Supporting Online Material for

Microtubule Stabilization Reduces Scarring and Causes Axon Regeneration After Spinal Cord Injury

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Movies S1 to S4
Materials and Methods

Surgical procedures. These studies were performed in accordance with the animal handling laws of the German government, and in accordance with protocols approved by the Institutional Animal Care and Use Committee at The Johns Hopkins University. Surgeries were performed on adult female Sprague-Dawley rats weighing 200-250 g.

Spinal cord injuries. Bilateral dorsal hemisection, or a dorsal column cut of the spinal cord was performed at thoracic level 8 using iridectomy scissors (1). Contusion injury was performed at thoracic level 9 using the Infinite Horizon (IH) impactor device (force of 150 kiloDynes; Precision Systems, Kentucky, IL) as previously described (2). Consistency between rats was achieved by recording the actual force applied to the cord and the cord displacement during injury. Sciatic nerve (conditioning) lesion. A complete transection of the right sciatic nerve was performed 2 weeks after dorsal hemisection as previously described (1). Anterograde neuronal tracing: Cholera Toxin subunit B (CTB) tracing was performed as described previously (1, 3).

Immunofluorescence. Tissue harvesting and processing was performed as previously described (4). Sections of the spinal cord were incubated overnight with different combinations of the following antibodies: monoclonal mouse anti-glial fibrillary acidic protein (GFAP, 1:200; Sigma), anti-collagen IV (1:500; Developmental Studies Hybridoma Bank), anti-Smad2/3 (1:500, R&D System), and polyclonal rabbit anti-laminin (1:100; Sigma), anti-fibronectin (1:500; Dako), anti-GFAP (1:400; Dako), anti-phospho-(Ser 423/425)-Smad2/3 (1:500; Santa Cruz), anti-NG2 (1:200; Chemicon), anti-phospho-(Ser19)-histone H3 (1:200; Upstate), anti-serotonin (1:500; Sigma). Sections were incubated for 3 hours with appropriate secondary antibodies conjugated
to Alexa 488, Alexa 350 or Alexa 568 (1:200; Invitrogen) fluorophores. DAPI (Invitrogen) was used as counterstain to visualize nuclei. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) staining was performed as previously described (5). Confocal images were acquired using a Leica SP2 confocal microscope system in sequential scanning mode. All images are shown with the rostral side to the left.

**Primary cell cultures.** Primary astrocytes were isolated from P2-5 rat brains as previously described (6). Cerebellar granule neurons were isolated from P8 mouse brains as described (7). Cortical neurons were obtained from P4 rat cortices as described (8) and purified by pre-plating them on non-coated tissue flasks in MEM with 10% fetal bovine serum (FBS) for 2 hours. Neurons were collected from the medium by centrifugation at 800 g for 5 minutes, and resuspended in neurobasal medium supplemented with 10% FBS, 2% B27, 2 mM Glutamine and 1% penicillin-streptomycin (all from Invitrogen). Neurons were plated onto 8-well dishes (10000 cells/well, NunC) coated with the following inhibitory substrates (all from Millipore): chondroitin sulfate proteoglycans (1.5 µg/ml), Semaphorin 3A (200 ng/ml), Myelin Associated Glycoprotein (MAG; 100 ng/ml) and NogoA (200 ng/ml) mixed with laminin (5 µg/ml; Roche). Taxol (5 nM) or DMSO was added to the cells 2 hours after plating. Neurons were fixed with 4% PFA at 48 hours post-plating and immuno-stained for β-III-tubulin (1:10000; Covance). Primary meningeal cells were obtained from cortical meninges of P2-3 rats and digested with Collagenase I (3000 U/ml; Cell System Biotechnology) during 45 minutes at 37°C (9). Cells were re-plated onto poly-lysine-coated coverslips and used for experiments after ten days in vitro (DIV). Before each experiment, the cells were serum starved for 48 hours. For the Smad2/3 translocation
experiment, cells were treated for 20 minutes with Taxol (100 nM) followed by TGF-β1 (10 ng/ml) for 1 hour. Cells were fixed with 4% PFA and immuno-stained for Smad2/3. For the double-staining with tubulin and Smad2/3, cells were simultaneously extracted and fixed with PHEM fixative to remove cytosolic components and components that only bind loosely to microtubules (10). The migration assay for meningeal cells and astrocyte was performed on confluent cultures using 2-well culture-inserts according to the manufacturer (Ibidi). Cells were subsequently treated with TGF-β1 (100 pg/ml), with DMSO (0.02%) or Taxol (10 nM) for three days. Finally, cells were fixed with 4% PFA and immuno-stained for fibronectin (1:200) and Cy3-conjugated actin phalloidin (1:50; Invitrogen). Quantitative analysis of outgrowth length was carried out by measuring the longest neurite per neuron on an average of 200 neurons per condition using Image J software as described (1).

**Dimethymethylene blue (DMMB) assay.** DMMB assay was performed as previously described (11) on conditioned serum-free media from meningeal cell and astrocyte confluent flasks after 3 DIV and from spinal cords 7 days after injury. Tissue was digested overnight at 37°C with Proteinase K (50 µg/ml; Sigma) prior to the assay. Samples treated with chondroitinase ABC (Associates of Cape Cod) were used as negative controls. Absorbance was measured at 656 nm. Sulfated GAG quantities were determined using a calibration curve of Chondroitin sulfate (Sigma) solutions treated as described (11).

**Videomicroscopy and Photoactivation.** Photoactivation and live-cell microscopy experiments were performed using a Leica TCS SP2 confocal scanning microscope equipped with an Argon Laser and two diode lasers (562 nm and 405 nm). Imaging was
performed under a Leica HCX PL APO 63x/1.40-0.60 oil immersion objective using 488 nm excitation and collecting emission with 500-550 nm bandwidths. HaCaT cells (kindly provided by Axel Ullrich, Max Planck Institute of Biochemistry, Martinsried, Germany) were plated onto 35 mm live-cell imaging dishes (Ibidi) and were transiently transfected (24 hours later) using lipofectamine LTX (Invitrogen) with the photoactivatable GFP-Smad2 construct (PAGFP-Smad2, kindly provided by Caroline Hill, London Research Institute, London, UK) (12-13). Cells were serum-starved for 24 hours before treatment with DMSO (0.02%) or Taxol (100 nM) and then stimulated 30 minutes later with 2 ng/ml TGF-β1. One hour after TGF-β1 stimulation, Smad2 translocation was monitored via photoactivation of the PAGFP moiety (in the entire cell cytoplasm excluding the nucleus) using a 405-410 nm excitation at 45% laser power. Images were acquired at 30 second intervals using the live-imaging mode of the Leica confocal software. Videos were compiled at 7 frames per second using Adobe Photoshop CS4 software.

**Inducible intracellular cargo-trafficking assay.** The activity of kinesin-1 and dynein was assessed using an intracellular cargo trafficking assay (14). This assay probes the motility of RFP-labeled peroxisomes to which the motor protein of interest is recruited during the experiment (fig. S7). COS-7 cells were transfected with two fusion constructs: (1) Peroxisomal biogenesis factor 3 (PEX) fused to RFP and FK506 binding protein (FKBP) which targets peroxisomes. (2) FKBP-rapamycin-binding domain (FRB) with either a truncated KIF5 motor construct or with a dynein/dynactin adaptor (BICDN). Upon addition of Rapalog the FRB-linked motor domain binds to the FKBP domain, which is linked to the peroxisomes. Generation of pβactin-PEX3-mRFP-FKBP, KIF5B(1-807)-GFP-FRB, and HA-BICD2(1-594)-FRB has been described.
previously (14). FKBP- and FRB-encoding fragments were obtained from Argent Regulated Heterodimerization kit (Ariad Pharmaceuticals).

Time-lapse live-cell imaging was performed on a Nikon Eclipse TE2000E (Nikon) equipped with an incubation chamber (Tokai Hit; INUG2-ZILCS-H2) mounted on a motorized stage (Prior). Coverslips (24 mm) were mounted in metal rings, immersed in 0.6 ml Ringer’s solution (10 mM Hepes, 155 mM NaCl, 1-2 mM CaCl₂, 1 mM MgCl₂, 2 mM NaH₂PO₄, 10 mM glucose, pH 7.2) and maintained at 37°C (and 5% CO₂). 4-6 cells were selected and imaged every 30 seconds for 30-60 minutes using a 40x objective (Plan Fluor, NA=1.3, Nikon) and a Coolsnap HQ camera (Photometrics). A mercury lamp (Osram) was used for excitation. Rapalog (AP21967) was dissolved to 0.1 mM in Ethanol. To induce motility during image acquisition, 0.2 ml of culture medium or Ringer’s solution (COS-7 cells) with Rapalog (0.4 μM) was added to establish a final Rapalog concentration of 0.1 μM.

Images of live cells were processed and analyzed using MetaMorph (Molecular Devices) or LabVIEW (National Instruments) software. Before analysis, cells were masked to exclude contributions from neighboring cells from the analysis. For the color plots in Fig. 2E all images of a time-lapse recording were thresholded to yield binary images that were subsequently overlaid non-transparently, starting with the final frame (first frame on top). Each frame was colored using a time-coded gradient that ran from blue to white before and from white to red after Rapalog addition at 0:00. To calculate the displacement curves shown in Fig. 2E, each pixel value above threshold was inserted into a histogram of intensity \( I \) versus distance from center for each video frame, using the pixel size (161 nm) as bin size. From such a histogram, the radius required to
include a fraction $p$ of peroxisomes could be obtained by finding the first bin $B_n$ for which $\sum_{i=B_n}^{\infty} I(B_i) \geq p \sum_{i=1}^{N} I(B_i)$, where $N$ is the total number of bins.

**Microtubule/Tubulin assay.** Determination of the ratio of microtubule content versus free tubulin was carried out using the Microtubules/Tubulin in vivo assay kit (Cytoskeleton). Dissected spinal cord lesions from vehicle and Taxol-treated animals 7 days post-injury as well as sample of spinal cord from non-injured animals ($n=6$ per group) were processed according to the manufacturer. Proteins from obtained supernatants (free tubulin) and pellets (polymerized microtubules) were resuspended in SDS-PAGE sample buffer, separated by electrophoresis on 4-12% and 4-8% gels as previously described (8) and probed with monoclonal $\beta$-tubulin (1:10000; Cytoskeleton) and polyclonal KIF5B (1:500; AbCam) antibodies. Quantification of the cellular tubulin was performed according to the manufacturer using tubulin standard as positive control.

**Far Western blot and co-immunoprecipitation.** Far western blot and co-immunoprecipitation were performed on brain and spinal cord extracts of non-injured animals ($n=5$). Far Western blot was performed as previously described (15). In brief, the extracts were homogenized in microtubule stabilizing buffer (35 mM PIPES (pH 7.4), 5 mM MgSO4, 5 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol (DTT) and 1X of protease inhibitor cocktail (Roche)) and centrifuged at 10000 g for 15 minutes. The fraction of the resulting low-speed spin supernatants and equal protein amount of albumin (negative control; Sigma) was separated on 4-8% Tris-Acetate gel (Invitrogen) and was electro-blotted onto nitrocellulose membrane (Biorad). Proteins were renatured in vitro by incubation of the membrane in graded solutions of guanidine/HCl 6-0 M (in
25 mM Tris buffer pH 7.5 supplemented with 100 mM NaCl, 1 mM EDTA, 0.1% Tween, 10% glycerol, 2% nonfat milk powder and 1 mM DTT), blocked with 5% non-fat dried milk in PBS, and incubated at 4°C with 10 µg of the His-tagged human recombinant protein Smad2 (Prospectbio) overnight. After washing, membranes were incubated with the monoclonal His antibody (1:1000, AbCam), followed by HRP-conjugated, goat anti-mouse IgG. Membranes were developed with ECL Plus substrate (Amersham Pharmacia Biotech) and exposed on Hyperfilm ECL. The membrane was stripped off and rebotted with the monoclonal anti-kinesin-1 (H2) antibody (1:500, Chemicon).

The co-immunoprecipitation was performed on enriched high density vesicle-bound kinesin complex of the low-speed spin supernatants (16). The low-speed spin supernatants were further centrifuged at 171000 g at 4°C for 30 minutes. The resulting pellet containing the vesicular fraction was resuspended in the microtubule stabilizing buffer supplemented with 0.5 M NaCl and 2% CHAPS. Detergent insoluble material was removed by centrifuging at 100000 g at 4°C for 30 minutes. The soluble fraction was adjusted to 20 µM Taxol, 2 mM AMP-PNP, 1 mM GTP to promote the binding between microtubules and kinesins, incubated at room temperature for 20 minutes prior addition of the polyclonal rabbit Smad2 antibody (1:50; Cell signalling). Irrelevant antibody at the same concentration (polyclonal rabbit anti-GFP; Chemicon) and beads alone were used as negative controls. After incubation at 4°C under gentle mixing overnight, samples were transferred to new tubes containing 30 µl of EZview red protein-G beads (Sigma) and further incubated at 4°C under gentle mixing for 3-4 hours. The immunocomplexes were recovered by centrifugation and washed with 50 mM Tris buffer pH 7.5 (150 mM NaCl, 5 mM EDTA). The immunocomplexes were
eluted by suspending the beads in 2X SDS-PAGE sample buffer, resolved by SDS-PAGE and blotted with the monoclonal anti-kinesin heavy chain antibody SUK4 (1:500, Covance).

**Taxol treatment in vivo.** Taxol was dissolved in a mixture of ethanol and cremophor EL oil (1:1; Sigma) and administered through an intrathecal catheter connected to an osmotic mini-pump (Alzet model 2004; Charles River). Immediately after injury, a second laminectomy was performed at thoracic level 12; a catheter was inserted through a small hole made in the Dura mater, and navigated up to the injury site ensuring that Taxol or vehicle (control) was delivered at the lesion site. Accurate working doses for Taxol infusions were defined by pilot dose-response studies within the nanogram range per day and per animal. For this study, mini-pumps were loaded with Taxol to achieve a daily dose of 256 ng per animal. Importantly, we did not observe any obvious adverse effect of Taxol treatment on the general health of the animals compared to controls.

**Grid walk test.** Locomotor function was assessed by testing the ability of rats to cross a 1 m long horizontal runway with round metal bars elevated approximately 15 cm from the ground (17-18). To prevent habituation to a fixed bar distance, the bars in this sector were placed irregularly (1-4 cm spacing) and were changed for every testing session. Each animal was videotaped while crossing the grid two times for proper identification and quantification of footfalls. Analysis was performed over a defined sector containing a fixed number of bars. Testing was done every other week starting 2 weeks after injury and up to 8 weeks. The test was performed in a blind fashion.

**Quantification and statistical analysis.** *Quantitative measurement of the fibrotic scar* and injury size at 7 days post-injury (dpi) as well as the glial scarring at 4 weeks after
injury were performed on 3 serial sagittal representative sections covering the left, middle and right sides of the spinal cord (250 µm interval between sections). Fibrotic scar evaluation was assessed by pixel counts of areas stained with laminin and fibronectin using Adobe Photoshop software. Injured areas were calculated by measuring the area of the lesion delineated by GFAP immunostaining using Image J software (19). Glial scar state was evaluated using a semi-quantitative scoring scale adapted from Hsu et al. (4, 20) over 1.5 mm long cord segments covering the rostral side of the lesion. Each segment was subdivided in 200 µm wide sectors starting from the lesion edge and scored by ranking the complexity level of the glial network from 0 (no glial scar) to 3 (hypertrophic scarring and formation of glia limitans excluding the lesion). Regeneration of CTB traced sensory fibers was measured as previously described (1). Animals with spared fibers were excluded. Quantitative measurement of 5HT-positive axons caudal to the lesion site was performed by counting the number of 5HT-positive fibers on 3 representative sections covering the left, middle and right sides of the spinal cord (250 µm intervals between sections). Only the positive fibers present on the dorsal part of the spinal cord caudal to the lesion were counted. All analysis and measurements were conducted in a blind fashion. Statistical analyses were performed using a Student’s t test with two-tailed distribution and unequal variances. For multiple group comparison, ANOVA was applied.

References

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Supplementary figure legends

Figure S1: Taxol decreases fibronectin, NG2 proteoglycan and collagen IV accumulation induced by spinal cord injury

(A) Representation of lesioned spinal cord (box). (B-D) Mid-saggital sections of lesion site from rat treated with Vehicle or Taxol at 7 days post-injury. Taxol decreases ECM deposit in the core of the lesion as assessed by fibronectin (B), collagen IV (C) and NG2 proteoglycan (D) immunostainings. The dashed lines outline the lesion site. Scale bar, 150 µm.

Figure S2: Taxol does not interfere with proliferation

(A) Representation of lesioned spinal cord (box). (B-C) Mid-saggital sections of lesion site from rats treated with Vehicle or Taxol (7 days post-injury) stained with DAPI (B) and phospho-histone H3 (C). Scale bar, 150 µm. (D) Taxol does not affect cell proliferation induced by spinal cord injury. Results expressed as mean ± SEM; p=0.591; two-tailed t test (n = 7 animals per group).

Figure S3: Taxol does not induce apoptosis in the injured spinal cord

(A) Representation of lesioned spinal cord (box). (B-C) Mid-saggital sections of lesion site from rats treated with Vehicle or Taxol stained with TUNEL at 3 (B) and 7 (C) days after dorsal hemisection injury. Scale bar, 150 µm. (D) Taxol does not affect the cell death induced by spinal cord injury. Results expressed as mean ± SEM; 3dpi: p= 0.628; 7dpi: p= 0.679; two-tailed t test (n = 7 animals per group).

Figure S4: Taxol sequesters Smad2 onto microtubules
Astrocytes treated with DMSO or Taxol (100 nM) 30 minutes prior to TGF-β1 (2 ng/ml) stimulation. Cells were permeabilised during fixation to extract soluble components of the cytoplasm and stained with Smad2/3 antibody. Taxol induces Smad2/3 co-localization with microtubules. Scale bar, 10 µm.

**Figure S5: Taxol promotes axonal outgrowth of primary neurons plated on inhibitory substrates**

(A) Cerebellar granule neurons (CGNs) plated on laminin or CSPGs at 2 DIV. (B) Taxol increases neurite growth of CGNs plated on laminin and CSPGs. Results are means ± SD from 3 independent experiments; \( n = 150-200 \) cells per condition; * \( p = 0.016 \); \( p = 0.038 \) respectively; two-tailed \( t \) test. (C) Taxol increases neurite outgrowth of primary cortical neurons plated on MAG, NogoA, Semaphorin 3A or CSPGs. Results are means ± SD from 3 independent experiments; \( n = 150-200 \) cells per condition; ** \( p = 0.005 \), \( p = 0.009 \), \( p = 0.007 \), \( p = 0.002 \) and \( p = 0.001 \), respectively; one way ANOVA).

**Figure S6: Taxol promotes formation of growth cone-like structures at the tip of the 5HT-positive axons**

Two representative examples of axonal tips of 5 HT-positive axons from Vehicle and Taxol treated animals 4 weeks after injury. Scale bar, 10 µm.

**Figure S7: Inducible intracellular cargo-trafficking assay**

Fusion construct of PEX, RFP and FKBP targets the peroxisomes. A fusion of FRB with a truncated KIF5 motor construct or with a dynein/dynactin adaptor (BICDN) is recruited to FKBP and consequently to the peroxisomes upon addition of Rapalog. For details, see Materials and Methods.
Supplementary movie S1: PAGFP-Smad2 translocates into the nucleus in response to TGF-β stimulation

HaCaT cells transfected with PAGFP-Smad2 after 2 DIV and treated with DMSO 30 minutes prior to TGF-β stimulation (2 ng/ml). PAGFP-Smad2 was photoactivated 1 hour post-stimulation. Images were acquired at 30 second intervals. Movie plays at 7 frames per second.

Supplementary movie S2: Taxol treatment prevents the translocation of PAGFP-Smad2 in response to TGF-β stimulation

HaCaT cells transfected with PAGFP-Smad2 after 2 DIV and treated with Taxol (100 nM) 30 minutes prior to TGF-β stimulation (2 ng/ml). PAGFP-Smad2 was photoactivated 1 hour post-stimulation. Images were acquired at 30 second intervals. Movie plays at 7 frames per second.

Supplementary movie S3: Gridwalk of a representative vehicle-treated animal 6 weeks after contusion injury

Vehicle-treated animal videotaped while crossing the grid from the Gridwalk test 6 weeks after contusion injury.

Supplementary movie S4: Gridwalk of a representative Taxol-treated animal 6 weeks after contusion injury

Taxol-treated animal videotaped while crossing the grid from the Gridwalk test 6 weeks after contusion injury.
Hellal et al, Figure S1

A

Rostral  Caudal

B

Vehicle  Taxol

Fibronectin

C

Collagen IV

D

NG2 proteoglycan
A

Rostral  Caudal

B

Vehicle  Taxol

DAPI

C

Phospho-histone H3

D

![Graph showing number of proliferative cells/mm² for Vehicle and Taxol, with no significant difference (n.s.)](image)

Hellal et al, Figure S2
Figure S3

A

Rostral    Caudal

B

Vehicle      Taxol

TUNEL

3dpi

C

7dpi

D

Vehicle  Taxol

n.s.

Number of TUNEL-positive cells/mm²

3dpi   7dpi
Hellal et al, Figure S5

A  
B

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<th></th>
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<td>Neurite length (µm)</td>
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- **PBS**
- **CSPG**

**PBS**

**CSPG**
Hellal et al, Figure S6
Peroxisomal biogenesis factor 3 (PEX)
FKBP-rapamycin-binding (FRB) domain fused to Kinesin
FKBP-rapamycin-binding (FRB) domain fused to N-terminus of Bicaudal D2 (BICDN)
Dynein

Hellal et al, Figure S7