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Fibronectin EDA forms the chronic fibrotic scar after contusive spinal cord injury

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Abstract

Gliosis and fibrosis after spinal cord injury (SCI) lead to formation of a scar that is an impediment to axonal regeneration. Fibrotic scarring is characterized by the accumulation of fibronectin, collagen, and fibroblasts at the lesion site. The mechanisms regulating fibrotic scarring after SCI and its effects on axonal elongation and functional recovery are not well understood. In this study, we examined the effects of eliminating an isoform of fibronectin containing the Extra Domain A domain (FnEDA) on both fibrosis and on functional recovery after contusion SCI using male and female FnEDA-null mice. Eliminating FnEDA did not reduce the acute fibrotic response but markedly diminished chronic fibrotic scarring after SCI. Glial scarring was unchanged after SCI in FnEDA-null mice. We found that FnEDA was important for the long-term stability of the assembled fibronectin matrix during both the subacute and chronic phases of SCI. Motor functional recovery was significantly improved, and there were increased numbers of axons in the lesion site compared to wildtype mice, suggesting that the chronic fibrotic response is detrimental to recovery. Our data provide insight into the mechanisms of fibrosis after SCI and suggest that disruption of fibronectin matrix stability by targeting FnEDA represents a potential therapeutic strategy for promoting recovery after SCI.

Keywords

spinal cord injury; fibrosis; gliosis; fibronectin; fibronectin EDA; matrix; scarring

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Introduction

Although the formation of fluid-filled cavities is a common feature of human spinal cord injury (SCI), not all human SCI leads to cavity formation. In fact, connective tissue scars characterized by accumulation of collagen, fibronectin, and fibroblasts dominate the injury sites in massive compression and laceration SCI cases (Bunge *et al.*, 1997; Kim, 2013). In these cases, a surprisingly small glial response and no cavity formation are observed (Bunge *et al.*, 1997; Kim, 2013). These findings suggest that fibrotic scarring may be an impediment to tissue repair and axonal regeneration after severe compression and laceration spinal cord injuries in humans. In the present study, we examined fibrotic responses after SCI using a severe mouse contusion model, which compresses the spinal cord for a prolonged period of time and results in significant fibrotic scarring (Jeong *et al.*, 2017).

Fibrotic scar formation is mediated by the assembly of a fibronectin matrix through a stepwise cell-mediated process (Erickson *et al.*, 1981; Allio and McKeown-Longo, 1988; Fogerty *et al.*, 1990; Aguirre *et al.*, 1994). Fibronectin is composed of multiple repeated domains: twelve type I domains, two type II domains, and fifteen type III domains (Erickson *et al.*, 1981; Erickson and Carrell, 1983; Rocco *et al.*, 1987; Fogerty *et al.*, 1990; Johnson *et al.*, 1999). Type III domains contain two alternatively spliced domains: extra domain A (EDA) and extra domain B (EDB). Hepatocytes in the liver produce plasma fibronectin that lacks both EDA and EDB (Tressel *et al.*, 1991; Wilson and Schwarzbauer, 1992; Magnusson and Mosher, 1998). By contrast, cellular fibronectin is synthesized by fibroblasts, endothelial cells, and myoblasts and undergoes alternative splicing to include EDA (FnEDA) and/or EDB domains (FnEDB) (Mao and Schwarzbauer, 2005).

In particular, the FnEDA splice variant has been implicated in pathological fibrosis in models of skin wound healing, idiopathic pulmonary fibrosis and scleroderma (Muro *et al.*, 2003; Muro *et al.*, 2008; Bhattacharyya *et al.*, 2014). FnEDA is virtually absent from normal adult tissues, and uninjured mice lacking FnEDA are indistinguishable from wildtype mice (Manabe *et al.*, 1997; Shiozawa *et al.*, 2001; Muro *et al.*, 2003; Muro *et al.*, 2008; Khan *et al.*, 2012; Bhattacharyya *et al.*, 2014). However, FnEDA-null mice demonstrate abnormal skin wound healing, ulceration, and inflammation at sites of injury (Muro *et al.*, 2003). Persistent accumulation of the FnEDA isoform is observed in the lungs of humans with idiopathic pulmonary fibrosis, and mice lacking FnEDA do not develop pulmonary fibrosis after bleomycin injection (Muro *et al.*, 2008). Additionally, a recent study observed significant increases of FnEDA levels in both serum and lesional skin biopsies of patients with scleroderma. The study also demonstrated that FnEDA-null mice show attenuated cutaneous fibrosis and reduced numbers of activated fibroblasts after bleomycin insult (Bhattacharyya *et al.*, 2013). These findings demonstrate that FnEDA plays an indispensable role in the pathogenesis of fibrotic scarring after injury to many organs. The detailed mechanism by which FnEDA facilitates fibrotic responses is still unknown, but recent studies suggest that FnEDA acts both as a structural component of the fibrotic scar and as a signaling molecule that contributes to fibroblast activation into myofibroblasts (Balza *et al.*, 1988; Borsi *et al.*, 1990; Bhattacharyya *et al.*, 2013).

The present study was undertaken to better understand the mechanisms regulating fibrotic scar formation after SCI by focusing on the role of FnEDA. We observed significant acute elevations of FnEDA in the injured spinal cord and persistent accumulation of FnEDA in the spinal lesion after SCI. Eliminating the FnEDA isoform did not affect acute fibrotic scarring after SCI but significantly reduced chronic fibrotic scarring by altering the dynamics of fibronectin matrix degradation. Importantly, FnEDA-null mice showed significantly improved behavioral recovery from SCI compared to wildtype mice. Thus, chronic fibrotic scarring in the injured spinal cord is mediated, at least in part, by the FnEDA isoform, and reducing chronic fibrotic scarring via targeting FnEDA promotes functional recovery.

Methods

Experimental Design

The present work adheres to the ARRIVE guidelines and the Minimal Information about a Spinal Cord Injury Experiment reporting standards (Kilkenny *et al.*, 2010; Lemmon *et al.*, 2014). The objective of this study was to study the role of FnEDA after SCI and to determine whether reducing fibrotic scarring by eliminating FnEDA improves functional recovery after SCI in mice. All experiments in this paper, including measuring functional recovery of the animals, were performed by investigators blinded to the identity of the animals and were replicated with three or more independent experiments using different experimental samples.

Mouse spinal cord injury and care

All animal procedures performed in this paper were approved by the Northwestern University Institutional Animal Care and Uses Committee and adhered to the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Spinal cord injury was performed using the Infinite Horizons Spinal Cord Impactor system (IH-0400 Precision Systems and Instrumentation) with 70 kdyn of impact force and a dwell time of 60s and our previously described techniques (Jeong *et al.* 2017). 8-10 week old male and female FnEDA-null and littermate wildtype mice were used for experiments involving FnEDA-null mice. Eight-week old female C57BL/6 mice (Charles River) were used for FnEDA mRNA and protein quantifications. Post-operational care was performed as previously described (Jeong *et al.*, 2017).

Immunohistochemistry

Immunohistochemical analyses was performed as previously described (Jeong *et al.*, 2017). Mice were euthanized via CO₂ inhalation and transcardially perfused with ice cold PBS followed by 4% paraformaldehyde in PBS. Spinal cords were removed and post-fixed for 2 hours in 4% PFA on ice. Samples were dehydrated overnight in 30% sucrose at 4°C and then embedded in O.C.T. matrix (Tissue-Tek). Embedded tissues were sectioned into 20µm thick sagittal sections and collected on microscope slides (Fisher Scientific Superfrost Plus Gold). Spaced serial sections were collected and every seventh section was placed onto the same slide. Thus, each adjacent section was 140µm from its neighbor. An average of six sections were collected onto each slide, so the width of spinal cord spanned on each slide was about 840 µm. We chose to divide the tissues across seven slides to ensure that each slide

contained at least one mid-sagittal section, identified by the presence of the central canal as a landmark, located near the center of the slide. Slides were washed in PBS-T (0.05% Triton X-100) and incubated with primary antibodies at 4°C overnight in blocking media (5% normal goat serum in PBS-T). Primary and secondary antibodies used are listed in Supplementary Table 1. Stained sections were mounted in ProLong Gold (Molecular Probes) and images were acquired with a Leica SP5 AOBS confocal microscope.

Image Quantification

Quantification of immunofluorescent images was performed by blinded investigators using the open-source “Icy” bioimage analysis software (de Chaumont *et al.*, 2012). As in our previous study (Jeong *et al.*, 2017), a single representative mid-sagittal section per animal, identified by the presence of the ~70 µm wide ependymal canal as a landmark, was used for image quantification of each stain. We chose to use mid-sagittal sections in order to minimize sampling variability and make consistent comparisons between cords. Since the spinal impactor tip was centered on the midline of the spinal cord, our mid-sagittal sections pass directly through the center of the lesion. Past experience has shown us that mid-sagittal sections through the lesion midline display the largest lesion areas, as compared to more lateral slices. When quantifying fibronectin intensity and lesion size, the lesion core was defined as the GFAP⁺ area surrounded by a bright GFAP⁺ border. The lesion rim was defined as any spinal tissue outside of the lesion core but within 250µm of the bright GFAP⁺ border that bounds the lesion center. These regions were outlined using Icy’s polygon ROI tool. Areas and signal intensities were automatically quantified using Icy’s native ROI Statistics tool.

The methods used for the 3-D reconstructions are provide in the supplementary material.

Western blot

Mice were euthanized via CO₂ inhalation and 1mm of spinal cord tissue containing the spinal lesion was dissected and mechanically lysed in Tissue Protein Extraction Reagent (T-PER) (Thermo Scientific) with 100 × HALT protease inhibitor cocktail (Thermo Scientific). Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Thermo Scientific) with a standard curve of bovine serum albumin. Protein lysates were combined with Laemmli buffer (9% SDS and 10% 2-Mercaptoethanol) and boiled at 95°C for 5 minutes except for the samples used for quantifying FnEDA protein levels. For blots used for quantifying FnEDA levels, the protein samples were boiled at 95°C for 20 minutes. After SDS-PAGE, blots were transferred to Immobilon-P membranes (Millipore) and washed with TBS-T (0.1% Tween 20). Membranes were blocked in 5% milk. Primary and secondary antibodies used are listed in Supplementary Table 1. Blots were developed using ECL Western Blotting Substrate (Thermo Scientific) and Amersham HyperFilm ECL (GE Healthcare). Membranes were stripped and re-probed using Restore Western Blot Stripping Buffer (Thermo Scientific). Western blot films were scanned using a CanoScan 9000F scanner (Canon) and the images quantified using the Analyze Gels tool in Fiji Is Just ImageJ (Fiji) (Schindelin *et al.*, 2012).

Deoxycholate (DOC) digestion

To separate matrix assembled fibronectin from unassembled fibronectin, we followed a previously published DOC digestion protocol (Wierzbicka-Patynowski *et al.*, 2004; Zhu *et al.*, 2015a). 4mm of lesion containing injured spinal cord tissue was harvested after perfusing the animals with ice-cold PBS. The tissues were mechanically digested in freshly prepared 2% DOC lysis buffer and centrifuged at 4°C to harvest DOC-soluble proteins, which includes soluble, unassembled, fibronectin that is not incorporated into the fibronectin matrix. The remaining pellet was further digested in freshly prepared 1% SDS lysis buffer to extract DOC-insoluble proteins, which include fibronectin that was assembled into a fibronectin matrix. The protein concentration of the resulting DOC-soluble and DOC-insoluble protein samples was determined by BCA protein assay as described above. Samples were reduced in Laemmli buffer (9% SDS and 10% 2-Mercaptoethanol) and boiled for 5 minutes at 95°C. The proteins were separated using SDS-PAGE and transferred to Immobilon-P membranes (Millipore) and washed with TBS-T (0.1% Tween 20). Membranes were blocked in 5% milk and incubated with an anti-fibronectin antibody (Millipore, AB2033, 1:1000) overnight at 4°C. Blots were developed with appropriate secondary antibodies (Supplementary Table 1) and band intensities were quantified as described above. To control for protein loading variability membranes were stained in a solution of 0.1% Coomassie blue in 1:1 methanol/water and then destained in a solution of acetic acid/ethanol/water (1:5:4) as previously described (Wierzbicka-Patynowski *et al.*, 2004; Zhu *et al.*, 2015b). Fibronectin band intensities were then normalized to total protein as measured by Coomassie blue staining. This Coomassie normalization procedure is necessary because the proteins typically used as loading controls for western blotting preferentially segregate to either the DOC or SDS extraction fraction.

Open field behavioral scoring

To measure behavioral outcomes after SCI, we used both the 9-point Basso Mouse Scale (BMS) and the 20-point modified Basso, Beattie and Bresnahan (mBBB) scoring systems (Basso *et al.*, 2006; Yuesheng Li *et al.*, 2006). Mice were scored at 24 hours post SCI by two blinded investigators, and animals with a combined BMS score (left hindlimb + right hindlimb) greater than one were excluded as improperly injured. After the initial screening, all mice were scored weekly by two blinded investigators.

Statistical analyses

GraphPad Prism software (version 7.03) was used to perform statistical analysis of the data and a predetermined significance level of 0.05 was used. No outlier values were excluded from any experiments. Comparisons between pairs of experimental groups were performed using Student's t-test. Comparisons among three or more groups were conducted using ANOVA with Tukey's Multiple Comparison test. Mouse BMS and BBB behavioral scores were compared between wildtype and FnEDA-null mice by two-way repeated measures ANOVA with Bonferroni testing to correct for multiple comparisons between each week. All data are presented as mean $\pm$ standard error of the mean (SEM).

Results

FnEDA and FnEDB levels increase significantly after SCI

Both splice variants of cellular fibronectin, FnEDA and FnEDB, are largely absent in adult tissues but are significantly upregulated after injury in many organs (Manabe *et al.*, 1997; Shiozawa *et al.*, 2001; Muro *et al.*, 2003; Muro *et al.*, 2008; Khan *et al.*, 2012; Bhattacharyya *et al.*, 2014). To determine whether FnEDA and FnEDB are expressed in the spinal cord before and after SCI, wildtype mice (8 wks old, C57Bl6, female) were subjected to contusive T11-spinal cord injury. At different time points after SCI, lesion sites were dissected to harvest RNA and protein samples. Levels of *FnEDA* and *FnEDB* mRNAs were quantitated by real-time quantitative polymerase chain reaction (qPCR) using validated primer sets (Muro *et al.*, 2003; Tan *et al.*, 2004). The qPCR results showed low but detectable levels of *FnEDA* and *FnEDB* mRNA in uninjured spinal cord. At 7 days post SCI, we observed a 70 fold increase in *FnEDA* mRNA and a 40 fold increase in *FnEDB* mRNA in the injured compared to control (uninjured) spinal cord. Levels of *FnEDA* and *FnEDB* declined thereafter but remained elevated even 13 weeks after the injury (Fig. 1A, B, * $p < 0.05$, ** $p < 0.01$, Student's t-test, $n = 8-10$ mice per group). Levels of FnEDA protein were measured by western blot analysis (Fig. 1C, D, $n = 3$ mice/group, *** $p < 0.001$, ANOVA followed by Tukey's multiple comparison test). We did not observe any change in levels of FnEDA at 3 days post SCI despite the ~10 fold increase in *FnEDA* mRNA at this time. However, by day 7 levels of FnEDA protein increased about 10-fold. FnEDA levels decreased thereafter but were still increased ~5-fold at 90 days post SCI, suggesting long-term persistent accumulation of FnEDA (Fig. 1F). FnEDB protein levels were not measured because reliable antibodies specific for the FnEDB protein isoform are not available. Levels of total fibronectin protein at 3 days post injury were increased ~8-fold at a time when FnEDA levels were unchanged (Fig. 1 E,G, $n = 3$ mice/group, ANOVA followed by Tukey's multiple comparison test). This suggests that in the acutely injured spinal cord, fibronectin is mainly composed of plasma fibronectin or other splice variants of cellular fibronectin. Levels of total fibronectin peaked at 7 days and declined thereafter. Together, these results show that the FnEDA spliceform is upregulated and deposited chronically in the lesion site after SCI.

Eliminating the FnEDA splice variant results in a smaller lesion area and reduced chronic fibrotic scarring after SCI

To explore the potential role of FnEDA in the injured spinal cord, we used mice with homozygous deletion of the FnEDA domain (FnEDA-null). FnEDA-null mice were viable and developed normally, consistent with previously published observations (Muro *et al.*, 2003). 8-10 week old male and female FnEDA-null and littermate wildtype mice were subjected to a contusion SCI. Exclusion of the EDA domain in FnEDA-null mice was verified using RT-PCR and western analysis at 7 days post SCI (Supplementary Fig. 1).

To determine whether fibrotic scarring is altered in FnEDA-null mice, we performed western blot analyses at 7 and 21 days post SCI to measure levels of total fibronectin in the injury sites (Fig. 2A, B). There were no significant differences in the levels of total fibronectin between wildtype and FnEDA-null mice at either 7 or 21 days post SCI (Fig. 2D, $n = 4-8$

mice/group, ANOVA with Tukey's multiple comparison test). However, at 90 days post SCI, the FnEDA-null group showed a significantly reduced levels of total fibronectin (Fig. 2C, D n=7 mice/group, *** $p<0.001$, ANOVA with Tukey's multiple comparison test).

IHC analysis of spinal cords from the FnEDA-null group also demonstrated smaller lesion size and significantly reduced mean intensity of total fibronectin staining inside the GFAP lesion core compared to wildtype mice at 90 days post SCI (Fig. 2E-G, n=6-7 mice/group, ** $p<0.01$, Student's t-test or ANOVA with Tukey's multiple comparison test). In contrast, there was no difference in GFAP levels as measured either by immunohistochemical analysis of the lesion rim (Fig. 2H) or by western blot (Fig. 2I) at 90 days post SCI, indicating that FnEDA is a critical contributor to the chronic fibrotic response, but not the glial response, after SCI.

In order to further characterize the changes in spinal lesion volume, 90 day post-injury spinal cords were imaged for three-dimensional tissue reconstruction. (Supplementary Fig. 2 and Supplementary Video 1-2) Comparison of estimated lesion volumes from these 3D reconstructions demonstrated significantly decreased lesion volumes in FnEDA-null mice. (Supplementary Fig. 2, * $p<0.05$, Student's t-test).

FnEDA-null mice show significantly attenuated levels of insoluble fibronectin matrix after SCI

Since FnEDA-null mice showed significantly diminished chronic fibrotic scarring, but unaltered acute and subacute fibrotic scarring, it was possible that the fibronectin matrix lacking the FnEDA isoform was more susceptible to long-term remodeling and degradation. To better understand the dynamics of fibrotic scarring, we used a deoxycholate (DOC) digestion protocol to separate assembled fibronectin matrix from unassembled soluble fibronectin. The DOC digest can distinguish insoluble fibronectin matrix, which is assembled to form the chronic fibrotic scar, from soluble fibronectin, which does not form the matrix and is washed away (Mosher, 1983; Wierzbicka-Patynowski *et al.*, 2004; Zhu *et al.*, 2015b). The DOC digest protocol involves lysing the tissue with two different detergents, DOC and SDS, to separate soluble fibronectin from insoluble fibronectin. At 7, 21, and 90 days post SCI, 4 mm of lesion sites were dissected, and the amounts of fibronectin in soluble or insoluble fractions were measured by separating the proteins using SDS-PAGE and probing the blots for fibronectin using a total fibronectin antibody. Band intensities were normalized to total protein measured by Coomassie blue staining (Supplementary Fig. 3). This Coomassie normalization procedure was necessary because proteins typically used as loading controls for western blotting preferentially segregate to either the DOC or SDS extraction fraction.

At 7 days post SCI, there were no differences between wildtype and FnEDA-null mice in the levels of fibronectin present in either the insoluble or the soluble fractions (Fig. 3A, B, n=7-8 mice/group, ANOVA with Tukey's multiple comparison test). At 21 days and 90 days post SCI, we observed significantly reduced levels of insoluble fibronectin in FnEDA-null compared to wildtype mice. By contrast, there were no significant differences in the levels of soluble fibronectin between the two groups (Fig. 3C-F, n=7-8 mice/group, ** $p<0.01$, ANOVA with Tukey's multiple comparison test). Our results indicate that deletion of the

FnEDA isoform selectively diminishes chronic fibrotic scarring by disrupting the stability of insoluble fibronectin matrix.

Mice lacking FnEDA show a higher axonal density in the injured spinal cord

Since FnEDA-null mice showed significantly reduced chronic fibrotic scarring, we investigated whether this made the microenvironment of the injured spinal cord more conducive to axonal regeneration or sparing. At 12 weeks post SCI, injured spinal cords of wildtype and FnEDA-null mice were harvested and sectioned into 20µm thick sagittal sections that were stained with SMI-312 to visualize axons. Previously published studies have shown that SMI-312 staining can be used to quantify neurofilaments in the injured rodent spinal cord (Bhalala *et al.*, 2012; Jeong *et al.*, 2017). FnEDA-null mice showed significantly higher SMI-312 intensities around and within the center of the lesion as compared to wildtype mice (Fig. 4A-C, $n=5$ mice/group. * $p < 0.05$, ** $p < 0.01$. ANOVA with Tukey's multiple comparison test). Interestingly, FnEDA-null mice showed only marginal decreases in the mean intensities of SMI-312 around and within the lesion sites whereas wildtype mice showed an almost twofold decrease in the SMI-312 staining intensity within and distal to the lesion (Fig. 4C).

Ablating FnEDA improves functional recovery after SCI

To investigate whether inhibiting the chronic fibrotic response by targeting FnEDA can improve behavioral recovery after SCI, FnEDA-null mice and littermate wildtype controls were subjected to a contusion SCI. Hindlimb locomotor recovery was assessed weekly using both the Basso Mouse Scale (BMS) and modified Basso, Beattie, and Bresnahan Scale (mBBB) open field scoring methods. One day after injury, there were no differences between the BMS and mBBB scores of wildtype and FnEDA-null mice. Beginning at 4 weeks post injury, FnEDA-null mice displayed significantly improved functional capacity as compared to their wildtype littermates. This statistical separation was sustained each week until the end of the study (10 weeks post injury, Fig. 5A-D, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with groups compared by two-way repeated measures ANOVA with Bonferroni testing to correct for multiple comparisons between each week).

At 10 weeks post SCI, FnEDA-null mice had a mean BMS score of 2.4 and mean mBBB score of 3.9, while wildtype mice remained at a mean BMS score of 0.6 and mean mBBB score of 1.6. Almost 80% of the FnEDA-null mice had mBBB scores of 2 or greater at 10 weeks compared to less than 25% of the wildtype mice (Fig. 5D). 70% of the FnEDA mice had BMS scores of 1 or greater compared to only 35% of the wildtype mice at 10 weeks. Further, by the end of the study almost 30% of the FnEDA mice had BMS scores of 2 or greater whereas no wildtype mice achieved that score threshold (Fig. 5B). The overall treatment effect of FnEDA ablation was highly significant when assessed by both BMS ($p=0.0038$) and mBBB ($p=0.0006$) scoring paradigms. Linear regression analysis demonstrated that reduced levels of fibronectin (as assessed by western blot, shown in Fig. 2D) correlate well with improved BMS locomotor scores at the last behavioral session 10 weeks post injury. Pearson correlation coefficient $r = -0.631$, coefficient of determination $R^2 = 0.3981$.

Discussion

Fibrosis is a general feature of SCI

Although injured human spinal cords typically contain fluid-filled cavities, major compression and laceration injuries to the human spinal cord lead predominantly to fibrotic scarring and surprisingly small glial responses (Bunge *et al.*, 1997; Hermanns *et al.*, 2006; Klapka and Muller, 2006; Kim, 2013; Jeong *et al.*, 2017). Similar to glial scars, fibrotic scars remain chronically for the life of the patient. Such injuries were observed in 14 out of 48 human SCI specimens in the classic study of Bunge *et al.* Moreover, all injuries that occurred at the thoracic level resulted from such major compression or laceration type injuries (Bunge *et al.*, 1997). Thus fibrotic scarring is a common but not well recognized feature of human SCI pathology, and a better understanding of the fibrotic scar could lead to new approaches for promoting repair and regeneration after SCI.

No animal model can recapitulate human SCI pathology perfectly. However, rodent compression models can be useful for mimicking the fibrotic and connective tissue scar responses that occur after massive compression injuries in humans. The mouse spinal cord shows a dense accumulation of connective tissue molecules, including fibronectin, collagen, tenascin-C and CSPG, in the lesion epicenter that are also found in human spinal cord lesions (Aldskogius, 2013; Jeong *et al.*, 2017). Similarly, we have observed that the rat spinal cord displays both cavities and fibrotic scarring after SCI (data not shown), an observation reported previously by others (Zhu *et al.*, 2015b; Didangelos *et al.*, 2016; Kjell and Olson, 2016; Hong *et al.*, 2017). Thus, fibrotic scarring appears to be a general feature of the SCI response in many species including humans.

Similar to other organs, fibrotic responses after SCI are initiated when fibroblasts migrate into the injured area and secrete ECM proteins that assemble into scar connective tissue. Goritz *et al.* (2011) reported that GLAST⁺ type A pericytes that line blood vessels in the spinal cord parenchyma migrate into SCI lesions and become scar-forming fibroblasts, secreting fibronectin and collagen. These fibroblasts express PDGFRb and CD13, but after injury they lose CD13 expression while maintaining PDGFRb expression (Goritz *et al.*, 2011). Soderblom *et al.* (2013) showed that Col1a1⁺PDGFRb⁺CD13⁺ fibroblasts of perivascular origin migrate into the lesion after SCI and are the source of fibronectin (Soderblom *et al.*, 2013). These two populations of fibroblasts express PDGFRb and fibronectin and demonstrate similar spatial and temporal patterns of migration after SCI. Given these similarities, it is possible that the GLAST⁺ type A perivascular fibroblasts and the Col1a1⁺PDGFRb⁺CD13⁺ fibroblasts are, in fact, the same population of cells. Regardless, these studies demonstrate that fibroblasts of perivascular origin are the primary source of fibronectin after SCI.

Complete ablation of GLAST⁺ pericytes/fibroblasts results in failure to seal the lesion and causes open tissue defects after SCI (Goritz *et al.*, 2011). This suggests that pericytes play a critical role during the tissue repair process. However, other studies have demonstrated that inhibiting fibroblast migration and polarization after SCI by systemic administration of taxol or epothilone B significantly reduces fibrotic scarring and improves functional recovery in mice (Hellal *et al.*, 2011; Sengottuvel and Fischer, 2011; Ruschel *et al.*, 2015). Additionally,

limiting the infiltration of inflammatory monocytes reduces chronic fibrotic scarring in the injured spinal cord and improves behavioral outcomes (Jeong *et al.*, 2017). These findings suggest that the fibrotic scar, similar to the glial scar, plays both detrimental and beneficial roles after SCI. Thus, a better understanding of the mechanism by which the fibrotic scar is formed and regulated may inform the future development of treatments for SCI.

Fibronectin EDA contributes to formation of a degradation-resistant fibrotic scar

In this paper, we examined mechanisms underlying fibrotic scarring after SCI by defining the role of the FnEDA splice variant. FnEDA was minimally expressed in the uninjured spinal cord, but its expression increased significantly after SCI and it remained in the lesion for at least 90 days after SCI. Thus, accumulation of FnEDA is part of the fibrotic response after SCI. Interestingly, the expression pattern of FnEDA after SCI mirrors the temporal migration pattern of pericytes/fibroblasts observed in other studies. Similar to pericyte/fibroblast migration patterns after SCI, we observed a delay of at least 72 hrs before FnEDA began to accumulate within the lesion site. RNA and protein expression of FnEDA in the lesion site peaked at 7 days post SCI, the same time at which the greatest number of pericytes/fibroblasts are seen in the lesion. Cultured fibroblasts express FnEDA when stimulated with TGF β , a signal strongly upregulated after SCI (Balza *et al.*, 1988; Borsi *et al.*, 1990; Muro *et al.*, 2008; Bhattacharyya *et al.*, 2013). This suggests that increased TGF β signaling within the injured spinal cord may lead to the expression of FnEDA by pericytes/fibroblasts after SCI.

We also found that mice lacking FnEDA have smaller lesions and significantly attenuated fibrotic scarring at 90 days post injury, but not at 7 and 21 days post injury. This suggests that plasma fibronectin or other isoforms of cellular fibronectin predominate during the acute and sub-acute phases of SCI. Using the DOC digest protocol, we found that FnEDA-null mice show reduced levels of matrix-assembled insoluble fibronectin starting at 21 days post SCI. Levels of soluble, unassembled, fibronectin remain unchanged in FnEDA-null mice at all times. Based on these observations, we suggest that FnEDA may contribute to the formation of a fibronectin network that is resistant to degradation and scar resolution over time. It has been previously suggested that the lack of the EDA domain in plasma fibronectin induces a closed conformation, in which the integrin binding RGD domain is unavailable for binding. Inclusion of the EDA domain, however, induces conformational changes that expose the RGD, or other cell surface receptor-binding epitopes, and facilitates matrix assembly (Leahy *et al.*, 1996; Manabe *et al.*, 1997; Johnson *et al.*, 1999). Thus, the lack of the EDA domain after SCI may lead to an unstable assembly of the fibronectin matrix, which fails to resist long-term remodeling and degradation. It is also possible that ablating the FnEDA variant inhibits migration and proliferation of pericytes/fibroblasts after SCI since FnEDA can act as a signaling molecule via activation of TGF β 1 and TLR4 signaling pathways in fibroblasts to drive fibroblast activation (Balza *et al.*, 1988; Borsi *et al.*, 1990; Bhattacharyya *et al.*, 2014). Therefore, it is possible that ablating FnEDA deters fibroblast activation, consequently diminishing fibronectin synthesis. However, we did not observe any differences in pericyte/fibroblast migration and/or activation in the lesion at 7, 21, and 90 days post SCI in FnEDA-null animals as assessed by the intensity of PDGFRb staining in the lesion core (Supplementary Fig. 4), a reliable marker for pericytes/fibroblasts after SCI

(Soderblom *et al.*, 2013). In addition, we also did not observe any significant differences in TLR4, MyD88 or Nfkb protein expression between wildtype and FnEDA-null mice at 7 and 21 days post SCI (data not shown). Thus, our data suggest that FnEDA acts as a structural component, rather than a signaling molecule, mediating the chronic fibrotic response after SCI. One limitation of the present study is the utilization of mice with a constitutive homozygous deletion of the FnEDA domain. Future work is needed to develop a mouse model of inducible FnEDA knockout under the control of a pericyte/fibroblast-specific Cre-promoter. Use of such a model would further strengthen the conclusions of this work.

Decreased fibrosis in FnEDA-null mice is associated with improved behavioral recovery

Importantly, mice lacking FnEDA showed increased axon densities in the lesion and improved behavioral recovery after SCI. We did not investigate whether this reflects axonal regeneration or sparing. Tract tracing experiments will be needed to investigate this question which is another area for future study. Regardless, our data suggest that eliminating FnEDA creates a more conducive environment for axonal regeneration or sparing by reducing the amount of chronic fibrotic scarring. Across animals, increased levels of total lesion fibronectin were inversely correlated with BMS score during each animal's last behavioral session. We observed no significant changes in GFAP⁺ astrocyte density at 21 and 90 days post SCI in FnEDA-null mice, indicating that changes in fibrotic but not glial scarring led to the improved behavioral outcomes in FnEDA-null mice. While this suggests that the chronic fibrotic scar and its matrix of assembled cellular fibronectin is detrimental to recovery after SCI, our findings also suggest that the fibrotic scar, similar to the glial scar, might have different roles depending on the stage of formation. The early stage of fibronectin deposition, mainly composed of plasma fibronectin, reportedly exerts neuroprotective effects after traumatic brain injury and focal cerebral ischemic injury in mice (Sakai *et al.*, 2001; Tate *et al.*, 2007). Given that ablating the FnEDA isoform did not change acute or subacute fibronectin deposition after SCI, but still resulted in improved functional recovery, we propose that the early fibrotic response may serve protective roles whereas the chronic stage acts as an impediment to recovery.

In summary, our study provides new insights into the mechanisms of fibrotic scar formation and remodeling after SCI. Our results demonstrate that FnEDA is an important building block of the insoluble fibronectin matrix after SCI and that deleting FnEDA results in significantly reduced chronic fibrotic scarring and enhanced functional recovery.

Accordingly, we suggest that dampening the expression of FnEDA could represent a new therapeutic strategy for promoting recovery after SCI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Fibronectin EDA (FnEDA) enhances fibronectin matrix stability after SCI.
- FnEDA-null mice display improved motor recovery after SCI.
- Eliminating FnEDA does not reduce the acute fibrotic response.
- Eliminating FnEDA reduces chronic fibrotic scarring without altering glial scarring.
- FnEDA-null mice exhibit smaller lesions with higher axonal density.

Figure 1. FnEDA and FnEDB expression increase significantly after SCI

(**A and B**) qPCR quantification of lesional tissue at different time points after SCI. *FnEDA* and *FnEDB* gene expression levels are significantly increased inside the lesions after SCI (* $p < 0.05$, ** $p < 0.01$, Student's t-test, $n = 8-10$ mice per group). Levels of expression of both genes in the uninjured spinal cord were low, but detectable ($C_t = 30-31$). (**C and E**) Western blot analysis of lesional tissues at different time points after SCI ($n = 3$ mice/group). (**D**) The antibody for FnEDA was validated using the lesional tissues harvested from wildtype and FnEDA-null mice at 7 days post SCI. (**F and G**) Western blot quantifications for FnEDA and total fibronectin ($n = 3$ mice/group, *** $p < 0.001$, ANOVA followed by Tukey's multiple comparison test).

Figure 2. FhEDA-null mice have smaller lesions and attenuated fibrotic scarring at a chronic time point after SCI

(A-C) Western blot analysis of fibronectin in injured spinal cords of wildtype (WT) and FhEDA-null (KO) mice at 7, 21, and 90 days post SCI. (D) Quantification of fibronectin western blots at 7, 21, and 90 days post SCI (n=4-8 mice/group *** $p < 0.001$, ANOVA with Tukey's multiple comparison test). At 90 days post SCI, 20 μ m thick mid-sagittal sections were obtained from wildtype (WT) and FhEDA-null (KO) mice and stained for fibronectin and GFAP. (E) Representative images of wildtype and FhEDA-null mouse injured spinal cords at 90 days post SCI. Scale bar=250 μ m. (F) Lesion areas were measured by outlining the GFAP⁺ border (n=6-7 mice/group, ** $p < 0.01$, Student's t-test). (G and H) Quantification of fibronectin mean staining intensity inside the GFAP⁺ lesion core (Lesion Core) and GFAP mean staining intensity within the 250 μ m wide GFAP⁺ glial border area that immediately surrounds the lesion (Lesion Rim) (n=6-7 mice/group, ** $p < 0.01$, ANOVA with Tukey's multiple comparison test). (I) Western blot analysis of GFAP in wildtype and FhEDA-null

mouse injured spinal cords at 90 days post SCI (n=7 mice/group, ANOVA with Tukey's multiple comparison test).

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Figure 3. FnEDA-null mice develop less stable fibronectin matrix after SCI

DOC digests were performed at 7, 21, and 90 days post SCI to measure the amount of matrix-assembled (insoluble FN) and soluble fibronectin present in the injured spinal cords of wildtype (WT) and FnEDA-null mice (KO). (**A and B**) At 7 days post SCI, the levels of insoluble and soluble fibronectin did not differ between wildtype and FnEDA-null mice. (**C-F**) At 21 and 90 days post SCI, the level of soluble fibronectin did not differ between wildtype and FnEDA-null mice, but FnEDA-null mice showed significantly reduced amounts of insoluble fibronectin ($n=7-8$ mice/group, $**p<0.01$, ANOVA with Tukey's multiple comparison test).

Figure 4. FnEDA-null mice have higher axonal densities in lesions at 90 days post SCI

(A) Representative images of 20 µm thick mid-sagittal sections of wildtype and FnEDA-null mice at 90 days post SCI. Scale bar=250 µm (B) Enlarged images from the outlined lesion areas. Scale bar=50 µm. (C) Quantification of SMI-312 staining intensity. The staining intensity at different distances from the lesion center was normalized to that of 2mm rostral to the lesion site (n=5 mice/group). * $p < 0.05$, ** $p < 0.01$ with groups compared by ANOVA with Tukey's multiple comparison test. Distances rostral to the lesion center are defined as negative numbers, while distances caudal to the lesion center are defined as positive.

Figure 5. FnEDA-null mice show significantly improved functional recovery after SCI
 Locomotor function of wildtype and FnEDA-null mice was evaluated once every week (A) using the Basso Mouse Scale (BMS). (B) A threshold histogram demonstrates the number of mice in each treatment group that were able to achieve the listed BMS score thresholds during the final week of behavioral testing. (C) Locomotor function was also measured using the modified Basso, Beattie, Bresnahan (mBBB) scale. (D) A threshold histogram for mBBB scores. All data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with groups compared by two-way repeated measures ANOVA with Bonferroni's multiple comparisons correction.