Deoxyribozyme-mediated knockdown of xylosyltransferase-1 mRNA promotes axon growth in the adult rat spinal cord

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In the injured spinal cord, proteoglycans (PGs) within scar tissue obstruct axon growth through their glycosaminoglycan (GAG)-side chains. The formation of GAG-side chains (glycosylation) is catalysed by xylosyltransferase-1 (XT-1). Here, we knocked down XT-1 mRNA using a tailored deoxyribozyme (DNAXTAs) and hypothesized that this would decrease the amount of glycosylated PGs and, consequently, promote axon growth in the adult rat spinal cord. A continuous 2-week delivery of DNAXTAs near the rostral border of a peripheral nerve graft bridging the transected dorsal columns in the thoracic spinal cord resulted in an 81% decrease in XT-1 mRNA, an average of 1.4-fold reduction in GAG-side chains of chondroitin sulphate or heparan sulphate-PGs and 2.2-fold reduction in neurocan and brevican core proteins in scar tissue. Additionally, compared to control deoxyribozyme, the DNAXTAs treatment resulted in a 9-fold increase in length and a 4-fold increase in density of ascending axons growing through the nerve graft and scar tissue present at the rostral spinal cord. Together our data showed that treatment with a deoxyribozyme against XT-1 mRNA decreased the amount of glycosylated PGs and promoted axon growth through scar tissue in the injured spinal cord. The deoxyribozyme approach may become a contributing factor in spinal cord repair strategies.

Keywords: scar; neurocan; brevican; XT; DNA enzyme

Abbreviations: CS = chondroitin sulfate; CTB = cholera toxin B subunit; DNA = deoxyribonucleic acid; DS = dermatan sulphate; GAG = glycosaminoglycan; GFAP = glial fibrillary acidic protein; HS = heparin sulphate; mRNA = messenger ribonucleic acid; PB = phosphate buffer; PBS = phosphate-buffered saline; PG = proteoglycan; XT-1 = xylosyltransferase-1


Introduction
Axon growth in the injured adult spinal cord is obstructed by a cellular and molecular barrier, the lesion scar (Preston et al., 2001). This scar may have a protective role in the damaged spinal cord (Bush et al., 1999; Faulkner et al., 2004) but its ability to prevent endogenous or therapy-induced axon growth is imperious and thought to be a crucial participant in failed attempts to repair the spinal cord. Therapies designed to decrease scar-mediated axon growth-inhibition are important for the development of effective spinal cord repair strategies.

Reactive astrocytes are involved in the growth-obstructive effects of the scar. These cells express a number of growth-inhibitory molecules such as tenascin-C (Zhang et al., 1997), semaphorin 3 (Pasterkamp et al., 2001), ephrin-B2 (Bundesen et al., 2003), slit proteins (Hagino et al., 2003) and chondroitin sulphate proteoglycans (CS-PGs, Grimpe and Silver, 2002; Jones et al., 2003; Rhodes and Fawcett, 2004; Silver and Miller, 2004). CS-PGs exert their growth-inhibitory effects through the glycosaminoglycan (GAG)-side chains (Preston et al., 2001; Laabs et al., 2007).

The formation of GAG-side chains (glycosylation) is catalysed by xylosyltransferase-1 (XT-1), which transfers β-D-xylosyl (from UDP-β-D-xylose) to hydroxyl in serine residues of CS-, its epimeric form dermatan sulphate (DS-) and heparan sulphate (HS-) PG core proteins (Götting et al., 2000) and heparin. XT-1 is crucial for the biosynthesis of glycosylated PGs in the scar after spinal cord injury.
(Gries et al., 2007). Earlier it was shown that administration of a deoxyribozyme (or DNA enzyme) that digests XT-1 mRNA (DNAXTas) improves axon growth of dorsal root ganglion neurons microtransplanted into the injured rat spinal cord (Grimpe and Silver, 2004).

Here, we investigated whether administration of DNAXTas would render scar tissue at a peripheral nerve graft–spinal cord interface conducive to growth of severed endogenous axons. We also quantitatively investigated the effects of DNAXTas treatment on GAG-side chain and deglycosylated PG core protein presence in vivo. We found that DNAXTas treatment resulted in a reduction in GAG-side chains on CS- and HS-PGs and core proteins of neurocan and brevican in the injured/transplanted spinal cord. This was accompanied by a significant improvement in axon growth through scar tissue. Our results confirm the notion that deoxyribozymes may become key components of repair strategies for the injured spinal cord.

Materials and Methods

Deoxyribozymes

The deoxyribozyme to XT-1 (DNAXTas) and the control deoxyribozyme (DNAXTmb) were synthesized as end-cap phosphorothioated oligonucleotide by MWG Biotech (High Point, NC). DNAXTmb was unable to bind and digest XT-1 mRNA. To demonstrate effective delivery of deoxyribozyme in the spinal cord repair model used in this study, we employed DNAXTas biotinylated at its first nucleotide of the 5' end (Grimpe and Silver, 2004).

Implantation of the peripheral nerve graft and administration of deoxyribozyme

Adult female Sprague-Dawley rats (200–225 g, n = 46; Harlan, Indianapolis, IN) were anaesthetized with an intramuscular injection of 60 mg/kg ketamine and 0.4 mg/kg medetomidine. A laminectomy was performed at the thoracic (T) 8 and T9 level to expose the spinal cord. Using iridectomy scissors, a 2-mm long gap was made in the dorsal columns, which was immediately filled with a 2-mm long piece of the distal part of the peroneal nerve, which was transected 7 days earlier (Fig. 1; Oudega and Hagg, 1996, 1999). Next, a custom-made metal cannula was inserted into the dorsal columns, 1 mm rostral to the nerve graft (Fig. 1). The cannula was fixed to the T6 dorsal spinous process and attached via a catheter to a subcutaneous osmotic minipump (Alzet Model 1007D, Durect, Cupertino, CA) filled with DNAXTas (10 μg/μl; n = 8), DNAXTmb (10 μg/μl; n = 7) or saline (n = 9). The wound area was rinsed with sterile phosphate-buffered saline (PBS) with 0.1% gentamicin (Sigma; St. Louis, MO), the overlying muscles were closed in layers with 4.0 sutures, and the skin was closed with metal wound clips. Rats received 10 ml of lactated Ringers’ solution subcutaneously and 0.25 mg of atipamezole hydrochloride (Antisedan®; Pfizer Animal Health, Exton, PA) intramuscularly and were kept in a small animal incubator at 30 ± 0.5°C until full recovery from anaesthesia. The rats were then returned to their cages with ad libitum access to food and water. Gentamicin (0.01 mg/kg body weight; Buck Inc., Owings Mills, MO) was given intramuscularly immediately after surgery and daily for 1 week to prevent infections.

In all rats, the minipump was replaced after 7 days to assure continuous deoxyribozyme delivery for 14 days. During the survival period, rats were monitored at least twice a day.

Verification of deoxyribozyme presence in the spinal cord

To confirm enzyme delivery to the spinal cord, biotinylated DNAXTas (10 μg/μl) was infused as described above in four rats. After 2 weeks, the rats were perfused with fixative (see below) and their spinal cord removed and histologically processed for immunostaining with Texas-Red-conjugated streptavidin (1:500, Invitrogen, Carlsbad, CA; Grimpe and Silver, 2004). This enabled the detection and localization of (biotinylated) DNAXTas after infusion into the dorsal columns.

Anterograde axon labelling

Eleven days after nerve implantation and start of the enzyme treatment, cholera toxin B subunit (CTB; List Biological Laboratory, Campbell, CA) was injected into the right sciatic nerve (Fig. 1). Three days later the rats were fixed and the spinal cords and brains removed for histology. Technical details on the tracing and histology have been described in detail previously (Oudega et al., 1994). Labelling with CTB enabled immunocytochemical detection and quantification of responding axons and therefore assessment of the effects of DNAXTas on their growth through scar tissue at the nerve graft–spinal cord interface.

Histological procedures

Rats were anaesthetized and transcardially perfused first with saline containing heparin (at room temperature) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4; ice-cold). The spinal cords and brains were removed and post-fixed overnight in the same fixative at 4°C. Next, a 12-mm long segment of the spinal cord with the nerve graft was kept in 30% sucrose in 0.1 M PB (pH 7.4) for 48 h, embedded in gelatin (Oudega et al., 1994), and cut into 40-μm thick horizontal sections on a freezing microtome. These sections were kept in 0.1 M PB at 4°C until further processing.

Fig. 1  Schematic representation of the spinal cord injury/repair model. Our model is a slightly modified version of that used by Oudega and colleagues (1994). The peroneal nerve is transected 7 days before harvesting the nerve graft from its distal part. The epineurium is removed from the nerve graft before transplantation into the transection gap in the dorsal columns at the T9 spinal cord level. Deoxyribozyme treatment starts immediately after graft placement through a metal cannula fixed to the T6 dorsal spinous process and connected to an osmotic minipump. The needle is inserted into the dorsal columns 1 mm rostral of the nerve graft.
**Immunohistochemistry**

Sections were blocked with PBS (pH 7.4) containing 0.1% bovine serum albumin and 5% normal goat or rabbit serum for 30 min and then rinsed three times for 10 min with PBS. Next, the sections were incubated overnight at 4°C with goat anti-CTB (List Biological Laboratory, Hornby, Canada), mouse anti-CS-side chains (IgM, clone CS-56; 1:200; Sigma, St. Louis, MO), mouse anti-heparan sulphate (IgM, clone F58-10E4; 1:200; Seikagaku, Falmouth, MA), rabbit anti-glia-l fibrillary acidic protein (GFAP; 1:500; Sigma, St. Louis, MO), mouse anti-neurocan (clone 1F6; 1:200; Developmental Hybridoma Bank, Idaho) or rabbit anti-brevican (clone 1058+; 1:1000 generous gift from Dr Rupert Timpl, Max Planck Institute of Biochemistry, Munich, Germany). All antibodies were diluted in PBS (pH 7.4) containing 0.1% bovine serum albumin and 5% normal goat or rabbit serum. After the overnight incubation, the sections were rinsed three times 10 min with PBS and incubated for 2 h at room temperature with one or a combination of the following secondary antibodies: goat anti-mouse, goat anti-rabbit, and rabbit anti-goat conjugated with Alexa 488 or Alexa 568 (Invitrogen, Carlsbad, CA). The sections were rinsed three times for 10 min with PBS, coverslipped with Gel-Mount (Electron Microscopy Sciences, Hartfield, PA), and analysed using an inverted LSM 510 confocal or Axio phot microscope (both from Carl Zeiss Inc, Jena, Germany) with Neurolucida (Virtual Slice Image application) and Stereoinvestigator software (both MBF Bioscience, Williston, VT).

**Analysis of CTB-labelled axons rostral to the nerve graft**

Stereoinvestigator (MBF Bioscience, Williston, VT) was used to determine the length (in mm) and density (per μm²) of CTB-labelled axons. Following stereological rules of random systematic sampling quantification was performed in 13 sections of 4 DNAXTas-treated rats, 12 sections of 4 DNAXTmb-treated animals and eight sections of three saline-treated rats. The region of interest between the nerve graft and the infusion site was outlined and the surface area measured by overlaying square grids (140 × 140 μm, Cavalieri Principle). A 3D probe (60 μm) for thick sections (Optical Dissector) in combination with isotropic virtual plains (Optical Fractionator method) was used to estimate the length and density of the axons.

**Preparation and analysis of dot blots and Western blots from spinal cord**

DNAXTas-, DNAXTmb- and saline-treated rats (n = 3 each) were transcardially perfused with PB at room temperature. The dorsal half of a 5-mm long piece of the spinal cord just rostral to the nerve graft was rapidly removed, frozen on dry ice, and stored at −80°C. For dot blot and Western blot preparation, spinal cord tissue was dissolved in buffer [10 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA (pH 8), 1% Triton X-100, 0.5% NP-40, 0.2 mM PMPS with or without 0.05% SDS and 10 mM DTT]. Protein concentration was measured using BCA (Pierce, Rockford, IL). Equal amount of proteins were loaded onto a nitrocellulose membrane using a dot blot apparatus (BioRad, Hercules, CA) and for Western blots the samples were digested in 30 mM Na-acetate, 100 mM HCl–Tris, pH 8 with 0.01U protease-free chondroitinase ABC (Seikagaku, Falmouth, MA) before being loaded into a 4–12% SDS–PAGE (BioRad). After separating the protein, the gel was blotted using 10 mM sodium borate buffer. The dot blots and Western blots were blocked with 5% bovine serum albumin and hybridized using primary antibodies against β-actin (1:1000, Abcam, Cambridge, MA), GAG-side chains of CS- or HS-PGs, neurocan and brevican core protein (see the section ‘Immunocytochemistry’). Goat anti-rabbit or goat anti-mouse conjugated to horseradish peroxidase were used as secondary antibodies (Invitrogen). The ECL kit with or without Super Sigma West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was used for detection according to the manufacturer’s instructions. Dot blots were densitometrically evaluated using TINA 2.0 (Raytest, Wilmington, NC).

**RNA preparation of and RT–PCR for XT-I mRNA in spinal cords**

From DNAXTas-treated, DNAXTmb-treated and saline-treated rat spinal cord tissue (n = 3 each) RNA was isolated using Trisolve (Biotecx, Houston, TX) and reversed transcribed into cDNA. The real-time RT–PCR conditions for the various primer pairs used in vivo are provided in Table 1. The High Capacity cDNA Reverse Transcription Kit and the Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA) were used according to the manufacturer’s instructions. Untreated and uninjured spinal cord mRNA was used to create a standard curve. For all PCRs, the standard curve ranged from 104 to 0.144 ng/μl and all real-time RT–PCRs were normalized to the housekeeping gene β-actin. The melting

**Table 1** Primer pair conditions used for real time RT–PCR experiments of spinal cords

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
<th>Step cycle</th>
<th>Expo. cycle</th>
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<tr>
<td>XT-I</td>
<td>Sense: 5’-GTC GCA CCT CTG ACC TTC TC-3’&lt;br&gt;Antisense: 5’-CCA GCC TCC TAG TGC TGT CC-3’</td>
<td>Denature: 95°C, 30 s&lt;br&gt;Annealing: 61°C, 30 s</td>
<td>32–42 cycles</td>
</tr>
<tr>
<td>Neurocan</td>
<td>Sense: 5’-CGA GCC GCC CAC CTG ACA A-3’&lt;br&gt;Antisense: 5’TCC TCC CCA CCT GCG AAG AAA-3’</td>
<td>Denature: 95°C, 30 s&lt;br&gt;Annealing: 66°C, 30 s</td>
<td>30–45 cycles</td>
</tr>
<tr>
<td>Brevican</td>
<td>Sense: 5’-CAT CGA GGG TGA CTT CCT GT-3’&lt;br&gt;Antisense: 5’-TTG CAG GGT ACA TCA CTC CA-3’</td>
<td>Denature: 95°C, 30 s&lt;br&gt;Annealing: 66°C, 30 s</td>
<td>32–42 cycles</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Sense: 5’-GCC CCC TCT GAA CCC TAA G-3’&lt;br&gt;Antisense: 5’-GTC TCC GGA GTC CAT CAC AAT-3’</td>
<td>Denature: 95°C, 30 s&lt;br&gt;Annealing: 63°C, 30 s</td>
<td>25–35 cycles</td>
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The length and density of the axons.

Virtual plains (Optical Fractionator method) was used to estimate the volume and density of the axons. 140 μm was outlined and the surface area measured by overlaying square grids. The dot blots and Western blots were blocked with 5% bovine serum albumin and hybridized using primary antibodies against β-actin (1:1000, Abcam, Cambridge, MA), GAG-side chains of CS- or HS-PGs, neurocan and brevican core protein (see the section ‘Immunocytochemistry’). Goat anti-rabbit or goat anti-mouse conjugated to horseradish peroxidase were used as secondary antibodies (Invitrogen). The ECL kit with or without Super Sigma West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was used for detection according to the manufacturer’s instructions. Dot blots were densitometrically evaluated using TINA 2.0 (Raytest, Wilmington, NC).
Axon growth in the adult rat spinal cord

Statistical analysis
All statistical evaluations were carried out using SPSS (SPSS Inc., Chicago, IL). For the RT–PCR results, we used the non-parametric two-tailed Mann–Whitney U-test with an alpha value of 0.05 to compare two groups with three measurements each. For this a between-subject design was used and it was assumed that the dependent variables were ordinal scaled, that the independent variable had two levels, and the groups were independent from each other. For the in vivo dot blot results, the independent-sample two-tailed t-test with an alpha value of 0.05 was used. It was assumed that the dependent variable was normally distributed (this was confirmed with a Q-Q plot), the two groups had equal variance on the dependent variable (Levene’s test), and the groups were independent of each other. With three groups, we used one-way ANOVA with least significant difference (LSD) and ad hoc comparison, because the data fulfilled the first two above-mentioned assumptions. All results are given with the standard error of the mean (SEM), which reflects the exactness of our sample mean.

Results
We used an established rat spinal cord injury model (Oudega and Hagg, 1996, 1999). This model involves a transection gap of the dorsal columns and the subsequent implantation of a predegenerated peripheral nerve graft. Deoxyribozyme to XT-1, control enzyme or saline was administered rostral to the transplant via a needle, connected to an osmotic minipump (Fig. 1). We investigated (i) the ability of endogenous severed axons to grow from the nerve graft into the rostral spinal cord; and (ii) the presence of neurocan and brevican PG core proteins and GAG-side chains of CS- and HS-PGs after these treatments.

DNAXTas treatment promotes axon growth in adult spinal cord
In our model the severed endogenous axons grow into but not beyond the nerve graft without additional treatment (Oudega and Hagg, 1996, 1999). Here, we show that the delivery of DNAXTas 1 mm rostral to the nerve graft increased the presence of (CTB-labelled) axons within scar tissue in the rostral spinal cord (Fig. 2A). This response was absent in control rats (Fig. 2B and C). Some of these axons were found close to the infusion site (Fig. 2D and E). We infused biotinylated DNAXTas into the injured/transplanted spinal cord and found that the deoxyribozyme was present between the infusion site and the rostral border of the nerve graft, i.e. where the responding axons were located (Fig. 2F).

The average length of the responding axons was 0.57 mm in the DNAXTas-treated (SEM: +/-0.15), 0.063 mm in the DNAXTmb-treated (SEM: +/-0.026), and 0.08 mm in saline-treated (SEM: +/-0.038) spinal cords. A statistical significant difference was found between the DNAXTas- and DNAXTmb-treated spinal cords (P=0.001; Fig. 2G) and between the DNAXTas- and saline-treated spinal cords (P=0.002; Fig. 2G). There was no significant difference between the DNAXTmb- and saline-treated spinal cords. The axon density in the region between the rostral graft-spinal cord border and the infusion site was 0.0078 axons/µm² in DNAXTas- (SEM: +/-0.0018), 0.00196 axons/µm² in DNAXTmb- (SEM: +/-0.000681) and 0.00078 axons/µm² in saline-treated (SEM: +/-0.00031) spinal cords. A statistical significant difference was found between the DNAXTas- and DNAXTmb-treated spinal cords (P=0.003; Fig. 2G) and between DNAas-XT and saline-treated spinal cords (P=0.001, Fig. 2G). The average axon density in DNAXTmb- and saline-treated spinal cords was similar. Thus, DNAXTas administration compared to the control deoxyribozyme administration resulted in 9-fold longer and in 4-fold more axons in the spinal cord rostral to the nerve graft.

To confirm that our dorsal column injury had lesioned the entire axon tracts we stained coronal sections of their brainstem at the level of the gracile nucleus with CTB antibodies (Lu et al., 2004). Our tracing protocol involved an injection of CTB into the right sciatic nerve where the peripheral projections of the neurons in the lumbar dorsal root ganglia are bundled. The central projections of these neurons ascend in the gracile fascicle and terminate in the gracile nucleus (Smith and Bennet, 1987). We did not find CTB-labelled axons in the gracile nucleus in DNAXTas-treated (Fig. 2H and H’) or control rats (Fig. 2I and I’). In uninjured rats, using the same labelling protocol, many CTB-labelled axons were found in the gracile nucleus (Fig. 2J and J’). These results demonstrated that the ascending axon tract had been completely transected.

DNAXTas decreases the amount of GAG-side chains and core proteins of PGs in scar tissue
Xylosyltransferase transfers xylose from UDP-xylose to initiate GAG-side chain synthesis of CS-, DS- and HS-PGs. We analysed the effects of DNAXTas treatment on GAG-side chains and core proteins of PGs in the spinal cord immediately rostral to the nerve graft using immunohistochemistry, dot blot and Western blot experiments. With the CS-56 antibody, which recognizes GAG-side chains of PGs, we found a decrease in staining intensity in DNAXTas-infused spinal cords (Fig. 3A) compared to DNAXTmb- (Fig. 3B) and saline-infused spinal cords (Fig. 3C). Using an antibody that recognizes HS-chains, F58-10E4, we observed a reduction of immunostaining around the injection site (IS) and in the graft of DNAXTas-treated animals (Fig. 3D) compared to control DNAXTmb- (Fig. 3E) or saline-treated (Fig. 3F) animals. With the 1F6 and 1085+ antibodies, which recognize the core proteins of neurocan and brevican, respectively, we found a decrease in staining intensity in the spinal cords treated with DNAXTas (Fig. 3G and J, respectively) compared to DNAXTmb- (Fig. 3H and K) or saline-treated (Fig. 3I and L) control.
spinal cords. These results indicated a decrease in the amounts of GAG-side chains, neurocan and brevican core proteins in DNAXTas-treated spinal cords.

To verify the results from the immunostainings we performed dot blot experiments and quantified the changes in the scar after DNAXTas, DNAXTmb and saline treatment. We found significant decreases in the average amount of GAG-side chains on CS-PGs in DNAXTas-treated spinal cords relative to DNAXTmb- and saline-treated spinal cords (1.4- and 1.6-fold, respectively. *P* = 0.05; Fig. 4A) and HS-PGs (1.2- and 1.4-fold, respectively. *P* = 0.05; Fig. 4B). Also the average amount of neurocan core protein (2.5- and 2.7-fold, *P* < 0.018; Fig. 4C) and brevican core protein (1.5- and 2.3-fold, *P* < 0.018; Fig. 4D) were reduced in DNAXTas-treated animals compared to DNAXTmb- and saline-treated spinal cords, respectively. There was no significant difference between DNAXTmb- and saline-treated animals. In Fig. 4E we expressed the amount of GAG-side chains and proteins in DNAXTas-treated and DNAXTmb-treated spinal cords as a percentage of those in saline-treated spinal cords. We also quantified, in Western blots, the reduction in the amount of neurocan (75%) and brevican (60%) core proteins in DNAXTas-treated spinal cords relative to the amounts in DNAXTmb-treated spinal cords (Fig. 4E).
To visualize the influence of DNAXTas and DNAXTmb treatment on brevican and neurocan core protein expression, we performed Western blotting of chondroitinase ABC-digested protein extracts (Fig. 4F). We observe that the 130 kDa neurocan and the 80 kDa brevican band were reduced in DNAXTas-treated compared to control DNAXTmb-treated animals. Furthermore, we observed additional bands in both Western blots likely representing core protein fragments. β-Actin (40 kDa) was used as control to demonstrate that similar amount of protein were loaded. Together, our quantitative dot blot and Western blot results confirmed the differences in GAG-side chain and PG core proteins after DNAXTas treatment as indicated by the immunostained spinal cord sections.

**DNAXTas reduces XT-1 and PG core protein mRNA**

With real-time RT–PCR we found that administration of DNAXTas to spinal cords that received the peripheral nerve transplant resulted in a significant decrease in XT-1 mRNA (P ≤ 0.001) compared to DNAXTmb-treated (81% decrease) and saline-treated (98% decrease) spinal cords (Fig. 5). Furthermore, a significant reduction in neurocan mRNA (P ≤ 0.0001; Fig. 5B) and brevican mRNA (P ≤ 0.001; Fig. 5C) was observed in DNAXTas-treated spinal cords compared to the control spinal cords. The relative expression rate for neurocan core protein was 0.56 ± 0.052 ng/ml and for brevican core protein 0.22 ± 0.076 ng/ml. These data supported our results from the immunohistochemistry, Western blot and dot blot experiments.

**Discussion**

XT-1 is crucial for the formation of GAG-side chains (glycosylation) of CS-, DS- or HS-PG core proteins, which are abundantly present in scar tissue in the injured spinal cord. These GAG-side chains participate in the axon growth-inhibitory actions of the PGs. We hypothesized that DNAXTas, a deoxyribozyme that digests XT-1 mRNA, would decrease the amount of GAG-side chains and as a result decrease the axon growth-inhibitory nature of scar tissue in the injured adult rat spinal cord. To test this
premise, we used a dorsal column injury/repair model involving the continuous delivery of DNAXTas just rostral to a peripheral nerve graft transplanted into an injury gap. Treatment resulted in an 81% decrease in XT-1 mRNA in DNAXTas treated spinal cords compared to DNAXTmb-treated animals. We also found a 1.6-fold decrease in the amount of neurocan and brevican core protein in scar tissue of DNAXTas-treated animals. Together, our results showed that DNAXTas treatment reduced the presence of GAG-side chains in the lesion scar and promoted axon growth through an otherwise inhibitory terrain in the injured adult rat spinal cord.

Spontaneous axon growth through scar tissue in the injured spinal cord does not occur due to the presence of growth-inhibitory molecules. Several approaches have been shown to elicit axon growth through scar tissue, i.e. beyond a lesion/transplant site, such as increasing the level of neurotrophic factors (Oudega and Hagg, 1996, 1999; Bamber et al., 2001; Lu et al., 2004; Taylor et al., 2006) and the administration of chondroitinase ABC, a bacterial enzyme that removes GAG-side chains from PG core proteins (Zuo et al., 1998; Moon et al., 2001; Bradbury et al., 2002; Yick et al., 2003; Chau et al., 2004; Caggiano et al., 2005; Houle et al., 2006; Cafferty et al., 2007). Treatment with NEP1-40, a competitive antagonist of NgR1,
the receptor of oligodendrocyte myelin-associated growth inhibitors (GrandPre et al., 2002), or Rho/Rho-associated kinase inhibitors (e.g. C3 enzyme) that block the signalling pathway of myelin-associated growth inhibitors (Dergham et al., 2002; Fournier et al., 2003) also results in axon growth beyond a lesion/transplant in the spinal cord. In different spinal cord repair model systems, transplantation of olfactory ensheathing glia close to an injury/transplant was also shown to elicit growth of axons across scar tissue (Li et al., 1997, 1998; Ramon-Cueto et al., 1998, 2000).

In the present study, we demonstrate that DNAXTas promotes endogenous axon growth from a nerve graft and through scar tissue in the adult rat spinal cord. DNAXTas presence resulted in a decrease in mRNA of XT-1, the enzyme that catalyses PG glycosylation and in the amount of GAG-side chains and PG core proteins. Our findings suggest that DNAXTas treatment decreased the axon growth-inhibitory actions of PG and thus promoted the observed growth response. It is known that glycosylated PGs are growth inhibitory because removal of GAG-side chains by chondroitinase ABC promotes axon growth through scar tissue (Moon et al., 2001; Bradbury et al., 2002). It is possible that the observed growth response was in part mediated by the reduction of core proteins after deglycosylation. This idea is supported by findings by Inatani et al., (2001) and Sango et al., (2003) who showed that core proteins from members of the lectican family are inhibitory after chondroitinase ABC digestion. In contrast, others demonstrated that core proteins can promote neurite outgrowth from (neo-)cortical or olfactory neurons in vitro (Iijima et al., 1991; Maeda and Noda, 1996; Garwood et al., 1999; Clarris et al., 2000). This was also demonstrated for the deglycosylated NG2 core protein (Fidler et al., 1999). The possible contribution of PG core proteins to axon growth in our in vivo model needs to be addressed in future experiments.

The decrease in the amounts of GAG-side chains, and neurocan and brevican core proteins occurred within the region in which DNAXTas was delivered. These changes were observed in spinal cord sections stained with antibodies specific for the respective molecules and quantified in dot blot and real-time RT–PCR. The 1.6-fold or 1.4-fold decrease in the amount of GAG-side chains from CS-PGs or HS-PGs, respectively, within the scar results from the DNAXTas-mediated decrease in XT-1, which would prevent the formation of GAG-side chains. The decrease (about 2-fold) in the amounts of neurocan and brevican core protein after DNAXTas treatment mirrors the reduced mRNA expression. Alternatively, the observed decrease in PG core protein presence may have resulted from poor integration of deglycosylated PGs into extracellular matrix. Milev and colleagues (1998) showed that CS-side chains were necessary for neurocan to become properly incorporated into extracellular matrix through linkage with heparin-binding growth-associated molecule, one of the several known ‘binding partners’ for PGs (Grumet et al., 1994; Milev et al., 1996; Retzler et al., 1996; Aspberg et al., 1999). Another possible explanation for the lower amounts of PG core proteins after DNAXTas-mediated prevention of glycosylation may be that the core proteins are more accessible for proteolytic degradation by matrix metalloproteinase (Hardingham and Fosang, 1995), ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs; Abbaszade et al., 1999; Tortorella et al., 1999) or plasmin/plasminogen activator system (Tsirka, 2002).

CS-PGs and HS-PGs are involved in various signalling pathways. CS-PGs are involved in pathways containing calcium (Snow et al., 1994), EGFR (Koprivica et al., 2005),
TGF-β1 (Smith and Strunz, 2005), TGF-β2 (Brambilla et al., 2005), interleukine-1β (Smith and Strunz, 2005), PKC (Sivasankaran et al., 2004) and NFκB (Brambilla et al., 2005). HS-PGs are involved in signaling pathways of TGF-β (Zhang et al., 2006, Yue et al., 2008), hedgehog (Zhang et al., 2007), FGF (Yayon 1991, Reuss and von Bohlen und Halbach, 2003) and Wnt (Intani et al., 2003). Several of these pathways lead to activation of transcription factors such as AP-1 (Beckerman, 2005), SP-1 (Law et al., 2004) or NFκB (Krohn et al., 1999). The consensus sequences of these transcription factors have been identified in the promoter regions of neurocan (Rauch et al., 1995; Grimpe, 1996) and brevican (Rauch et al., 1997). Consequently, the loss of GAG-side chains after DNAXTas treatment followed by decreased amount of these PGs in the extracellular space may directly or indirectly influence the transcription of the neurocan and brevican genes through these pathways.

In sum, we provide evidence that treatment with DNAXTas, which efficiently digests mRNA of XT-1 that catalyses PG glycosylation, decreases the inhibitory nature of scar tissue and, as a result, elicits axon growth from an intraspinal nerve graft into the contiguous spinal cord. This response was accompanied by a decrease in mRNA and protein of GAG-side chains, neurocan and brevican core protein within the scar, i.e. where DNAXTas was delivered. Our findings demonstrate the potential of deoxyribozymes to specifically interfere in the life cycle and/or functioning of molecules with a crucial role in the failure of repair in the injured spinal cord. The deoxyribozyme technology may become a relevant component of strategies aimed at spinal cord repair.

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References
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