

Neuronal glycosylation differentials in normal, injured and chondroitinase-treated environments

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Abstract

Glycosylation is found ubiquitously throughout the central nervous system (CNS). Chondroitin sulphate proteoglycans (CSPGs) are a group of molecules heavily substituted with glycosaminoglycans (GAGs) and are found in the extracellular matrix (ECM) and cell surfaces. Upon CNS injury, a glial scar is formed, which is inhibitory for axon regeneration. Several CSPGs are upregulated within the glial scar, including NG2, and these CSPGs are key inhibitory molecules of axonal regeneration. Treatment with chondroitinase ABC (ChABC) can neutralise the inhibitory nature of NG2. A gene expression dataset was mined *in silico* to verify differentially regulated glycosylation-related genes in neurons after spinal cord injury and identify potential targets for further investigation. To establish the glycosylation differential of neurons that grow in a healthy, inhibitory and ChABC-treated environment, we established an indirect co-culture system where PC12 neurons were grown with primary astrocytes, Neu7 astrocytes (which overexpress NG2) and Neu7 astrocytes treated with ChABC. After 1, 4 and 8 days culture, lectin cytochemistry of the neurons was performed using five fluorescently-labelled lectins (ECA MAA, PNA, SNA-I and WFA). Usually α -(2,6)-linked sialylation scarcely occurs in the CNS but this motif was observed on the neurons in the injured environment only at day 8. Treatment with ChABC was successful in returning neuronal glycosylation to normal conditions at all timepoints for MAA, PNA and SNA-I staining, and by day 8 in the case of WFA. This study demonstrated neuronal cell surface glycosylation changes in an inhibitory environment and indicated a return to normal glycosylation after treatment with ChABC, which may be promising for identifying potential therapies for neuronal regeneration strategies.

Keywords: Spinal cord injury, lectins, glycosylation, chondroitin sulphate proteoglycans, neurons, *in silico* analysis

1. Introduction

Complex carbohydrates are found ubiquitously throughout the CNS and are involved in many developmental and functional processes in the nervous system, including cell-cell and cell-ECM interactions, adhesion and axonal guidance and neuronal migration [1; 2]. *In vivo*, they interact with lectins, non-enzymatic carbohydrate-binding proteins of non-immune origin that precipitate glycoproteins or polysaccharides and agglutinate cells [3; 4]. The expression of carbohydrates and their corresponding lectins is differentially regulated both temporally and spatially in the developing CNS [1; 2; 5] and cell surface glycosylation is known to be altered during cell differentiation [6] and disease states such as cancer [7].

After CNS injury, a glial scar is formed at the injury site which creates an inhibitory environment for axonal regeneration and remyelination [8]. This scar contains several growth-inhibitory compounds including myelin-associated glycoprotein (MAG, siglec-4), Nogo, semaphorines and CSPGs, of which NG2, neurocan and versican are the major components [9; 10]. CSPGs are a group of molecules consisting of a protein core heavily substituted with covalently attached GAGs. CSPGs are normally found in the ECM and on cell surfaces, playing roles in barrier formation and axonal guidance [8].

Potential therapies for repair and regeneration after CNS injury include the manipulation or removal of GAGs from the injury site [8; 11] by treatment with ChABC [12]. Numerous studies have demonstrated that ChABC treatment promotes functional recovery and axon regeneration [11; 12; 13]. Perineuronal nets (PNNs) are composed of CSPGs and hyaluronic acid and encase neurons in the spinal cord. As ChABC digests GAGs, it has been hypothesised that digestion of the PNNs contributes to the recovery of plasticity, improving functional recovery after peripheral nerve injury [14]. In addition, dermatan sulfate disaccharide, one possible product of ChABC degradation of CS, promoted neurite outgrowth

in immortalised rat pheochromocytoma PC12 cells and primary cultures of hippocampal neurons and promoted neuronal survival *ex vivo* and *in vivo* [15].

Given the importance of glycosylation in the CNS, there has been little attention to glycosylation changes of neurons in the injured environment or to the effect of ChABC treatment upon neuronal glycosylation. A multidisciplinary approach was taken to address these questions. Initially, *in silico* mining of a publicly available gene expression dataset was done to verify that glycosylation-related genes were differentially regulated after spinal cord injury and to identify potential targets for further investigation. An *in vitro* model with PC12 cells was then used to examine glycosylation expression in simulated normal, injured and ChABC-treated environments.

The PC12 cell line differentiates into neuron-like cells upon treatment with neurotrophins [16], and has been extensively used as a model for studying neuronal functions and responses including neurite outgrowth [17; 18] and neuroprotective effects [19; 20]. PC12 cells were co-cultured with various astrocytes [17; 20] (normal primary astrocytes, Neu7 astrocytes and Neu7 astrocytes treated with ChABC) to model the different environments [21]. The Neu7 astrocytic cell line is an inhibitory cell line that has been engineered to overproduce NG2, versican and the CS-56 antigen [22] and is used to mimic the inhibitory environment which occurs following spinal cord injury [21; 22; 23]. The glycosylation expression profile of the PC12 cell surface was examined at intervals using lectin cytochemistry. Lectins bind specifically to distinct carbohydrate moieties, but may also contain one or more non-carbohydrate ligand sites [24]. Plant lectins have long been used as an analytical tool in tissue and cell histochemistry and particular carbohydrate motifs have been associated with otherwise indistinguishable cell types and stages of cell differentiation [4; 6; 25].

We present *in silico* mining and lectin profiling results of a healthy, injured and ChABC-treated model to profile cell surface glycosylation of neurons and attribute any glycosylation changes to the presence and removal of one class of glial scar inhibitory molecule, GAGs on CSPGs.

2. Materials and Methods

2.1 Materials

Culture trays, transwells and cell culture plastics were from BD Falcon. Lectins were purchased from EY Labs (CA, USA). ProLong Gold antifade was from Invitrogen (Biosciences, Dublin, Ireland). PC12 cells were from ECACC (Salisbury, UK). All other reagents were from Sigma Aldrich Co. (Dublin, Ireland) unless otherwise indicated, and were of the highest grade available.

2.2 Cell cultures and environmental models

PC12 cells were cultured on poly-L-lysine (PLL; 10 µg/ul for 3 h) coated coverslips in 12 well trays in Dulbecco's Modified Eagle's Medium (D-MEM, high glucose with L-glutamine) supplemented with 10% horse serum, 5% foetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S) at 37 °C in a 5% humidified CO₂ atmosphere. The medium was supplemented with nerve growth factor (50 ng/mL) at intervals of three days for PC12 cell differentiation.

Primary cerebral astrocytes were obtained from P2 Sprague Dawley rat pups, purified and cultured as previously described [21]. Neu7 astrocytes were cultured in D-MEM supplemented with 10% horse serum, 1% L-glutamine and 1% P/S.

For 'normal' condition simulation, PC12 cells were co-cultured with primary astrocytes. PC12 cells were seeded at 5,000 cells for 8 days *in vitro* (DIV), 10,000 cells for 4

DIV, and 50,000 cells for 1 DIV per PLL coated transwell in a 12 well tray. Astrocytes were seeded at the same density as above onto sterile coverslips in a 12 well tray. The PC12 cells were grown on the transwell to allow CSPGs secreted from the astrocytes grown in the same well to enter the media and interact with the PC12 cells, but not allow the two cell types to interact. For ‘injured’ condition, PC12 cells were grown with Neu7 cells. The ‘treated’ model consisted of PC12 cells grown with Neu7 cells with media treated with 0.1 unit/mL ChABC every 2 days, i.e. treated day 0 and every two days thereafter.

2.3 Lectin cytochemistry

Lectin cytochemistry at room temperature was performed on PC12 cells after growth at 1, 4 and 8 DIV as follows. Cells were fixed with 4% paraformaldehyde for 10 min and washed four times in 10 mM Tris-HCl, 1 mM CaCl₂, 1mM MgCl₂, pH 7.4 (TBS). Cells were blocked with 2% periodate-treated [26] bovine serum albumin (BSA) in TBS for 30 min. The cells were washed four times in TBS and then incubated with fluorescein isothiocyanate (FITC)-labelled lectins (Table 1) for 1 h in the dark at the following concentrations: SNA-I, MAA, PNA and ECA at 20 µg/mL and WFA at 10 µg/ml in TBS. Inhibitory controls were also carried out in parallel by preincubation of the lectins with 100 mM concentrations of the appropriate haptenic carbohydrates in TBS for 1 h prior to cell staining as follows: SNA-I, MAA and PNA were prepared in lactose, ECA in galactose (Gal) and WFA in *N*-acetylgalactosamine (GalNAc) and staining was carried out in the presence of the sugar. The cells were washed twice in TBS, counterstained with 1 µg/mL DAPI in TBS for 5 min, washed four times in TBS and mounted on glass slides with a drop of ProLong Gold antifade.

2.4 Image and statistical analysis

Cells were imaged on an Olympus IX81 fluorescent microscope using Perkin-Elmer Velocity[®] image acquisition software. Observed intensity of staining for PC12 cells were

tabulated using a scale of no binding (-), slight binding (+), moderate binding (++) , intense binding (+++) and very intense binding (++++).

2.5 *In silico data mining*

A gene expression dataset of the response of rat motor neurons 0, 2, 7, 21 and 60 days after spinal cord injury (GeneChip rat genome 230 3.0 array, Affymetrix Inc., Santa Clara, CA) was downloaded from the Gene Expression Omnibus (accession number GSE19701, www.ncbi.nlm.nih.gov/geo/) [27]. Gene expression analyses were performed using GeneSpring 11.5 (Agilent Technologies, Cork, Ireland) and further detailed in supplementary data.

3. Results

3.1 *In silico analysis*

In silico mining was done on a study of gene expression response of rat motor neurons following spinal cord injury [27]. After statistical and gene expression analyses, 6,790 out of 31,099 probes were identified as having a 1.2-fold change above or below control day 0. From these probes, glycosylation-related genes were extracted and genes associated with the sialic acid pathway, glycosaminoglycans and chondroitin sulfate proteoglycans, galactose and lectins, including galectins, were differentially regulated and their products have been reported to have altered expression after spinal cord injury [9; 28; 29; 30] (see supplementary data). Neuronally expressed β -galactoside α -2,3-sialyltransferase (ST) genes were both up- and down-regulated after injury, with two of the initially up-regulated post-injury genes down-regulated again at 21 days post-injury. Only one α -2,6- and one α -2,8-ST was extracted and both were down-regulated at all timepoints post-injury (Fig.

1A). *N*-acetylgalactosaminyltransferases (GalNAcTs) and galactosyltransferases (GalTs) were both significantly up- and down-regulated post-injury, with one GalNAcT going from up-to down-regulated at 7 days post-injury and returning to up-regulated by 21 days. One GalT (a β -1,3GalT) went from initial down-regulation at day 2 to subsequent up-regulation at all later timepoints and conversely, the only significant α -1,3GalT (AI178222) went from initial up-regulation to down-regulation from 7 days onwards (Fig. 1B).

C-type lectin family genes were all upregulated at all time points, confirming the presence of carbohydrate recognition domains on cell surface. Galectins are β -galactoside binding lectins [1; 31]. The majority of extracted galectin genes were up-regulated at all timepoints following injury except for a soluble galectin-3 (NM_031832), which was down-regulated at day 7 post-injury but upregulated at all other timepoints, and a galectin-related protein (NM_057187), which was downregulated at all timepoints but upregulated at day 7 post-injury (Fig. S-1, supplementary).

3.2 *In vitro* profiling

The cell surface glycosylation changes of differentiated PC12 cells was analysed after 1, 4 and 8 DIV using fluorescently-labelled lectins selected to detect sialic acid-, galactose- and chondroitin sulfate-related motifs. The intensity of staining was recorded and tabulated (Table 2). All lectin cytochemistry was also carried out in parallel in the presence of haptenic sugars. A reduction in binding intensity was noted in these cases (not shown), demonstrating that lectin binding was carbohydrate-mediated [25].

Slight staining of PC12 cells with ECA was observed at 1 and 8 days in the ‘healthy’ primary astrocyte co-culture (Figures 2A and G, respectively), but increased staining intensity was observed at 4 days (Fig. 2D and Table 2). Cells grown in the ‘inhibitory’ environment (Fig. 2B) showed more intense staining than cells growing in a normal astrocyte

environment at 1 DIV, and maximum staining intensity was reached at 8 DIV (Fig. 2H), delayed compared to the 'healthy' environment. The ChABC-treated group showed no change in intensity of staining over time. At 1 DIV in the 'treated' environment, the staining intensity was moderate and highest intensity was observed on the cell surface, at 'caps' on the cells (Fig. 2C). At 4 DIV, the intensity remained moderate but was dispersed throughout the cell and was increased by 8 DIV, comparable to 4 DIV in the normal environment (Fig. 2F, 2I and Table 2).

PNA staining of PC12 cells was observed under all conditions, at all timepoints, differing in intensity and staining dispersal and was similar to that of ECA for normal condition. Cells grown in 'normal' and 'treated' conditions stained slightly and very slightly, respectively, at 1 DIV. The intensity increased by day 4 and decreased again to moderate binding by day 8. Day 4 of 'normal' co-culture cells appeared to have maximal binding at the cell surface, especially where cells touched one another (Fig.S-2 and Table 2). In contrast, cells grown in 'injured' conditions only reached maximal intensity by day 8.

In the 'normal' co-culture group, WFA bound with moderate intensity to PC12 cells at 1 DIV, with greatest intensity observed where cells touched one another. Binding intensity decreased to slight binding at day 4 and increased to very intense at 8 DIV, in common with both injured and treated environments (Fig. S-3 and Table 2). The binding intensity variation of the injured and treated environments were similar, with very slight intensity binding of WFA at 1 DIV, slightly increased binding at day 4 and greater intensity observed where cells touched one another in the 'injured' environment (Fig. S-3E), and moderate to intense binding at day 8. The slight binding intensity observed at 4 DIV in the 'injured' co-culture environment was most apparent at the point where the cells touched or at the cell surface.

MAA stained PC12 cells under all conditions at each timepoint, and staining intensity increased over time reaching greatest intensity at 8 DIV for all environments (Fig. S-4 and Table 2).

SNA-I did not stain the PC12 cells at any timepoint or condition (Fig. 3A-G, 3I), except at 8 DIV (Figure 3H and Table 2) in the ‘injured’ co-culture group where cell bodies and neurites were moderately stained.

4. Discussion

The retrieved genes from the *in silico* analysis verified that the expression of glycosylation-related genes was altered at various timepoints post-injury and identified STs, GalNAcTs, GalTs and galectins as potential targets for differential expression in injury conditions. The *in vitro* model used to simulate normal, injured and ChABC-treated environments suggested that neuronal glycosylation changes occurred in these conditions.

The lectin ECA binds with greatest affinity to unsialylated terminal *N*-acetyllactosamine (LacNAc) structures, which are a major component of glycoprotein *N*-linked oligosaccharides and glycolipids (Table 1). Zhang, *et al.* [28] found that ECA binding increased after brain injury in mice, correlating to our observations at 8 DIV in the ‘injured’ environment. ECA staining where the cells touch may indicate a role for this carbohydrate motif in cell-cell adhesion. Galectins are involved in cell-cell interactions, adhesion, differentiation, apoptosis and axonal guidance [1; 31], and bind to motifs elucidated by ECA. The majority of galectins with altered expression post-injury were upregulated at all timepoints. Expression of the potential galectin receptor evidenced by ECA binding seemed to be time-dependent, and reached a maximum in the ‘healthy’ culture at day 4, which may in turn reflect temporal expression of galectins in the CNS. However, expression of this receptor

appeared to be delayed in the injured environment as maximum expression was at day 8, in common with the treated environment.

In common with ECA, PNA also stained at areas of cell-cell contact and staining intensity was temporally regulated, except for the treated environment which was more similar to the healthy condition rather than the injured (Table 2). In the *in silico* analysis, all significant β -GalTs were upregulated by day 7 post-injury, which may correlate with the intense PNA and ECA staining of the PC12 cells at 8 DIV.

WFA lectin is commonly used as a marker for PNNs [11; 32]. In all three culture conditions maximal WFA intensity was noted at the latest timepoint, and was most intense where the cells touched, which may indicate a role for the elucidated motif in adhesion. However, by day 7 post-injury, only one β 1,4GalNAcT (BF561001) was upregulated in the *in silico* analysis but the majority of CSPG-related significant genes were upregulated at all timepoints post-injury (supplementary data).

Sialic acids are the mainly terminal residues of complex *N*- and *O*-linked oligosaccharides of glycoproteins and glycolipids and comprise polysialic acid in an α (2,8)-linkage on neural cell adhesion molecule (NCAM). Sialylated motifs on the cell surfaces of vertebrates are involved in cell-cell communication, development and adhesion [33], interacting with siglecs and galectins [31]. The lectins MAA and SNA-I have binding specificity for α (2,3)- and α (2,6)-linked sialic acid, respectively, and MAA is also known to bind to Gal-3-SO₄ [34] (Table 1). In the brain, the occurrence of α (2,3)-linked sialic acid is predominant with little to no α (2,6)-linkage expected [2]. SNA-I binding was observed in the ‘inhibitory’ environment at 8 DIV, but was not seen in the ChABC-treated condition, where cell glycosylation was comparable to the ‘normal’ environment. The altered expression of significant α 2,6- and α 2,3-STs post-injury did not correlate with staining intensities, and interestingly, expression of the α 2,8-ST, relevant to PSA synthesis, was downregulated at all

timepoints. Electrical signaling in neurons, skeletal muscle cells and cardiomyocytes is modulated by the sialic acid content of particular isoforms of ion channels [35]. Altered or aberrant sialic acid expression could impact neuron polarization [35], which may be consistent with altered excitability of neurons post-injury [33]. Exposure of $\alpha(2,6)$ -linked sialic acid and binding to SNA-I has been observed on apoptotic and necrotic cells [36], and $\alpha(2,6)$ -sialylation has been identified as blocking binding to galectins, hence functioning as a biological ‘off switch’ [31]. Interestingly, the expression of $\alpha 2,6$ -ST was downregulated at all timepoints post-injury while galectin-1 expression, which has been associated with pathogenesis in the injured spinal cord [30], was upregulated.

ChABC treatment promotes functional recovery and reduces the inhibitory effect of CSPGs but anatomical regeneration post-treatment is limited [28]. This may be due in part to the potentially immunogenic carbohydrate ‘stub’ structures created by ChABC action [13]. The NG2 protein core has also been suggested to have an inhibitory effect on axonal growth [9]. However, in the model system, treatment with ChABC appeared to be successful in returning the neuronal glycosylation to normal conditions at all timepoints in the case of MAA, PNA and SNA-I staining, and by day 8 in the case of WFA.

A multidisciplinary approach allowed the *in silico* verification of differentially regulated glycosylation-related genes in neurons and targeting of altered carbohydrate motifs. The model system demonstrated neuronal cell surface glycosylation changes in an inhibitory environment and may be the first indication of the occurrence of abnormal sialylation in an injured environment. The limited number of cell types in a controlled environment can help attribute any changes to specific molecules and their degradation products alone, and help fit this information into a wider injury picture. In addition, this model indicated a return to normal neuronal glycosylation after treatment with ChABC which may be promising for identifying points of intervention or potential therapies for neuronal regeneration strategies.

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Figure and Table legends

Fig. 1. Up- and down-regulated glycosylation related genes compared to control day 0. **(A)** Sialic acid pathway related genes where the accession numbers of β -galactoside α -2,3-STs are blue, α -2,6-ST is red, α -2,8-ST is green and others which include sialic acid transporters are black. **(B)** Galactose-related genes where the accession numbers of polypeptide GalNAcTs are black, GalNAcTs are blue and GalTs are red. See supplementary data for full assignment of GenBank accession numbers.

Fig. 2. Photomicrographs of ECA-FITC stained PC12 cells at 1 DIV (A-C), 4 DIV (D-F) and 8 DIV (G-I) co-cultured with primary astrocytes ('normal'), Neu7 cells ('inhibitory') and Neu7 cells treated with ChABC ('treated'), respectively. Scale bar = 30 μ m.

Fig. 3. Photomicrographs of SNA-I-FITC stained PC12 cells at 1 (A-C), 4 (D-F) and 8 DIV (G-I) co-cultured with primary astrocytes ('normal'), Neu7 cells ('inhibitory') and Neu7 cells treated with ChABC ('treated'), respectively. Scale bar = 30 μ m.

Table 1. Lectins and their corresponding carbohydrate binding specificity.

Table 2. Intensity of lectin binding to PC12 cells. 'Normal' condition was simulated by PC12/ primary astrocyte co-culture, 'Injured' condition by PC12/ Neu7 astrocyte co-culture and 'Treated' condition by treatment of media of PC12/ Neu7 astrocyte co-culture with ChABC.

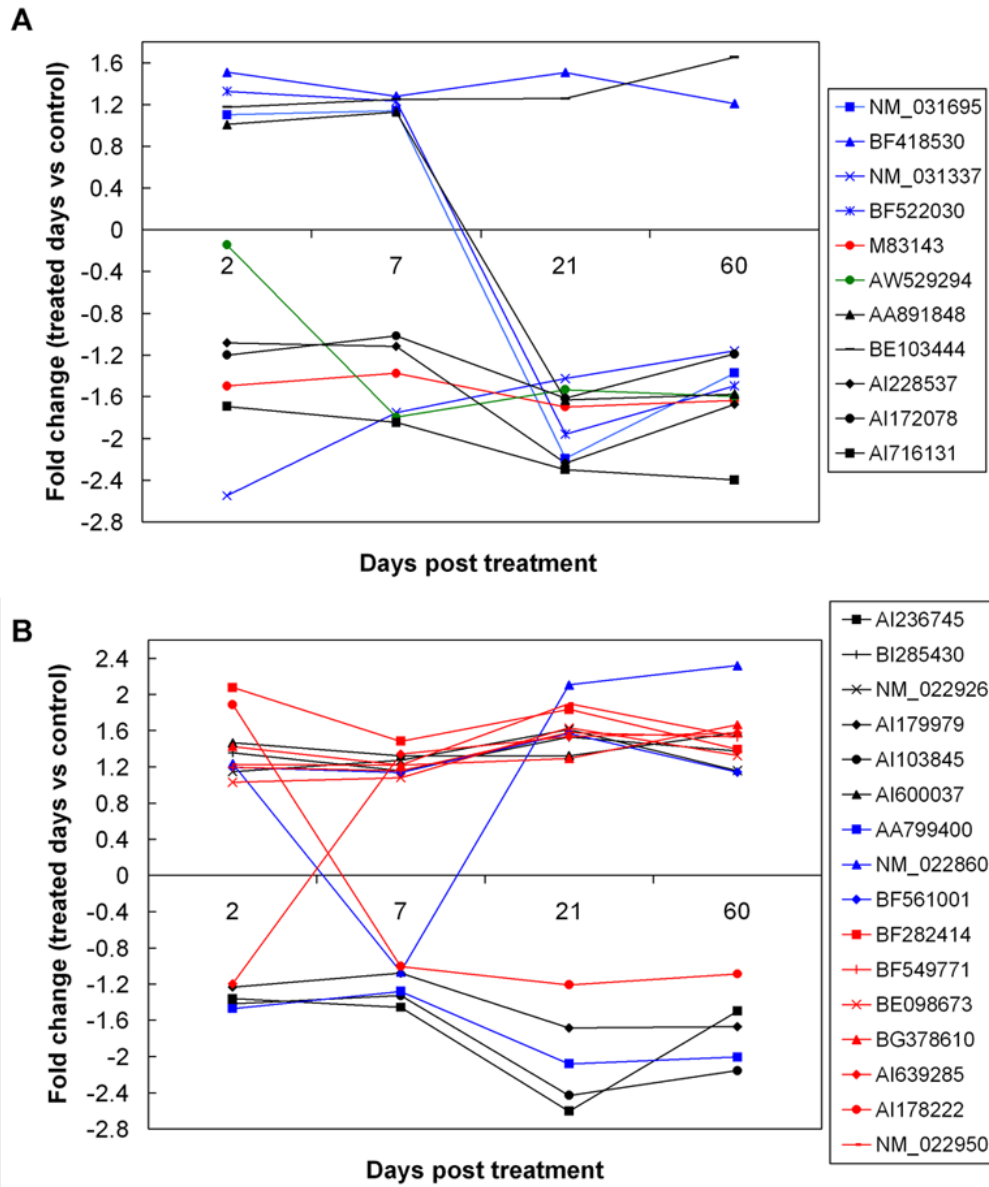


Figure 1

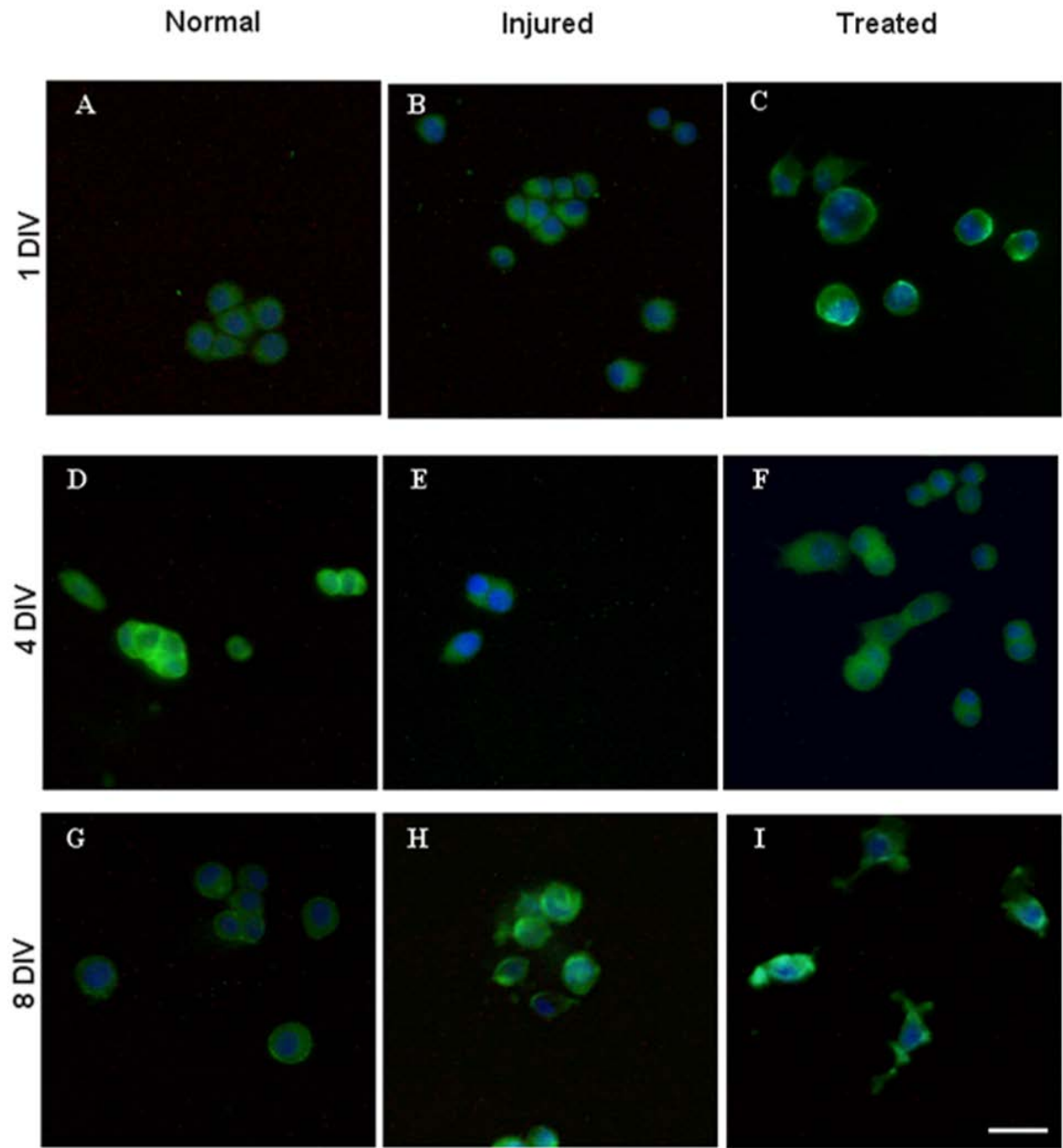


Figure 2

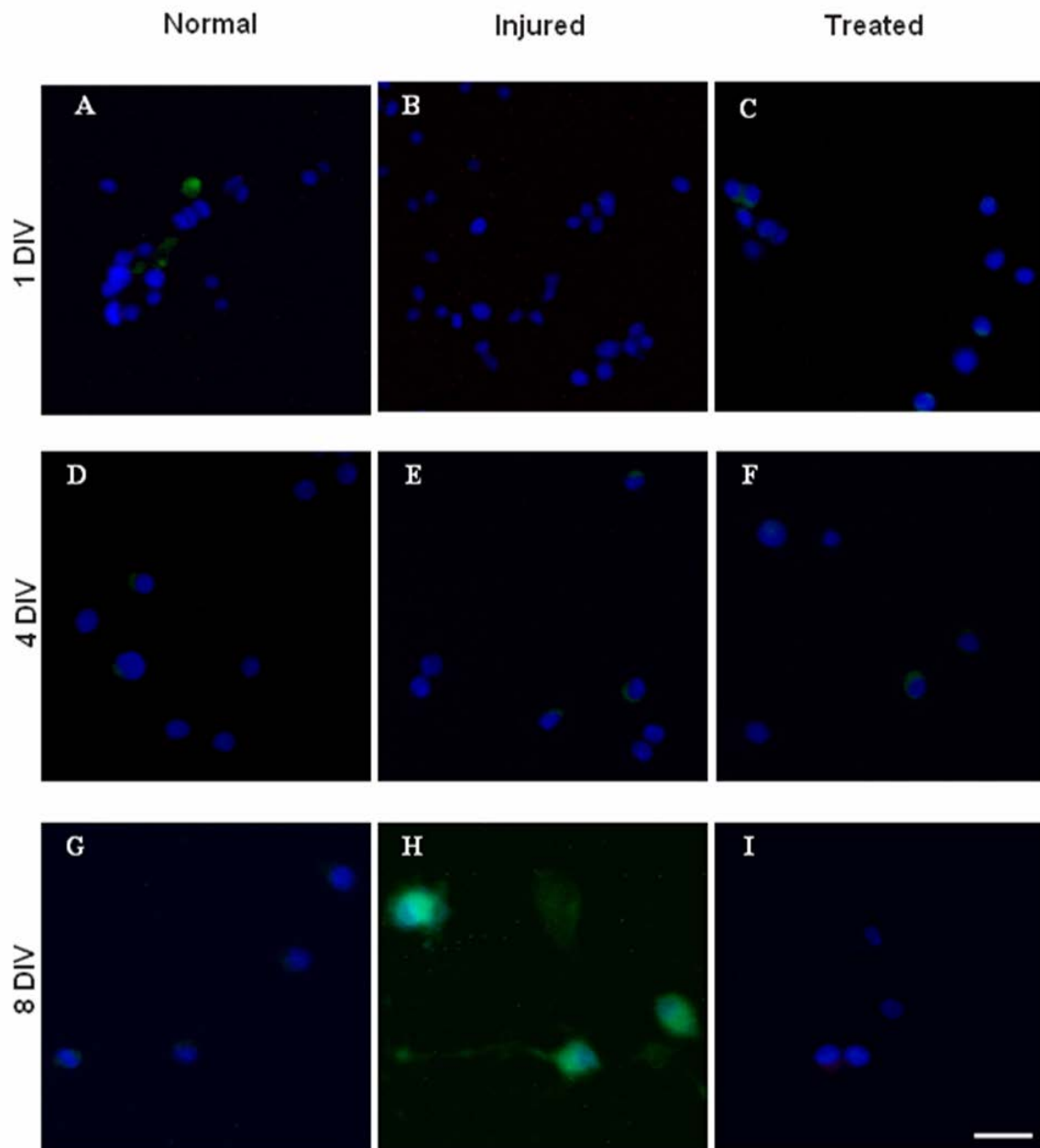


Figure 3

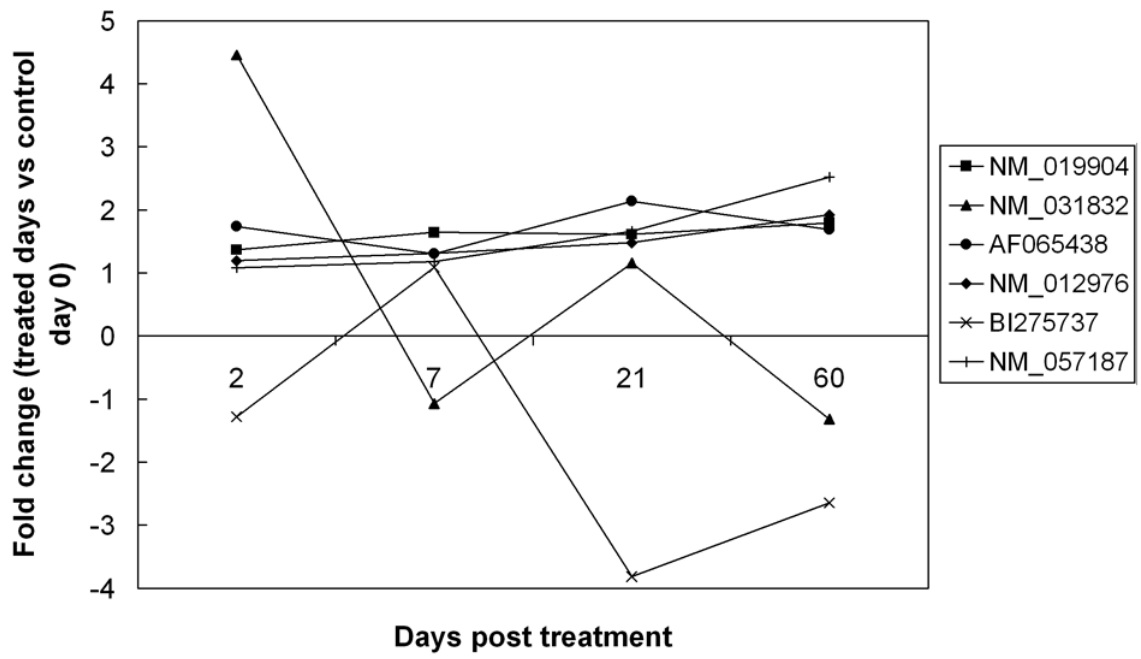


Figure S1

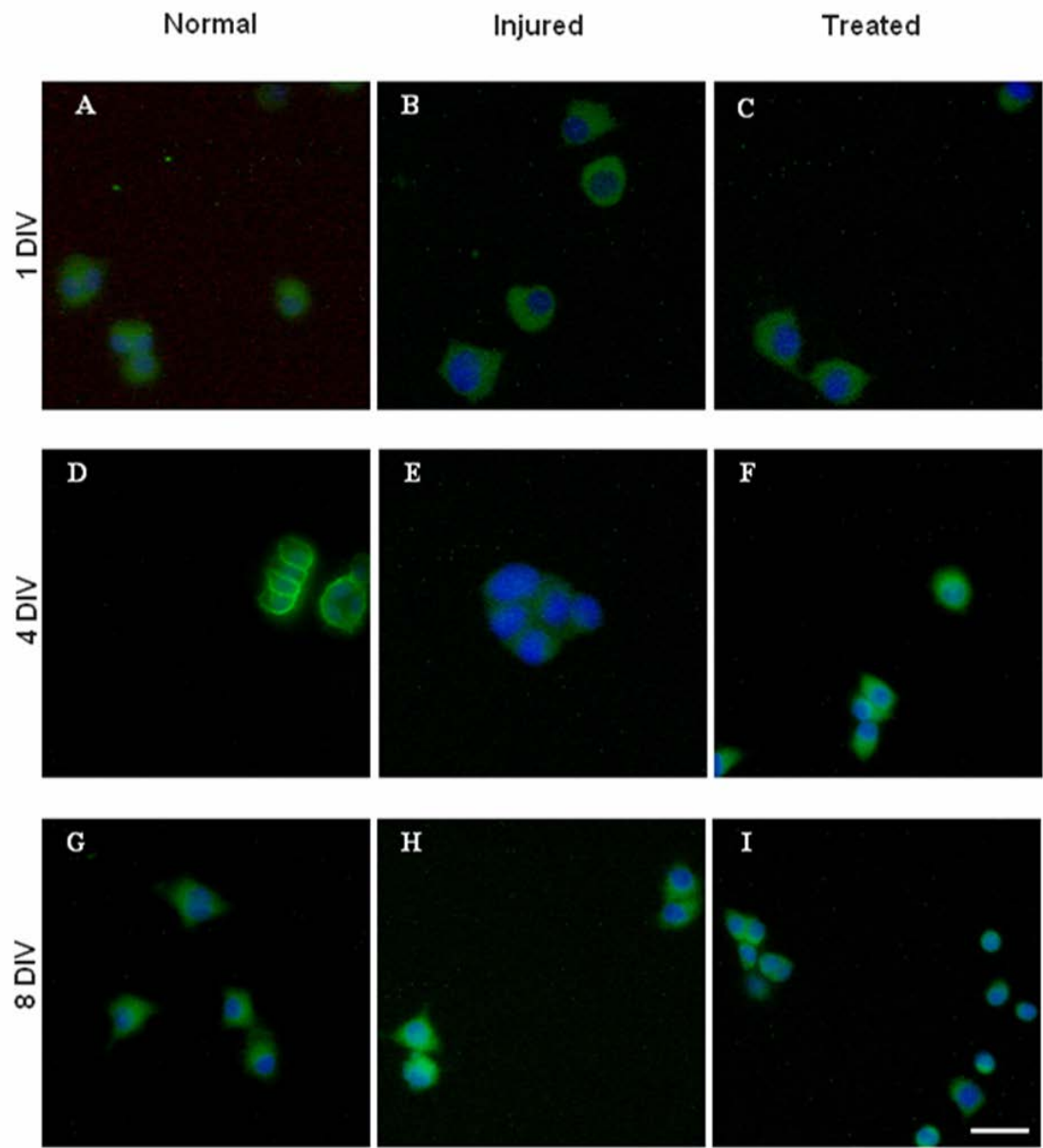


Figure S2

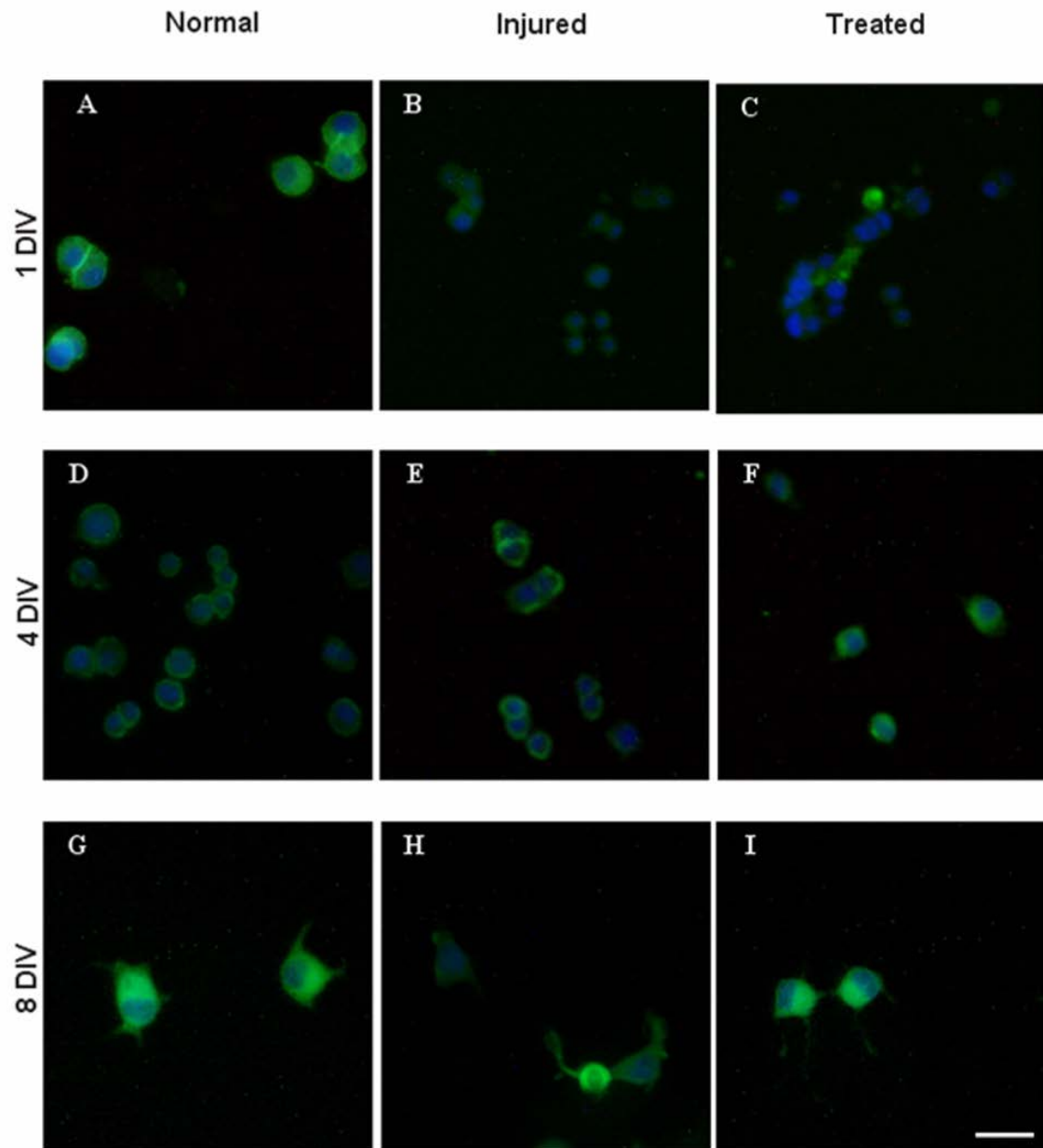


Figure S3

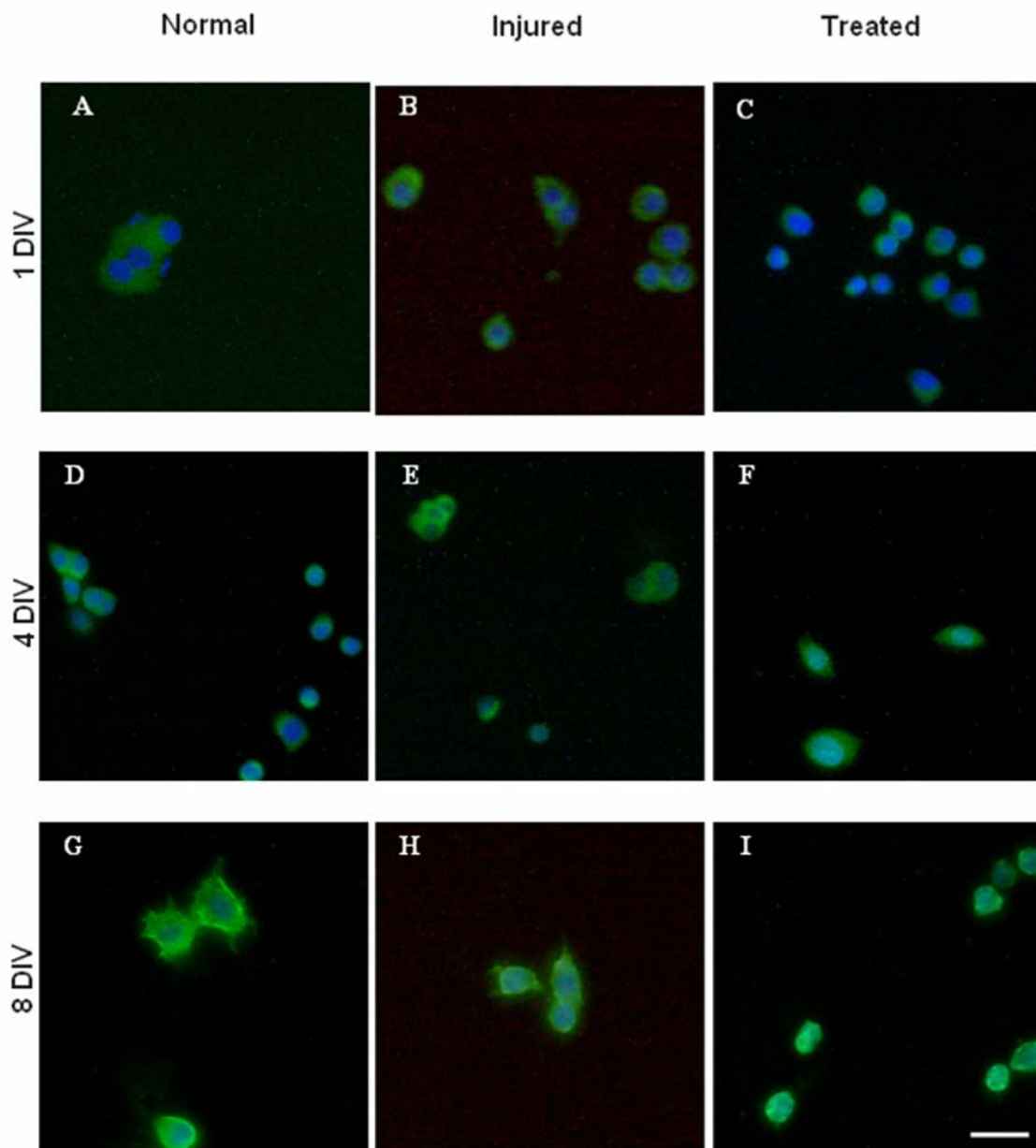


Figure S4