 2B). Because fibulin-1 peaked on day 5, changes in expression levels in areas distant from the lesion site were examined using day 5 animals. In axial sections, 2 mm rostral (Fig. 2) and caudal (data not shown) from the lesion site, fibulin-1 immunoreactivity was observed around the lesion site in both gray and white matter. In naive spinal cord, fibulin-1 was generally expressed in the nuclei and cytoplasm (Fig. 2A–C).

Figure 3 Double immunofluorescence labeling for fibulin-1 and different phenotype-specific markers in spinal cord. At 5 days after SCI, horizontal sections 2 mm from the epicenter were labeled with fibulin-1 (red) and different specific markers (green; NF-200, GFAP, and CD11b). Images from the gray matter (A–C) and white matter (D–I) are shown. (J) Quantitative analysis of different cell types expressing fibulin-1 (%) in naive spinal cord and 5 days after SCI. The changes in fibulin-1 expression after SCI were prominent in neurons and microglia but not in astrocytes. * $P < 0.05$ indicates significant difference compared with naive. Error bars represent SEM. Scale bars: 20 μ m.

However, the range of expression changed following SCI. Fibulin-1 was predominantly expressed in the cytoplasm on day 5 (Fig. 2E,F) and returned to normal in gray and white matter at 2 weeks (Fig. 2H,I).

Colocalization of fibulin-1 with different phenotype-specific markers after SCI

To determine which cell types were associated with peak fibulin-1 expression following contusion, we performed double immunofluorescent labeling. In gray matter, fibulin-1 expression overlapped with NF200-positive neurons in the ventral and dorsal horns (Fig. 3A–C).

In spinal cord white matter, fibulin-1 was clearly co-expressed in CD11b-positive microglia (Fig. 3D–F). However, only weak colocalization could be observed with fibulin-1 and glial fibrillary acidic protein (GFAP) (Fig. 3G–I). This finding indicates that there was no obvious change of fibulin-1 expression in the astrocytes of injured spinal cord.

Colocalization of fibulin-1 with pathological markers after SCI

It is well known that neuronal apoptosis occurs following severe spinal cord contusion in adult rats. There is

Figure 4 Double immunofluorescence staining for caspase-3 and fibulin-1 or NF200 in spinal cord. At 5 days post-SCI, tissue sampled 2 mm from the epicenter contained apoptotic neurons that were labeled with active caspase-3 and fibulin-1 (A–C) and NF-200 (D–F). Scale bars: 20 mm.

evidence that caspase-3 mRNA and protein expression levels are positively correlated with apoptosis after SCI.^{17,19} Thus, we used caspase-3 as an apoptotic marker to investigate whether delayed cell death occurred in fibulin-1-expressing cells at 5 days after contusion. First, merged images were generated to identify the types of the dying cells in spinal cord sections, and these results demonstrated colocalization of caspase-3 with NF200 (Fig. 4D–F), suggesting delayed neuronal death at 5 days after SCI. Second, we observed that fibulin-1/caspase-3-labeled cells were mainly neurons (Fig. 4A–C). These morphological observations suggest that up-regulation of fibulin-1 expression is correlated with delayed cell death in the process of secondary SCI.

Based on the colocalization of fibulin-1 and CD11b, we speculated that fibulin-1 is involved in the activation of microglia. Thus, F4/80 was used as a marker of activated microglia to investigate if fibulin-1 was expressed in activated microglia. As shown in Fig. 5, significant fibulin-1 expression was observed in many activated F4/80-positive microglia. These findings indicate that fibulin-1 might play a crucial role in the process of microglial activation.

Discussion

In the current study, temporospatial patterns of fibulin-1 expression were investigated after SCI in adult rats. We performed immunoblotting and double immunofluorescence and found that protein levels of fibulin-1 were significantly increased at day 5 after SCI, then gradually declined over a 2-week period. These changes were striking in neurons and microglia. In contrast, we observed lower expression of fibulin-1 in astrocytes, and expression was not affected by SCI. These findings indicate that the astrocytic ECM and the response of these cells to injury may be distinct from those of microglia and neurons. This is the first report demonstrating that fibulin-1 levels increased both in caspase-3-positive neurons and activated microglia in the injured rat spinal cord. These changes could be involved in secondary SCI pathogenesis. According to the immunohistochemistry results, fibulin-1 was expressed in the nuclei and cytoplasm of naive spinal cord cells; cytoplasmic staining peaked on day 5 and then gradually declined for 2 weeks. These results provide important clues to the biological functions of fibulin-1.

Figure 5 Double immunofluorescence staining for fibulin-1 (A) and F4/80 (B) in spinal cord. At 5 days post-SCI, sections were made 2 mm rostral to the epicenter. Colocalization is shown in (C). Scale bar: 20 mm.

Ohsawa *et al.*²⁴ reported that the secreted form of amyloid precursor protein (sAPP) stimulates the proliferation of rat neocortical neural stem cells. Their findings suggest that fibulin-1 inhibits sAPP-mediated proliferation of cultured neural stem cells via binding to the N-terminal domain of APP.²³ Also, a previous report by Kobayashi *et al.*²⁵ demonstrated that APP expression is up-regulated from day 1 and peaks 7 days after spinal cord hemisection. From the above evidence, we can speculate that the SCI-induced increase in APP expression stimulates the growth of nerve cells, and this effect can be suppressed by increased fibulin-1 following SCI. These pathways may be involved in irreversible spinal cord damage. We also showed the colocalization of fibulin-1 and caspase-3 in neurons, suggesting that fibulin-1 might be involved in the process of apoptosis during the secondary response after SCI. Previous studies have shown that caspase-3 participates in the downstream functions of the mitochondrial pathway and that the activation of caspase-3 might result in irreversible apoptosis.^{26,27} In a previous study, we found that the expression of RBMX, a nuclear ribonucleoprotein, was increased and colocalized with active caspase-3 in neurons after SCI.²⁸ These findings indicate that the regulatory networks involved in neuronal apoptosis after SCI are complex and utilize both extracellular and nuclear components.

Extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinase family that participates in numerous intracellular signaling pathways that can initiate reparative processes or cell death.²⁹ Curtis and Finkbeiner³⁰ have clearly demonstrated that ERK pathways promote cell survival and proliferation. It has been reported that ERK1/2 is activated in models of reperfusion and excitotoxic SCI,^{31,32} and ERK activation is involved in post-SCI neuronal survival.^{33,34} Fibulin-1 was previously established as a modulator of FN-stimulated ERK signaling, and it suppresses FN-regulated cell adhesion and motility. Furthermore, research by Selwyn *et al.* showed that fibulin-1 can alter ECM-mediated cell signaling through inhibiting ERK activation.³⁵ Collectively, these findings provide evidence that fibulin-1 plays a crucial role in neuronal apoptosis after SCI by blocking ERK signaling pathways in neurons.

Consideration of other known ligands of fibulin-1 leads us to propose that fibulin-1-mediated apoptosis may also occur through additional signaling pathways. One ligand of fibulin-1 is versican.³⁶ This proteoglycan is found in the ECM of many tissues, and recent work indicates that a cleaved form of versican regulates

apoptosis of tumor cells *in vitro*.³⁷ Moreover, in mouse embryos, the presence of versican fragments affects regression of the interdigital web.³⁸ Regression requires apoptosis and resorption of the ECM. Cleavage of versican is mediated by metalloproteases of the ADAMTS family, which are thought to require fibulin-1 as co-factors.³⁹ Studies *in vitro* indicate that the JNK and GSK-3 β pathways direct the cellular response to versican.³⁷

In response to certain stimuli, microglia cells assume an active phenotype associated with adhesion, proliferation, migration to the injury site, phagocytosis of cellular debris, and expression of both neurotoxic and neurotrophic factors.⁴⁰ If these cells are activated excessively, they release large amounts of toxic substances and inflammatory cytokines, both of which aggravate the nerve damage. Our study provides some of the first evidence that injury-evoked fibulin-1 is expressed in reactive microglia cells of the spinal cord. Moreover, our results also demonstrate that fibulin-1 is expressed in activated F4/80-positive microglia. Therefore, we conclude that fibulin-1 might also play a role in microglia activation after SCI.

In conclusion, our results demonstrate the expression profile of fibulin-1 after SCI. Based on these findings, we propose that fibulin-1 is involved in neuronal apoptosis and microglial activation. Further work is needed to elucidate how these processes affect post-SCI changes.

Disclaimer statements

Contributors GX and YC contributed equally to this work.

Funding This work was supported by the National Natural Science Foundation of China (No. 81271367).

Conflicts of interest None.

Ethics approval All animal experiments were carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was approved by the institutional review board of the Second Affiliated Hospital of Nantong University (Nantong, China).

References

- 1 Dumont RJ, Okonkwo DO, Verma S, Hurlbert RJ, Boulos PT, Ellegala DB, *et al.* Acute spinal cord injury, part I: pathophysiologic mechanisms. Clin Neuropharmacol 2001;24(5):254–64.
- 2 Crowe MJ, Bresnahan JC, Shuman SL, Masters JN, Beattie MS. Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. Nat Med 1997;3(1):73–6.

- 3 Liu XZ, Xu XM, Hu R, Du C, Zhang SX, McDonald JW, *et al.*, Neuronal and glial apoptosis after traumatic spinal cord injury. *J Neurosci* 1997;17(14):5395–406.
- 4 Young W. Secondary injury mechanisms in acute spinal cord injury. *J Emerg Med* 1993;11(Suppl 1):13–22.
- 5 Byrnes KR, Faden AI. Role of cell cycle proteins in CNS injury. *Neurochem Res* 2007;32(10):1799–807.
- 6 Lu J, Ashwell KW, Waite P. Advances in secondary spinal cord injury: role of apoptosis. *Spine (Phila Pa 1976)* 2000;25(14):1859–66.
- 7 Park E, Velumian AA, Fehlings MG. The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. *J Neurotrauma* 2004;21(6):754–74.
- 8 Jana A, Hogan EL, Pahan K. Ceramide and neurodegeneration: susceptibility of neurons and oligodendrocytes to cell damage and death. *J Neurol Sci* 2009;278(1–2):5–15.
- 9 Cheng IH, Lin YC, Hwang E, Huang HT, Chang WH, Liu YL, *et al.*, Collagen VI protects against neuronal apoptosis elicited by ultraviolet irradiation via an Akt/phosphatidylinositol 3-kinase signaling pathway. *Neuroscience* 2011;183:178–88.
- 10 Hadass O, Tomlinson BN, Gooyit M, Chen S, Purdy JJ, Walker JM, *et al.* Selective inhibition of matrix metalloproteinase-9 attenuates secondary damage resulting from severe traumatic brain injury. *PLoS ONE* 2013;8(10):e76904.
- 11 Hu J, Deng L, Wang X, Xu XM. Effects of extracellular matrix molecules on the growth properties of oligodendrocyte progenitor cells *in vitro*. *J Neurosci Res* 2009;87(13):2854–62.
- 12 Tassi E, Walter S, Aigner A, Cabal-Manzano RH, Ray R, Reier PJ, *et al.* Effects on neurite outgrowth and cell survival of a secreted fibroblast growth factor binding protein upregulated during spinal cord injury. *Am J Physiol Regul Integr Comp Physiol* 2007;293(2):R775–83.
- 13 Argraves WS, Greene LM, Cooley MA, Gallagher WM. Fibulins: physiological and disease perspectives. *EMBO Rep* 2003;4(12):1127–31.
- 14 de Vega S, Iwamoto T, Yamada Y. Fibulins: multiple roles in matrix structures and tissue functions. *Cell Mol Life Sci* 2009; 66(11–12):1890–902.
- 15 Kohl J. Anaphylatoxins and infectious and non-infectious inflammatory diseases. *Mol Immunol* 2001;38(2–3):175–87.
- 16 Pan TC, Sasaki T, Zhang RZ, Fassler R, Timpl R, Chu ML. Structure and expression of fibulin-2, a novel extracellular matrix protein with multiple EGF-like repeats and consensus motifs for calcium binding. *J Cell Biol* 1993;123(5):1269–77.
- 17 Reinhardt DP, Sasaki T, Dzamba BJ, Keene DR, Chu ML, Gohring W, *et al.* Fibrillin-1 and fibulin-2 interact and are colocalized in some tissues. *J Biol Chem* 1996;271(32):19489–96.
- 18 Roark EF, Keene DR, Haudenschild CC, Godyna S, Little CD, Argraves WS. The association of human fibulin-1 with elastic fibers: an immunohistological, ultrastructural, and RNA study. *J Histochem Cytochem* 1995;43(4):401–11.
- 19 Zhang HY, Timpl R, Sasaki T, Chu ML, Ekblom P. Fibulin-1 and fibulin-2 expression during organogenesis in the developing mouse embryo. *Dev Dyn* 1996;205(3):348–64.
- 20 Greene LM, Twal WO, Duffy MJ, McDermott EW, Hill AD, O'Higgins NJ, *et al.* Elevated expression and altered processing of fibulin-1 protein in human breast cancer. *Br J Cancer* 2003; 88(6):871–8.
- 21 Twal WO, Czirok A, Hegedus B, Knaak C, Chintalapudi MR, Okagawa H, *et al.* Fibulin-1 suppression of fibronectin-regulated cell adhesion and motility. *J Cell Sci* 2001;114(Pt 24):4587–98.
- 22 Timpl R, Sasaki T, Kostka G, Chu ML. Fibulins: a versatile family of extracellular matrix proteins. *Nat Rev Mol Cell Biol* 2003;4(6): 479–89.
- 23 Ohsawa I, Takamura C, Kohsaka S. Fibulin-1 binds the amino-terminal head of beta-amyloid precursor protein and modulates its physiological function. *J Neurochem* 2001;76(5):1411–20.
- 24 Ohsawa I, Takamura C, Morimoto T, Ishiguro M, Kohsaka S. Amino-terminal region of secreted form of amyloid precursor protein stimulates proliferation of neural stem cells. *Eur J Neurosci* 1999;11(6):1907–13.
- 25 Kobayashi S, Sasaki T, Katayama T, Hasegawa T, Nagano A, Sato K. Temporal-spatial expression of presenilin 1 and the production of amyloid-beta after acute spinal cord injury in adult rat. *Neurochem Int* 2010;56(3):387–93.
- 26 Sunkaria A, Wani WY, Sharma DR, Gill KD. Dichlorvos exposure results in activation induced apoptotic cell death in primary rat microglia. *Chem Res Toxicol* 2012;25(8):1762–70.
- 27 Ullah N, Naseer MI, Ullah I, Lee HY, Koh PO, Kim MO. Protective effect of pyruvate against ethanol-induced apoptotic neurodegeneration in the developing rat brain. *Neuropharmacology* 2011;61(8):1248–55.
- 28 Zhang J, Li D, Shen A, Mao H, Jin H, Huang W, *et al.* Expression of RBMX after spinal cord injury in rats. *J Mol Neurosci* 2013; 49(2):417–29.
- 29 Ding Q, Wang Q, Deng J, Gu Q, Hu S, Li Y, *et al.* Sevoflurane preconditioning induces rapid ischemic tolerance against spinal cord ischemia/reperfusion through activation of extracellular signal-regulated kinase in rabbits. *Anesth Analg* 2009;109(4): 1263–72.
- 30 Curtis J, Finkbeiner S. Sending signals from the synapse to the nucleus: possible roles for CaMK, Ras/ERK, and SAPK pathways in the regulation of synaptic plasticity and neuronal growth. *J Neurosci Res* 1999;58(1):88–95.
- 31 Shackelford DA, Yeh RY. Activation of extracellular signal-regulated kinases (ERK) during reperfusion of ischemic spinal cord. *Brain Res Mol Brain Res* 2003;115(2):173–86.
- 32 Yu CG, Yeziarski RP. Activation of the ERK1/2 signaling cascade by excitotoxic spinal cord injury. *Brain Res Mol Brain Res* 2005; 138(2):244–55.
- 33 Genovese T, Esposito E, Mazzon E, Muia C, Di Paola R, Meli R, *et al.* Evidence for the role of mitogen-activated protein kinase signaling pathways in the development of spinal cord injury. *J Pharmacol Exp Ther* 2008;325(1):100–14.
- 34 Yune TY, Park HG, Lee JY, Oh TH. Estrogen-induced Bcl-2 expression after spinal cord injury is mediated through phosphoinositide-3-kinase/Akt-dependent CREB activation. *J Neurotrauma* 2008;25(9):1121–31.
- 35 Williams SA, Schwarzbauer JE. A shared mechanism of adhesion modulation for tenascin-C and fibulin-1. *Mol Biol Cell* 2009;20(4): 1141–9.
- 36 Aspberg A, Adam S, Kostka G, Timpl R, Heinegard D. Fibulin-1 is a ligand for the C-type lectin domains of aggrecan and versican. *J Biol Chem* 1999;274(29):20444–9.
- 37 Du WW, Yang BB, Yang BL, Deng Z, Fang L, Shan SW, *et al.* Versican G3 domain modulates breast cancer cell apoptosis: a mechanism for breast cancer cell response to chemotherapy and EGFR therapy. *PLoS ONE* 2011;6(11):e26396.
- 38 McCulloch DR, Nelson CM, Dixon LJ, Silver DL, Wylie JD, Lindner V, *et al.* ADAMTS metalloproteases generate active versican fragments that regulate interdigital web regression. *Dev Cell* 2009;17(5):687–98.
- 39 Lee NV, Rodriguez-Manzanique JC, Thai SN, Twal WO, Luque A, Lyons KM, *et al.* Fibulin-1 acts as a cofactor for the matrix metalloprotease ADAMTS-1. *J Biol Chem* 2005;280(41): 34796–804.
- 40 Garden GA, Moller T. Microglia biology in health and disease. *J Neuroimmune Pharmacol* 2006;1(2):127–37.