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## Transient activation of Wnt/ $\beta$ -catenin signaling reporter in fibrotic scar formation after compression spinal cord injury in adult mice

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### Abstract

After traumatic spinal cord injury (SCI), a scar may form with a fibrotic core (fibrotic scar) and surrounding reactive astrocytes (glial scar) at the lesion site. The scar tissue is considered a major obstacle preventing regeneration both as a physical barrier and as a source for secretion of inhibitors of axonal regeneration. Understanding the mechanism of scar formation and how to control it may lead to effective SCI therapies. Using a compression-SCI model on adult transgenic mice, we demonstrate that the canonical Wnt/ $\beta$ -catenin signaling reporter TOPgal (TCF/Lef1-lacZ) positive cells appeared at the lesion site by 5 days, peaked on 7 days, and diminished by 14 days post injury. Using various representative cell lineage markers, we demonstrate that, these transiently TOPgal positive cells are a group of Fibronectin(+);GFAP(-) fibroblast-like cells in the core scar region. Some of them are proliferative. These results indicate that Wnt/ $\beta$ -catenin signaling may play a key role in fibrotic scar formation after traumatic spinal cord injury.

### Keywords

Traumatic spinal cord injury (SCI); spinal cord compression; fibrotic scar; Wnt/ $\beta$ -catenin signaling; TOPgal; transgenic mice

### Introduction

Wnt signaling is activated in neural stem cells and progenitors as well as in glial cells, and is critical for neurogenesis in the embryonic and adult CNS [1–6]. Wnt signaling also plays an indispensable role in neurite outgrowth and axon guidance during neural development and regeneration [7–11]. In the adult spinal cord, the expression of various Wnt signaling

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molecules is normally low, but is induced by spinal cord injury (SCI) in rodents or zebrafish [12–15]. Reinduction of repulsive Wnt/Ryk signaling molecules in the adult spinal cord has been shown to suppress axonal regeneration after SCI [16, 17]. Conversely, Wnt3a administration enhances axonal regeneration and functional recovery after SCI in rats [18, 19], while overexpression of an inhibitory ligand Dkk inhibits axon regeneration in zebrafish [15]. Nevertheless, the identities of the Wnt responsive cells in the injured spinal cord and the cellular mechanisms by which Wnt signaling influences recovery from SCI remain poorly understood.

The potential of axons to regenerate is likely conserved in the adult spinal cord, but it is blocked by various inhibitory factors generated at the lesion site [20–22]. Immediately after SCI, oligodendrocyte/myelin-derived axon growth inhibitors, such as Nogo, MAG, and OMG are produced [23–25]. Subsequently, a scar forms at the lesion site that becomes a physical barrier and also generates chemical inhibitors, such as chondroitin sulfate proteoglycans (CSPGs), further limiting the regenerative potential of the adult CNS axons [26–28]. The core scar region consists of dominantly fibroblasts that secrete extracellular matrix molecules, including Fibronectin, Laminin, and collagen type IV (Col IV) [29, 30], but the cellular and signaling mechanisms responsible for fibrotic scar formation are not well understood.

In this study, we determined that the injury-induced Wnt responsive cells are neither neuronal nor glial lineages, but a group of fibroblasts at the lesion site. Many of them are proliferative. These results provide new insights into the function of Wnt/ $\beta$ -catenin signaling in fibrotic scar formation after adult neural injury and suggest Wnt signaling as a novel therapeutic target for control of scar formation and enhanced regeneration after SCI.

## Materials and methods

### Animals

Wnt signaling reporter TOPgal transgenic mice (*Tg(Fos-lacZ)34Efu/J*) [31] were obtained through the Jackson Laboratory and expanded/housed in the UC Davis vivarium. Two- to four-month-old adult mice were used in this study. All animal procedures were approved by the Institutional Animal Care and Use Committee at UC Davis and conformed to NIH guidelines.

### Surgical procedures

All surgeries were performed under aseptic conditions. Spinal cord injury was carried out using the compression or crush method as described previously with minor modifications [32–34]. Animals were anesthetized with Ketamine/Xylazine (100/10 mg/kg) administered intraperitoneally (i.p.). Depth of anesthesia was assured by monitoring lack of response to a foot pinch prior to the surgery. The back of the mouse was shaved and disinfected with povidone-iodine (Betadine) and 70% alcohol. A skin incision was made above the lower thoracic vertebrae. Paravertebral muscles on both sides of the T10-T12 vertebrae level were cut, and the vertebrae were exposed. A laminectomy was then performed between T10-T12, and the spinal cord was compressed by inserting a sterilized No.5 Dumont forceps (Fine

Science Tools) with or without 0.5 mm spacer [32] for 30 seconds at the T11 level. After the compression injury, muscles and skin were sutured, and animals were placed on a heat pad until ambulatory. Each animal was given systematic postoperative analgesia (Buprenorphine, 0.05mg/kg i.p.) to prevent pain. Animals were examined daily to record their body weight. After surgery, the mouse bladder was manually expressed twice daily until the recovery of spontaneous bladder function, which generally occurred between 7 and 14 days post-injury (dpi).

### **X-gal staining and immunohistochemistry**

For X-gal staining to detect TOPgal activity, the injured mice were perfused transcardially with phosphate-buffered saline (PBS, pH7.4). The spinal cord was dissected out and immersed into X-gal (5-bromo-4-chloro-3-indolyl-beta-d-galactopyranoside) substrate solution (with 1 mg/ml X-gal in 5 mM  $K_4Fe(CN)_6$ , 5 mM  $K_3Fe(CN)_6$ , and 5 mM  $MgCl_2$ ) for 12–24 hours in the dark to detect  $\beta$ -galactosidase enzymatic activity. The stained sample was washed three times with PBS and postfixed with 4% paraformaldehyde (PFA), then processed for histological analysis. For co-labeling of X-gal and immunohistochemistry, the X-gal-stained and fixed tissue was washed with PBS and incubated in 30% sucrose at 4°C and embedded in O.C.T. compound (Sakura Finetek). Fourteen-micrometer thick longitudinal frozen sections were prepared. Bright-field immunohistochemistry was performed using an HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) and color-developed with diaminobenzidine (DAB) (Sigma). The injury site and adjacent spinal cord tissue (2.5 mm caudal and rostral from the epicenter, 5 mm total length) was dissected out and post-fixed in 4% PFA. Primary antibodies including the Goat anti-Nestin (1:100, Santa Cruz), mouse anti-BrdU (1:100, Dako), rat anti-MBP (Dako, 1:200), rabbit anti-NG2 (1:200, Millipore), rabbit anti-Fibronectin (1:1000, Millipore), rabbit anti-GFAP (1:1000, Dako), mouse anti-GFAP (1:500, Covance), rabbit anti-Laminin (1:1000, Millipore), rabbit anti-p75 (1:500, Millipore), rabbit anti-PDGFR $\beta$  (1:100, Upstate), and rabbit anti-Vimentin (1:1000, kindly provided by P. Fitzgerald at UC Davis) were used in this study. Stained sections were examined and photographed using Olympus BX61 microscope for bright fields.

## **Results**

### **Spinal cord injury induced transient activation of the Wnt signaling reporter TOPgal**

In order to investigate the effects of SCI on canonical Wnt/ $\beta$ -catenin signaling, we performed complete compression injury at the lower thoracic level to induce mechanical and ischemic injuries in adult TOPgal reporter mice. Wnt signaling reporter TOPgal activity in the injured spinal cord was detected by X-gal staining, and showed a dynamic change within the first two weeks after SCI (Fig. 1). X-gal positive cells began to appear at the lesion site by five days post injury (5 dpi, Fig. 1C,F), reached peak numbers by 7 dpi (Fig. 1G,J), were substantially diminished by 14 dpi (Fig. 1H,K), and had almost disappeared by 21 dpi (Fig. 1I,L). These observations indicated that Wnt/ $\beta$ -catenin signaling is transiently induced by SCI in a group of cells at the lesion site.

### The SCI-induced Wnt responsive cells were proliferative non-neural stem cells

To determine the cell identity of the SCI-induced TOPgal(+) cells at the lesion site, we performed immunohistochemistry with antibodies for representative cell lineage marker proteins on the spinal cord sections after X-gal staining (Fig. 2). As Wnt/ $\beta$ -catenin signaling is crucial in the proliferation and differentiation of neural stem cells that may be responsive to SCI, we used anti-Nestin antibodies to mark the possible neural stem cell population. We found no overlapped labeling of the Nestin(+) cells and X-gal stained cells (Fig. 2A), indicating that these SCI-induced TOPgal(+) cells are not neural stem cells. BrdU incorporation and immunolabeling assay demonstrated that one half of the BrdU(+) proliferating cells were X-gal stained Wnt responsive cells (Fig. 2B). Many X-gal stained cells were intermingled with the crushed MBP(+) myelinated axonal fibers and oligodendrocytes or NG2 positive polydendrocytes around the lesion site (Fig. 2C,D).

### The SCI-induced Wnt responsive cells were Fibronectin(+);GFAP(-) fibroblast-like cells in the core scar region

We found that most X-gal stained cells at the epicenter of the lesion site were co-immunolabeled with antibodies against the fibroblastic markers Fibronectin, Laminin, and Vimentin (Fig. 3), but did not express GFAP (Fig. 4), whereas cells surrounding this core were X-gal negative but GFAP positive (Fig. 4A,B). These results indicated that the Wnt signaling reporter TOPgal was activated in the fibroblast-like cells that formed the core fibrotic scar after SCI. Moreover, we demonstrated that the core fibroblast-like cells were not co-immunolabeled for p75, a protein normally expressed in Schwann cells and induced in oligodendrocytes by SCI [35] (Fig. 4C,D), nor for PDGFR $\beta$ , a marker for pericytes [36, 37] (Fig. 4E,F). These observations suggest a lineage-specific activation of Wnt/ $\beta$ -catenin signaling in fibroblast-like cells at the epicenter of the lesion site.

## Discussion

Our study, which used a murine spinal cord compression model, demonstrates that Wnt signaling is activated by compression injury of spinal cord. These results are consistent with prior reports that Wnt ligands and receptors are upregulated in the injured spinal cord after hemisection or contusion lesions [12–14, 16], but adds to those studies by showing that Wnt signaling, as demonstrated with the Wnt/ $\beta$ -catenin signaling reporter TOPgal, is induced specifically in the fibrotic scar. Our finding of the fibrotic activation of Wnt signaling reporter TOPgal is completely different from a previous study that demonstrates induction of another Wnt signaling reporter BATgal in neurons of the dorsal horn and cells of the central canal of uninjured spinal cords after hemisection [14]. Notably, many of the SCI-induced TOPgal active cells are proliferative, suggesting a potential role of Wnt/ $\beta$ -catenin signaling in the expansion of fibroblasts and the formation of the fibrotic scar. SCI-induced scar formation interrupts regeneration physically and chemically [38, 39]. This study is the first to address the role of Wnt signaling in scar formation in the injured spinal cord, but is consistent with a recent publication showing that Wnt signaling is critical in the formation of fibrosis in skin, liver, and lung, and that overexpression of the inhibitory ligand Dkk1 is sufficient to reduce fibrosis in the skin [40]. These results suggest a general function of Wnt signaling in fibrosis in both injured CNS and peripheral organs/tissues.

The involvement of Wnt signaling in SCI has been previously reported at different aspects. The expression of Wnt ligands and receptors are upregulated in the injured spinal cord after hemisection or contusion lesions in rats or mice [12–14, 16]. However, the effects of this upregulated Wnt signaling with respect to myelin loss, modulation of axonal regeneration, and neural precursor differentiation are controversial, and may vary with the injury model [16, 18, 19]. Spinal cord compression injury (also referred to as crush injury) that we used in this study has been recognized as a reliable experimental approach in a wide range of SCI studies [32, 34, 41–44]. In this study, the compression injury induced a defined area of fibrosis, which will allow us to examine the effect of Wnt signaling in fibrotic scar formation in future studies.

We observed that the injury-induced Wnt signaling reporter TOPgal activity was transiently present only in a subpopulation of the fibrotic cells at the lesion site. A recent study showed that a subpopulation of pericytes is the main source of the glial-fibrotic scar after dorsal funiculus incision or hemisection [37]. These pericyte-originated cells seem to fill the core of the injury and are negative for GFAP. However, we did not detect TOPgal activation in PDGFR $\beta$ (+) pericytes at 7 dpi. The TOPgal reporter is regulated by three sets of Wnt responsive elements in the promoter region, which may visualize only the most intensively activated Wnt signaling in the cells. Therefore, only those cells with high levels of Wnt signaling may transiently become TOPgal positive, which may possibly represent intermediates between pericytes and fibroblasts. Nevertheless, the cellular origins of the TOPgal(+) cells and the role of Wnt signaling in the initiation of the potential transformation from pericytes to fibroblasts after SCI remain to be addressed in future studies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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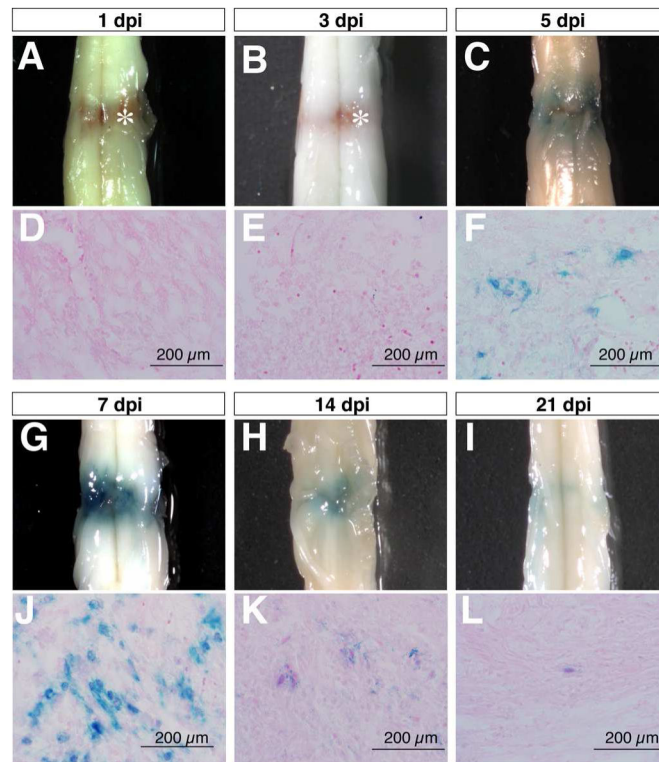


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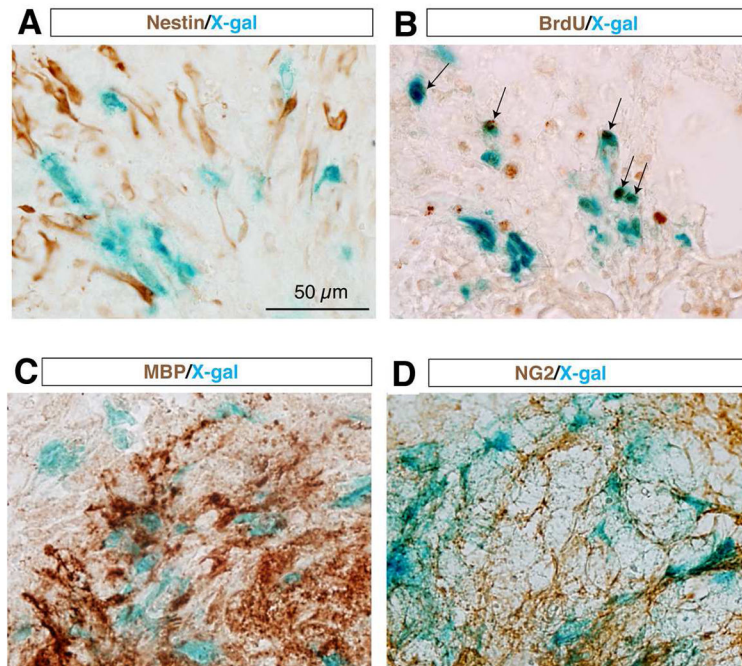


**Highlights**

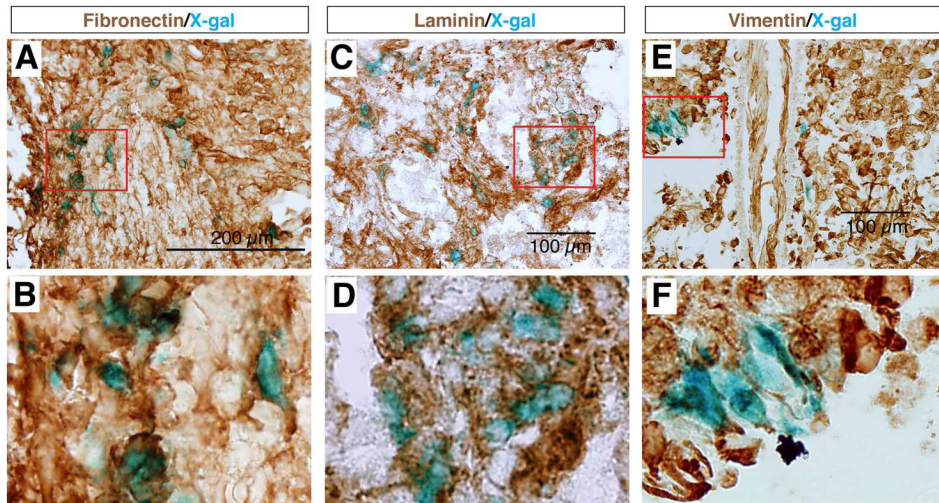
- The Wnt/ $\beta$ -catenin signaling reporter TOPgal is transiently activated at the lesion site with a peak on 7 days after compression spinal cord injury in adult mice.
- The SCI-induced TOPgal positive cells are fibrotic cells in the core scar region.
- Some SCI-induced TOPgal positive fibroblast-like cells are proliferative.



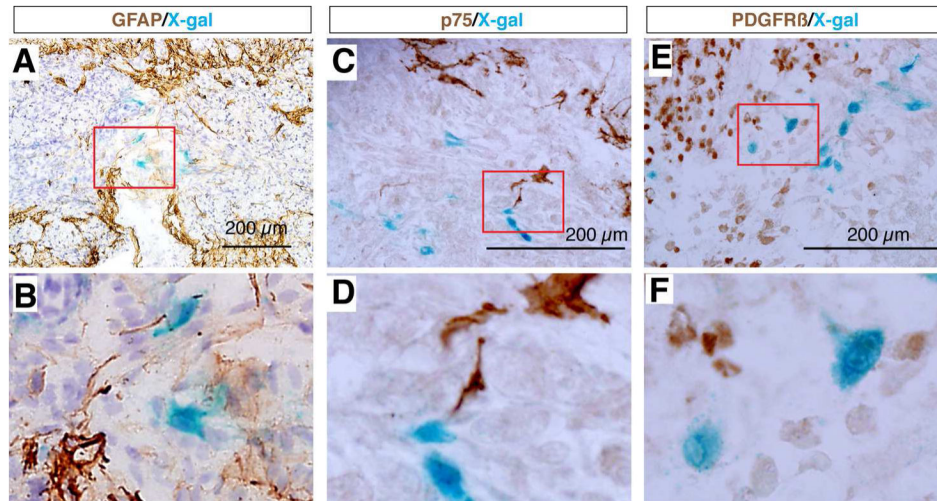
**Fig. 1.** Compression spinal cord injury activates the Wnt signaling reporter TOPgal in a time-dependent manner. The wholemount spinal cords (A-C,G-I) and respective transverse sections (D-F,J-L) show the X-gal staining results for the TOPgal activities in the adult TOPgal mice at 1, 3, 5, 7, 14, and 21 days post injury (dpi). Note that the blue X-gal staining appears at 5 dpi (C,F), peaks at 7 dpi (G,J), and diminishes dramatically at 14 (H,K) and 21 dpi (I,L). Asterisks (A,B) indicate the reddish-brown substance caused by hemorrhage in the lesion site at 1 and 3 dpi. The sections were counterstained with Eosin solution.



**Fig. 2.** The SCI-induced TOPgal positive cells are proliferative and non-neural stem cells. The injured spinal cord at 7 dpi were stained with X-gal solution, and then immunolabeled with antibodies against the neural stem cell marker Nestin (A), incorporated BrdU (B), myelin basic protein MBP (C), and polydendrocyte marker NG2 (D). Arrows in B indicate the cells co-labeled by X-gal staining (blue) and BrdU immunoreactivities (brown).



**Fig. 3.** The SCI-induced TOPgal positive cells are fibroblast-like cells. The X-gal stained cells are co-immunolabeled with the fibroblastic marker antibodies against Fibronectin (A,B), Laminin (C,D), and Vimentin (E,F). Red rectangles in the upper panels indicate the area enlarged in the respective lower panels.



**Fig. 4.**

The SCI-induced TOPgal positive cells neither glia nor pericytes. X-gal stained cells do not express the astrocyte marker GFAP (A,B), injured oligodendrocyte marker p75 (C,D), and pericyte marker PDGFR $\beta$  (E,F). Red rectangles in the upper panels indicate the area enlarged in the respective lower panels.