Editors' Choice

ORIGINAL PAPER

Nagoya J. Med. Sci. **87**. 305–319, 2025
doi:10.18999/nagjms.87.2.305

Meflin/Islr is a marker of fibroblasts that arise in fibrotic regions after spinal cord injury

Yoshinori Morita[1,2], Yukihiro Shiraki[1], Akira Kato[3], Yasuhiro Nagatani[1,2], Ryota Ando[1], Hiroaki Nakashima[2], Shiro Imagama[2] and Atsushi Enomoto[1,4]

[1]*Department of Pathology, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[2]*Department of Orthopedic Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[3]*Institute of Innovation for Future Society, Nagoya University, Nagoya, Japan*
[4]*Center for One Medicine Innovative Translational Research (COMIT), Nagoya University, Nagoya, Japan*

ABSTRACT

Scar formation after spinal cord injury (SCI) hampers axonal regeneration and functional recovery. Previous studies have shown that scar formation is attributable to both gliosis and fibrosis, the latter requiring fibroblast proliferation and extracellular matrix deposition. In this setting, there are essentially two cell types generating new fibroblasts: pericytes and tissue-resident fibroblasts. Here, we showed that Meflin, a glycosylphosphatidylinositol-anchored protein (a specific marker of fibroblasts across multiple organs) is expressed by fibroblasts in the normal mouse spinal cord. An in situ hybridization analysis showed that Meflin$^+$ cells arose from the meninges and perivascular region of the spinal parenchyma after spinal cord compression injury. That finding was corroborated by single-cell transcriptomic data from normal and injured mouse spinal cords. Interestingly, Meflin$^+$ cells are positive for the fibroblast markers collagen type I and platelet-derived growth factor receptor (PDGFR) α but not for pericyte markers such as PDGFRβ and chondroitin sulfate proteoglycan 4 in the normal spinal cord. Those findings are consistent with the recent view that tissue-resident fibroblasts play a central role in many other types of fibrotic disease. A lineage-tracing experiment using a knock-in mouse line that expressed inducible Cre recombinase under the control of the Meflin promoter showed that Meflin$^+$ cells yield PDGFRβ$^+$ myofibroblasts but not glial cells positive for glial fibrillary acidic protein. These findings suggest that the Meflin$^+$ population contains the cells of origin of myofibroblasts that are involved in scar formation after SCI.

Keywords: spinal cord injury, fibrosis, Meflin, immunoglobulin superfamily containing leucine rich repeat, Islr

Abbreviations:
SCI: spinal cord injury
PDGFR: platelet-derived growth factor receptor
Glast: Glutamate/Aspartate Transporter 1
CAF: cancer-associated fibroblast
LSL: loxP-stop-loxP
dpi: days post-injury

Received: September 8, 2024; accepted: October 28, 2024
Corresponding Authors: Atsushi Enomoto, MD, PhD; Yukihiro Shiraki, MD, PhD
Department of Pathology, Nagoya University Graduate School of Medicine,
65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
Tel: +81-52-744-2093,
E-mail: enomoto@iar.nagoya-u.ac.jp (Enomoto); shiraki.yukihiro.p8@f.mail.nagoya-u.ac.jp (Shiraki)

INTRODUCTION

Spinal cord injury (SCI) is a complex pathologic condition resulting from various etiologies, including mechanical damage to the spinal cord resulting in profound neurologic consequences.[1,2] Contributing to the serious problems in SCI is the fact that the injured spinal cord has a limited ability to regenerate lost neurons. The failure of axon regeneration is due to blocking by extensive proliferation of reactive astrocytes, a process called gliosis or astrogliosis.[3-7] The proliferation of astrocytes and glial scar formation are physiological efforts to repair the damaged spinal cord and restore structural integrity, but these processes can result in defects in functional recovery.[3-7] Accordingly, understanding the mechanisms of gliosis after SCI and development of therapeutics that could reverse gliosis have been the focus of extensive research.

Another prominent cause of scar formation after SCI is fibrosis.[8-10] Fibrosis is defined as excessive deposition of extracellular matrix (ECM) components, such as collagen and fibronectin that are produced by proliferating fibroblasts. Consequently, it is crucial to understand the mechanism underlying spinal cord fibrosis and to examine the characteristics of fibroblasts that are responsible for scar formation.[8,9] A pioneering study demonstrated that a subset of pericytes that express the Glutamate/Aspartate Transporter 1 (*Glast*)-CreERT2 transgene (type A pericytes) give rise to scar-forming stromal cells in the adult spinal cord.[11] Inhibiting the proliferation of type A pericytes decreased fibrosis and enhanced regeneration of the raphespinal and corticospinal tracts after SCI in mice.[12] These studies have proposed that type A pericytes initiate the fibrotic response in the damaged spinal cord as well as in other diverse central nervous lesions.[13] However, another line of studies that used some other transgenic mice to analyze SCI-associated injuries showed that perivascular fibroblasts positive for collagen type I genes (collagen type I alpha 1 chain [*Col1a1*] and collagen type I alpha 2 chain [*Col1a2*]) and platelet-derived growth factor receptor α (*Pdgfra*) but not classical pericytes expressing chondroitin sulfate proteoglycan 4 (*Cspg4*; a.k.a *Ng2*) and *Pdgfrb* were responsible for the induction of spinal cord fibrosis.[8,9,14,15] Thus, it is possible that perivascular fibroblasts substantially overlap with type A pericytes (expressing either collagen genes or the Glast-CreERT2 transgene) and that they constitute the primary fibroblastoid population that proliferates and forms a scar in the injured spinal cord. A more recent study showed that Col1a1-CreERT2 and Glast-CreERT2 mouse lines label perivascular fibroblasts and type A pericytes in the white and gray matter regions of the spinal cord, respectively.[16] Those results suggest the presence of anatomically distinct populations of perivascular stromal cells within the perivascular niche.

We previously described Meflin, a glycosylphosphatidylinositol-anchored membrane protein that is encoded by the immunoglobulin superfamily containing leucine rich repeat (*Islr*) gene.[17,18] It is a marker of mesenchymal stem/stromal cells (MSCs) in the bone marrow.[17,18] Given that MSCs share many phenotypic characteristics with fibroblasts and perivascular stromal cells,[19] it is not surprising that Meflin is a specific marker of tissue-resident fibroblasts across many organs and tissues.[20-24] The roles of Meflin and Meflin$^+$ fibroblasts have been investigated in Meflin-deficient mice and Meflin reporter mice in the context of numerous disease models. They include pancreatic, colon and lung cancers, pancreatitis, acute and chronic heart diseases and renal disease.[20-39] The results of these studies have suggested context-dependent roles of Meflin in both cancer and fibrotic disease. Importantly, the fibroblasts that proliferate in tumor stroma (cancer-associated fibroblasts or CAFs) are functionally and transcriptionally heterogeneous.[25,40] Thus, fibroblasts highly positive for Meflin seem to be cancer restraining whereas those negative

or weakly positive for Meflin promote cancer progression.[21,25,28]

In the present study, we examined the expression of Meflin in normal and injured spinal cords using in situ hybridization (ISH). We found that in normal spinal cords, Meflin expression was very weak and almost undetectable at the mRNA level. However, expression increased in activated fibroblastic cells that arose around the perivascular area and meninges in response to SCI induced in a clip-compression model. A single-cell transcriptomic analysis using a publicly available data set showed that Meflin is a marker of fibroblasts or type A pericytes but not classical pericytes. A lineage-tracing experiment was also performed using a Meflin reporter mouse that we previously generated.[21] The data showed that Meflin$^+$ cells are a source of activated myofibroblasts involved in scar formation after SCI.

MATERIALS AND METHODS

Animals

All animal protocols were reviewed and approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine (approval numbers M220014-001 and M230053-001), and studies were conducted in compliance with institutional and national guidelines. Meflin-CreERT2 knock-in mice expressing tamoxifen (TAM)-inducible Cre recombinase under the Meflin promoter were previously generated in our laboratory.[21] The Meflin-CreERT2 mice were crossed with Rosa26-loxP-stop-loxP (LSL)-tdTomato mice (7909, Jackson Laboratory, Bar Harbor, ME, USA) and used for lineage tracing and imaging of Meflin$^+$ cells and their lineage cells. All mice were maintained on the C57BL/6J background (Charles River Laboratories Japan, Yokohama, Japan). Female mice (8–10 weeks old) were used in this study. All mice were kept in autoclaved cages and provided sterile drinking water and chow *ad libitum*.

Compression model of SCI

Spinal cord compression injury at the vertebral level T10 was performed as previously described.[41-43] We used 8- to 10-week-old female C57BL/6J mice and Meflin-CreERT2; Rosa26-LSL-tdTomato mice. Medetomidine (0.3 mg/kg), midazolam (4.0 mg/kg) and butorphanol (5.0 mg/kg) were used for general anesthesia. After inducing anesthesia, the skin was incised, the muscles were dissected, and the laminae at vertebral level T10 were exposed and removed. The spinal cord was then exposed and compressed with a clip (KN-353 cat. no, AM-1; Natsume Seisakusho, Tokyo, Japan) for 30 seconds at a force of 60 g/mm^2. The muscle layers and skin were closed with sutures. After SCI, their bladders were manually squeezed to enable daily urination.

Lineage tracing experiments

To visualize Meflin$^+$ cells in the normal spinal cord and to perform lineage tracing after the induction of SCI, Meflin-CreERT2 mice were crossed with Rosa26-LSL-tdTomato mice. Female mice heterozygous for both Meflin-CreERT2 and Rosa26-LSL-tdTomato alleles (Meflin-CreERT2; LSL-tdTomato mice) were subjected to three alternate-day intraperitoneal (i.p.) administrations of TAM (T5648, Sigma-Aldrich, St. Louis, MO, USA) diluted in corn oil (C8267, Sigma-Aldrich) at 100 mg/kg body weight at 8–10 weeks of age (Day 0, Day 2, and Day 4), followed by subjecting the mice to SCI on Day 11. The mice were sacrificed 1, 7 and 14 days after the injury, followed by spinal cord isolation, tissue processing, and histological analyses.

ISH

ISH analyses on formalin-fixed paraffin-embedded (FFPE) tissue samples using RNAscope technology (RNAscope 2.5 HD Detection Kit; Advanced Cell Diagnostics) and custom-designed probes were performed as described previously.[20,21,22,44] Briefly, tissue sections were baked in a dry oven (HybEZ II Hybridization System; Advanced Cell Diagnostics) at 60 °C for one h, deparaffinized, and incubated with a H_2O_2 solution (Pretreat 1 buffer) for 10 min at room temperature (RT). The slides were boiled in a target retrieval solution (Pretreat 2 buffer) for 15 min and incubated with a protease solution (Pretreat 3 buffer) for 30 min at 40 °C. Slides were then incubated with the mouse *Islr* probe (NM_012043.4, region 763–1690, cat. no. 450041; Advanced Cell Diagnostics) for two h at 40 °C and successively incubated with Amp1–6 reagents (Advanced Cell Diagnostics). Staining was visualized with 3,3-diaminobenzidine (DAB), followed by counterstaining with hematoxylin.

Analysis of publicly available single-cell RNA sequence datasets

Publicly available single-cell RNA sequencing (scRNA-seq) data from normal and injured mouse spinal cords were obtained from the Gene Expression Omnibus (GEO) under accession number GSE162610.[45] The Seurat package (version 5.0.0) in R (version 4.3.1) was employed for data processing and analysis, adhering to the package's guidelines.

Cell types within each cluster were identified by examining the expression levels of specific markers. Thus, *Col1a1* and *Pdgfra* were used as fibroblast markers. In addition, *Mcam*, *Cspg4* and *Pdgfrb* were used as pericyte markers while *Pecam1* indicated the presence of endothelial cells. Similarly, *Gfap*, *Sox9* and *Slc1a3* were astrocyte markers, whereas *Ly6g*, *S100a8*, and *S100a9* were used as neutrophil markers. Moreover, *Cd68*, *Msr1*, and *Mrc1* constituted macrophage markers, whereas *Cd68* and *Cx3cr1* indicated the presence of microglia. In addition, *Mog* and *Sox10* were oligodendrocyte markers and *Pdgfra*, *Olig2* and *Cspg4* showed the presence of oligodendrocyte precursor cells (OPC). Finally, *H2-Ab1*, *H2-Aa* and *H2-Eb1* acted as dendritic cell markers. *Map2*, *Nefl* and *Nefm* were neuron markers and *Cd3e* was a lymphocyte marker.

Data visualization was performed using Uniform Manifold Approximation and Projection (UMAP) plots generated by the "FeaturePlot" and "DimPlot" functions. To isolate fibroblast clusters, the "subset" function was applied. Subsequently, the harmony package (version 1.2.0) integrated and analyzed data across the different time points after SCI.

Immunohistochemistry and immunofluorescence

Immunohistochemical (IHC) analyses of FFPE tissue sections were performed following methods described previously.[46,47] Briefly, the sections were deparaffinized and rehydrated, followed by antigen retrieval, as described above. After incubation with a blocking solution for 10 min at RT, the samples were incubated with rabbit anti-red fluorescent protein (RFP) antibody (polyclonal, dilution 1:1000, 600-401-379, Rockland Immunochemicals, Pottstown, PA, USA) overnight at 4 °C. After washing, the slides were incubated with horseradish peroxidase (HRP)-polymer secondary antibody ImmPRESS reagent HRP goat anti-rabbit IgG (MP-7451, Vector Laboratories, Newark, CA, USA) for 30 min at RT. The sections were washed and incubated with Liquid DAB$^+$ Substrate Chromogen System (K3468, Dako) for 5 min at RT, followed by counterstaining with hematoxylin. For immunofluorescence (IF) study, the sections were incubated with rabbit anti-RFP antibody (polyclonal, dilution 1:1000, 600-401-379, Rockland Immunochemicals, Pottstown, PA, USA), mouse anti-glial fibrillary acidic protein (GFAP) antibody (monoclonal, dilution 1:1000, 13-0300, Invitrogen, Carlsbad, CA, USA), or mouse anti-PDGFRβ antibody (polyclonal, dilution 1:1000, AF1042, R&D Systems, Minneapolis, MN, USA) overnight at 4 °C. After washing, the slides were incubated with Alexa fluor plus 647- or 555-conjugated secondary antibodies and

4′,6-diamidino-2-phenylin-dole (DAPI) for 30 min at RT. Finally, the slides were washed and mounted with aqueous mounting medium PermaFluor (TA-030-FM, Thermo Fisher Scientific). Fluorescent microscopic images were obtained using an all-in-one fluorescence microscope BZX-700 with optical sectioning (Keyence, Osaka, Japan).

RESULTS

Meflin⁺ cells arise around the perivascular area and the meninges after SCI induction

We first examined changes in Meflin gene (*Islr*) expression in a mouse model of SCI using extradural clip compression.[41-43] After laminectomy at the tenth thoracic vertebra (T10), the clip was closed at 60 g/mm^2 around the spinal cord for 30 sec to produce an acute injury, followed by isolation of the spinal cord tissues and histological analyses 1, 5, 7 and 14 days post-injury (dpi) (Fig. 1A). ISH analysis showed almost no expression of Meflin mRNA in any of the cells obtained from normal uninjured spinal cords (Fig. 1Ba). Meflin mRNA⁺ cells appeared around the perivascular space and in the meninges at the epicenter of the injured spinal cord at 1 dpi,

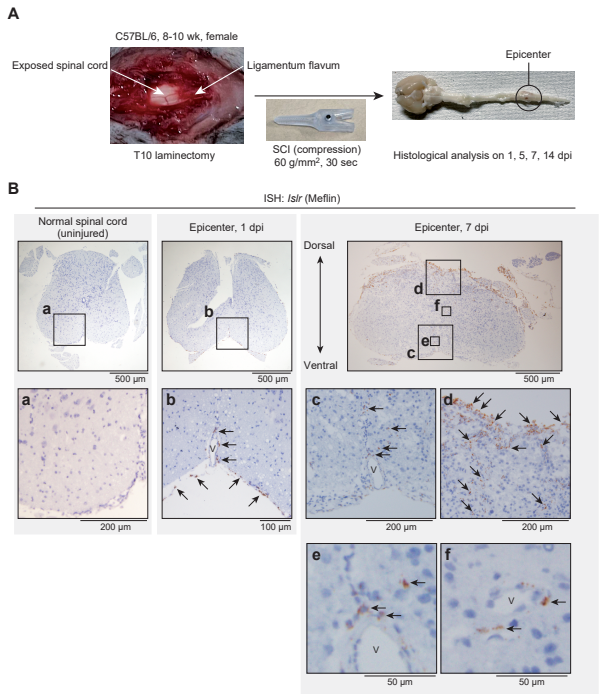

Fig. 1 Meflin⁺ cells appear around the perivascular area and the meninges after induction of SCI

Fig. 1A: A diagram showing the experimental process for the induction of SCI by the extradural clip compression method. See Materials and Methods for details of the protocol.

Fig. 1B: Tissue sections prepared from uninjured spinal cord at the T10 level (left) and the epicenter of injured spinal cord at 1 (middle) and 7 (right) dpi were stained for Meflin (*Islr*) mRNA by ISH. The boxed areas (**a**–**f**) are magnified in lower panels. Arrows denote Meflin⁺ cells.

V: vessel

SCI: spinal cord injury

ISH: in situ hybridization

Islr: immunoglobulin superfamily containing leucine rich repeat

and their number was increased at 7 dpi (Fig. 1Bb–f). Although not compared statistically, it seemed that Meflin mRNA$^+$ cells were most often observed around the meninges (Fig. 1Bd). The data suggested that Meflin expression was activated in some types of cells in response to SCI, although the proliferative capacity of those cells was not examined in the present study.

Fibroblasts expressed Meflin in the normal mouse spinal cord

We next explored the expression of Meflin in normal spinal cords by examining a publicly available data set in which single-cell transcriptomic data were analyzed.[45] The results showed that Meflin mRNA was indeed expressed in fibroblasts that were also positive for *Col1a1* and *Pdgfra* but not in conventional pericytes that express *Pdgfrb*, *Acta2* and *Cspg4* (Fig. 2A, B). Given that type A pericytes positive for the Glast-CreERT2 transgene are a major source of proliferating stromal cells in spinal cord fibrosis,[11,12,16] we examined the endogenous expression

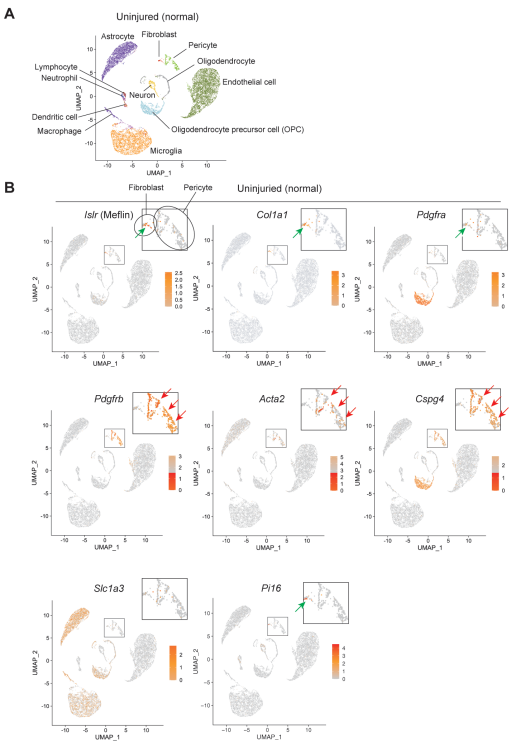

Fig. 2 Meflin expression in fibroblasts in the normal mouse spinal cord

Fig. 2A: A publicly available data set based upon a single-cell transcriptomic analysis of cells isolated from the normal mouse spinal cord (GSE162610) was analyzed, and the selected cell clusters were visualized by the UMAP algorithm.

Fig. 2B: The data were examined for the expression of Meflin (*Islr*) and marker genes of fibroblasts (*Col1a1*, *Pdgfra*), conventional pericytes (*Pdgfrb*, *Acta2, Cspg4*), type A pericytes (*Slc1a3*) and universal fibroblasts (*Pi16*). The fibroblast clusters positive for Meflin (*Islr*), *Col1a1*, *Pdgfra* and *Pi16* and the pericyte clusters positive for *Pdgfrb*, *Acta2* and *Cspg4* are indicated by green and red arrows, respectively.

Islr: immunoglobulin superfamily containing leucine rich repeat
Col1a1: collagen type I alpha 1 chain
Pdgfra: platelet derived growth factor receptor alpha
Pdgfrb: platelet derived growth factor receptor beta
Acta2: actin alpha 2, smooth muscle
Cspg4: chondroitin sulfate proteoglycan 4
Slc1a3: solute carrier family 1 member 3
Pi16: peptidase inhibitor 16
UMAP: Uniform Manifold Approximation and Projection

of the *Slc1a3* gene that codes for Glast. Glast expression was most evident in macrophages and glial cells, including astrocytes, microglia and OPCs. Lower levels of expression were also detected in fibroblasts (Fig. 2B), consistent with the view that type A pericytes and fibroblasts share common features and may in fact be the same cells.[14] Interestingly, peptidase inhibitor 16 (*Pi16*), reportedly a universal marker of fibroblasts with the potential to give rise to various fibroblasts across many diseases and organs,[48] was also specifically expressed by Meflin mRNA$^+$ fibroblasts. Those data are consistent with the notion that Meflin is a potential indicator for the stemness of MSCs.[17,49] In this analysis, a cell cluster that represents meningothelial cells or meningeal fibroblasts could not be identified due to the lack of a specific marker but may be included in the fibroblast cluster.

Persistent expression of Meflin in fibroblasts after SCI induction

The single-cell transcriptomic analysis of cells collected from the injured spinal cord at 7 dpi showed that Meflin mRNA expression was still restricted in fibroblasts (Fig. 3A, B). In

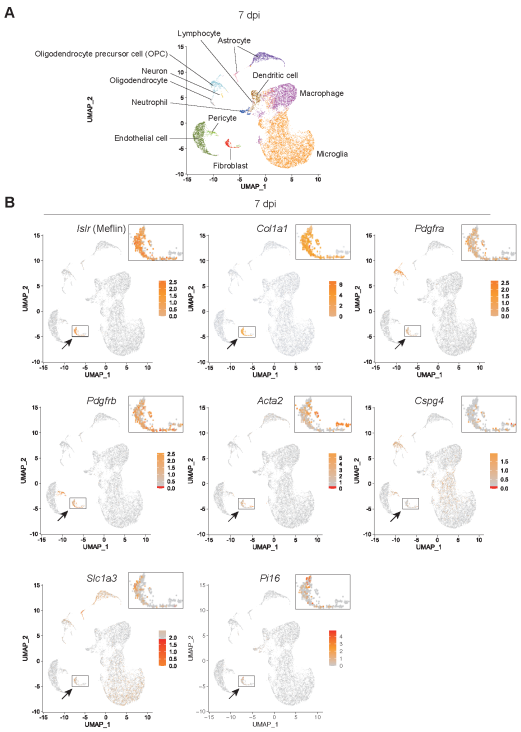

Fig. 3 Meflin expression in fibroblasts in the injured mouse spinal cord

Fig. 3A: The data set representing a single-cell transcriptomic analysis of cells isolated from the mouse spinal cord at 7 dpi (GSE162610). The selected cell clusters were visualized by the UMAP algorithm.

Fig. 3B: The data were examined for the expression of Meflin (*Islr*) and marker genes of fibroblasts (*Col1a1*, *Pdgfra*), myofibroblasts (*Pdgfrb*, *Acta2*), conventional pericytes (*Cspg4*), type A pericytes (*Slc1a3*) and universal fibroblasts (*Pi16*). Arrows indicate the fibroblast clusters.

Islr: immunoglobulin superfamily containing leucine rich repeat
Col1a1: collagen type I alpha 1 chain
Pdgfra: platelet derived growth factor receptor alpha
Pdgfrb: platelet derived growth factor receptor beta
Acta2: actin alpha 2, smooth muscle
Cspg4: chondroitin sulfate proteoglycan 4
Slc1a3: solute carrier family 1 member 3
Pi16: peptidase inhibitor 16
UMAP: Uniform Manifold Approximation and Projection

contrast to a normal spinal cord, most of these Meflin mRNA$^+$ fibroblasts were positive for *Pdgfrb* and *Acta2*, suggesting that these fibroblasts had been activated, becoming myofibroblasts that contributed to fibrosis. These fibroblasts/myofibroblasts expressed the type A pericyte marker *Slc1a3* more robustly than did fibroblasts in the uninjured spinal cord, suggesting that *Slc1a3* expression was induced in activated fibroblasts during SCI (Fig. 3B).

Downregulation of Meflin expression in activated fibroblasts/myofibroblasts in injured spinal cords

The heterogeneous expression of *Acta2* in individual fibroblasts in the injured spinal cords suggested differential activation or variable differentiation states (Fig. 3B). We used UMAP plotting to cluster all *Col1a1*$^+$ fibroblasts from the four groups of cells (uninjured, 1 dpi, 3 dpi and 7 dpi). The data showed that the fibroblasts could be divided into four distinct states, termed clusters 0, 1, 2 and 3 (Fig. 4). Meflin mRNA expression was observed in cluster 0,

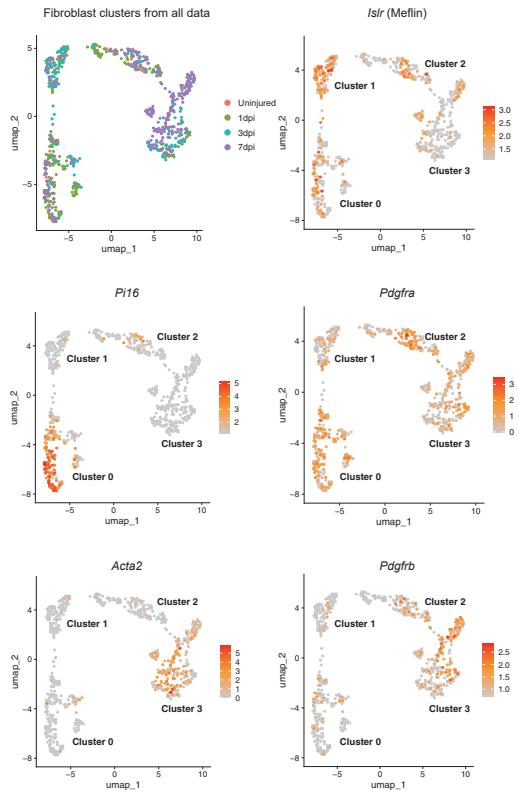

Fig. 4 Features of fibroblasts from both normal and injured spinal cords

Col1a1$^+$ fibroblasts were collected from a data set of a single-cell transcriptomic analysis performed on all cells collected from a normal spinal cord and injured spinal cords after the induction of SCI (1, 3, and 7 dpi). The identified fibroblast clusters (clusters 0, 1, 2, and 3) were visualized with the UMAP algorithm, followed by an expression analysis of the indicated genes.

Islr: immunoglobulin superfamily containing leucine rich repeat

Pi16: peptidase inhibitor 16

Pdgfra: platelet derived growth factor receptor alpha

Acta2: actin alpha 2, smooth muscle

Pdgfrb: platelet derived growth factor receptor beta

UMAP: Uniform Manifold Approximation and Projection

which comprises *Pi16*$^{+}$ universal fibroblasts and is therefore considered to be the root of all fibroblasts.[48] Meflin mRNA expression in clusters 1 and 2 was variable. In cluster 3, which expressed the myofibroblast marker *Acta2,* Meflin mRNA expression appeared downregulated. Although we could not determine the direction of differentiation by pseudotime analysis, this inverse correlation between Meflin mRNA and *Acta2* expression was consistent with our previous studies of other fibrotic diseases and cancer. In those settings, we showed that Meflin mRNA^{+} fibroblasts differentiate into *Acta2*$^{+}$ myofibroblasts that are themselves negative or weakly positive for Meflin mRNA.[20,21]

Lineage tracing confirmed the involvement of Meflin^{+} *fibroblasts following SCI-mediated fibrosis*

Finally, to demonstrate the contribution of Meflin^{+} fibroblasts to spinal cord fibrosis, we intraperitoneally injected TAM into Meflin-CreERT2; LSL-tdTomato female mice to label some Meflin^{+} fibroblasts with the RFP tdTomato. That step was followed by a one-week TAM washout period and the induction of SCI (Fig. 5A). Consistent with ISH analysis, IHC for tdTomato

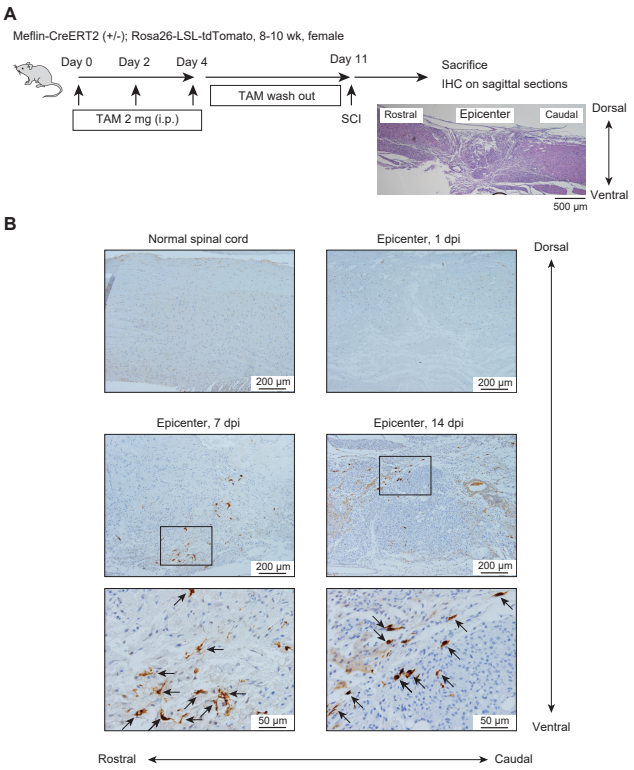

Fig. 5 Analysis of Meflin lineage cells after SCI induction in a Meflin reporter mouse line

Fig. 5A: Female mice (8-10-week-old Meflin-CreERT2; LSL-tdTomato) were administered TAM (2 mg) three times every other day, followed by a one-week washout period and the induction of SCI.

Fig. 5B: Sagittal sections prepared from the spinal cords at 0 (normal spinal cord), 1, 7, and 14 dpi were stained for tdTomato by IHC to visualize Meflin lineage cells. Boxed areas are magnified in lower panels. Arrows denote tdTomato^{+} cells.

TAM: tamoxifen
i.p.: intraperitoneal injection
SCI: spinal cord injury
IHC: immunohistochemistry
dpi: days post-injury

detected no apparent tdTomato expression in the normal spinal cord, suggesting that there are only a few Meflin$^+$ fibroblasts or that Meflin expression was very low under normal conditions (Fig. 5B). However, we observed many tdTomato$^+$ cells (Meflin lineage cells) in spinal cord tissues prepared from the epicenter at 7 and 14 dpi (Fig. 5B). The fibroblasts located near the meninges were not labeled by tdTomato, which may be attributable to the low recombination efficiency of Meflin-CreERT2; LSL-tdTomato mice. IF analysis showed that these Meflin cells were negative for the glial cell marker GFAP (Fig. 6A) but positive for the myofibroblast marker PDGFRβ (Fig. 6B). Those data further support the view that after SCI, Meflin$^+$ cells and their lineage are involved in the development of fibrosis but not gliosis.

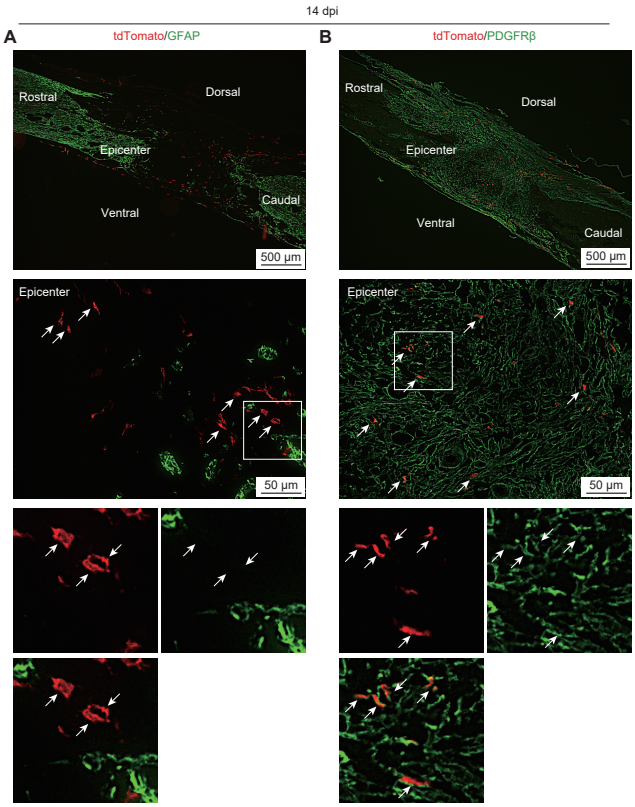

Fig. 6 Meflin lineage cells become myofibroblasts following spinal cord injury
Sagittal sections prepared from spinal cords at 14 dpi were stained by IF for tdTomato (red, arrows) and either GFAP **(A)** or PDGFRβ **(B)** (green). Boxed areas are magnified in lower panels. Arrows denote tdTomato$^+$ cells.
dpi: days post-injury
GFAP: glial fibrillary acidic protein
PDGFRβ: platelet derived growth factor receptor beta
IF: Immunofluorescence

DISCUSSION

Previously, we showed that Meflin was a marker of undifferentiated fibroblasts or MSCs.[17,18] Here, we showed that it was expressed in at least some subsets of fibroblasts that are most

likely equivalent to type A pericytes, in both normal and injured spinal cords (Fig. 7). Data from a single-cell transcriptomic analysis showed that Meflin was expressed by fibroblasts but not conventional pericytes in normal uninjured spinal cords. We found that in injured spinal cords, Meflin expression persisted in activated myofibroblasts, but not in pericytes or glial cells. The transition of Meflin$^+$ fibroblasts to myofibroblasts was proven by a lineage tracing experiment using Meflin-CreERT2; LSL-tdTomato mice. Taken together, the data indicate that Meflin is a marker of fibroblasts that are involved in the development of spinal cord fibrosis (Fig. 7).

Previous studies have explicitly shown the perivascular origin of fibrosis following SCI. Still, the cellular terminology has led to arguments and confusion about whether the fibrotic scar consists of fibroblasts derived from type A pericytes or perivascular fibroblasts that generally reside in the perivascular space.[9] The term "type A pericytes" was initially defined as cells that were recombined in Glast-CreERT2 transgenic mice and invariably located abluminal to type B pericytes. It is plausible that type A pericytes and perivascular fibroblasts are in fact the same cells.[9,11] This issue could be addressed by breeding several reporter mice expressing CreERT2 under the promoters of the type A pericyte/fibroblast marker genes.[9] However, differences in recombination efficiencies between different alleles may hamper conclusions. Meflin may be an excellent marker of type A pericytes/perivascular fibroblasts in this setting, given its expression profile specificity (Fig. 7).

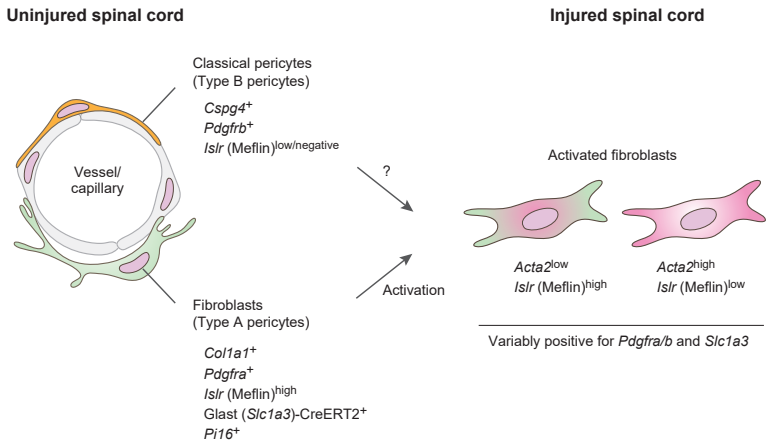

Fig. 7 Schematic illustration of heterogeneous features of stromal cells in normal and injured spinal cords
The data presented here suggest that Meflin is preferentially expressed by *Col1a1*$^+$*Pdgfra*$^+$ fibroblasts, which substantially overlap with type A pericytes, but not *Cspg4*$^+$*Pdgfrb*$^+$ classic pericytes in the normal spinal cord. The single-cell transcriptomic analysis and clustering showed fibroblast activation retaining Meflin expression in the injured spinal cord, where an inverse correlation between Meflin and *Acta2* was observed. The activation of fibroblasts, but not classic pericytes, by spinal cord injury was proven by the lineage tracing experiment performed in the present study.
Cspg4: chondroitin sulfate proteoglycan 4
Pdgfrb: platelet derived growth factor receptor beta
Islr: immunoglobulin superfamily containing leucine rich repeat
Col1a1: collagen type I alpha 1 chain
Pdgfra: platelet derived growth factor receptor alpha
Glast: Glutamate/Aspartate Transporter 1
Slc1a3: solute carrier family 1 member 3
Pi16: peptidase inhibitor 16
Acta2: actin alpha 2, smooth muscle

The present study showed that Meflin$^+$ cells arose around the meninges in injured spinal cords. The data imply that some meningothelial cells or meningeal fibroblasts may be very weakly positive for Meflin, and its expression in those cells could be activated after SCI. Consistent with this, in normal spinal cords, single-cell transcriptomic analysis showed very weak expression of Meflin in at least some subset(s) of fibroblasts or fibroblast-like cells. Our previous studies of other tissues showed perivascular localization of Meflin$^+$ fibroblasts, with most of them being located abluminal to endothelial cells of capillaries and the adventitia of mid- and large-sized vessels.[17,24,32] Thus, we speculate that Meflin is weakly expressed in perivascular fibroblasts both in the parenchyma and meninges of normal spinal cords.

The presence of fibroblasts in the central nervous system (CNS) and their roles in disease have attracted considerable interest in the neurosciences.[8,9,14-16,50,51] The analysis of a publicly available database suggested that Meflin is expressed in fibroblasts of the adult mouse brain,[52] implying that Meflin$^+$ fibroblasts may be involved in the pathogenesis of CNS diseases such as brain tumors, strokes and chronic inflammatory diseases. Our previous studies showed that Meflin$^+$ fibroblasts include CAFs in many types of cancer, and that Meflin$^+$ CAFs may have a role in suppressing cancer progression or augmenting tumor responses to chemotherapeutics and immune checkpoint inhibitors.[21,23,28,29,33] Interestingly, recent studies have revealed the presence of Meflin$^+$ CAFs in gliomas.[53] Importantly, those CAFs promote an immune-evasive cancer stem cell-like phenotype in glioblastoma. Thus, it will be important to determine how Meflin$^+$ CAFs are involved in the progression of brain tumors in future studies.

We recently showed that the oral administration of Am80, a synthetic retinoid, upregulated Meflin expression in CAFs in mouse models of cancer. This treatment significantly improved tumor responsiveness to anti-cancer therapeutics.[29,33,54] The possible mechanisms for Am80-mediated anti-cancer effects include a decrease in tumor stiffness and increases in tumor vascularity and drug delivery.[29,33] Interestingly, previous studies reported beneficial effects of Am80 administration on the recovery of motor function in a mouse model of SCI.[55] Given these findings, it will be of interest to investigate whether Meflin expression in fibroblasts is induced by Am80 administration in a SCI mouse model and how Am80-mediated induction of Meflin$^+$ expression in fibroblasts is involved in the amelioration of SCI-induced motor dysfunction.

ACKNOWLEDGMENT

This study was funded by the Japan Agency for Medical Research and Development (AMED) through grants 23gm1210009s0105 and 23ck0106779h0002 (to A.E.), the Ministry of Education, Culture, Sports, Science, and Technology of Japan through grants 22H02848 and 22K18390 (to A.E.), the Naito Foundation (to A.E.), the Takamatsunomiya Cancer Foundation (to A.E.), and the Toyoaki Foundation (to A.E.).

DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

REFERENCES

1 Quadri SA, Farooqui M, Ikram A, et al. Recent update on basic mechanisms of spinal cord injury. *Neurosurg Rev*. 2020;43(2):425–441. doi:10.1007/s10143-018-1008-3

2 Segi N, Nakashima H, Machino M, et al. Epidemiology of Cervical Fracture/Cervical Spinal Cord Injury and Changes in Surgical Treatment Modalities in Elderly Individuals During a 10-year Period: A Nationwide Multicenter Study in Japan. *Global Spine J*. 2024;14(5):1583–1594. doi:10.1177/21925682231151643
3 Burda JE, Sofroniew MV. Reactive gliosis and the multicellular response to CNS damage and disease. *Neuron*. 2014;81(2):229–248. doi:10.1016/j.neuron.2013.12.034
4 Gaudet AD, Fonken LK. Glial Cells Shape Pathology and Repair After Spinal Cord Injury. *Neurotherapeutics*. 2018;15(3):554–577. doi:10.1007/s13311-018-0630-7
5 Bradbury EJ, Burnside ER. Moving beyond the glial scar for spinal cord repair. *Nat Commun*. 2019;10(1):3879. doi:10.1038/s41467-019-11707-7
6 Tran AP, Warren PM, Silver J. New insights into glial scar formation after spinal cord injury. *Cell Tissue Res*. 2022;387(3):319–336. doi:10.1007/s00441-021-03477-w
7 Zheng B, Tuszynski MH. Regulation of axonal regeneration after mammalian spinal cord injury. *Nat Rev Mol Cell Biol*. 2023;24(6):396–413. doi:10.1038/s41580-022-00562-y
8 Dorrier CE, Jones HE, Pintarić L, Siegenthaler JA, Daneman R. Emerging roles for CNS fibroblasts in health, injury and disease. *Nat Rev Neurosci*. 2022;23(1):23–34. doi:10.1038/s41583-021-00525-w
9 Fehlberg CR, Lee JK. Fibrosis in the central nervous system: from the meninges to the vasculature. *Cell Tissue Res*. 2022;387(3):351–360. doi:10.1007/s00441-021-03491-y
10 Dias DO, Göritz C. Fibrotic scarring following lesions to the central nervous system. *Matrix Biol*. 2018;68-69:561–570. doi:10.1016/j.matbio.2018.02.009
11 Göritz C, Dias DO, Tomilin N, Barbacid M, Shupliakov O, Frisén J. A pericyte origin of spinal cord scar tissue. *Science*. 2011;333(6039):238–242. doi:10.1126/science.1203165
12 Dias DO, Kim H, Holl D, et al. Reducing Pericyte-Derived Scarring Promotes Recovery after Spinal Cord Injury. *Cell*. 2018;173(1):153–165.e22. doi:10.1016/j.cell.2018.02.004
13 Dias DO, Kalkitsas J, Kelahmetoglu Y, et al. Pericyte-derived fibrotic scarring is conserved across diverse central nervous system lesions. *Nat Commun*. 2021;12(1):5501. doi:10.1038/s41467-021-25585-5
14 Soderblom C, Luo X, Blumenthal E, et al. Perivascular fibroblasts form the fibrotic scar after contusive spinal cord injury. *J Neurosci*. 2013;33(34):13882–13887. doi:10.1523/JNEUROSCI.2524-13.2013
15 Dorrier CE, Aran D, Haenelt EA, et al. CNS fibroblasts form a fibrotic scar in response to immune cell infiltration. *Nat Neurosci*. 2021;24(2):234–244. doi:10.1038/s41593-020-00770-9
16 Holl D, Hau WF, Julien A, et al. Distinct origin and region-dependent contribution of stromal fibroblasts to fibrosis following traumatic injury in mice. *Nat Neurosci*. 2024;27(7):1285–1298. doi:10.1038/s41593-024-01678-4
17 Maeda K, Enomoto A, Hara A, et al. Identification of Meflin as a Potential Marker for Mesenchymal Stromal Cells. *Sci Rep*. 2016;6:22288. doi:10.1038/srep22288
18 Hara A, Kato K, Ishihara T, et al. Meflin defines mesenchymal stem cells and/or their early progenitors with multilineage differentiation capacity. *Genes Cells*. 2021;26(7):495–512. doi:10.1111/gtc.12855
19 Haniffa MA, Collin MP, Buckley CD, Dazzi F. Mesenchymal stem cells: the fibroblasts' new clothes? *Haematologica*. 2009;94(2):258–263. doi:10.3324/haematol.13699
20 Hara A, Kobayashi H, Asai N, et al. Roles of the Mesenchymal Stromal/Stem Cell Marker Meflin in Cardiac Tissue Repair and the Development of Diastolic Dysfunction. *Circ Res*. 2019;125(4):414–430. doi:10.1161/CIRCRESAHA.119.314806
21 Mizutani Y, Kobayashi H, Iida T, et al. Meflin-Positive Cancer-Associated Fibroblasts Inhibit Pancreatic Carcinogenesis. *Cancer Res*. 2019;79(20):5367–5381. doi:10.1158/0008-5472.CAN-19-0454
22 Nakahara Y, Hashimoto N, Sakamoto K, et al. Fibroblasts positive for meflin have anti-fibrotic properties in pulmonary fibrosis. *Eur Respir J*. 2021;58(6):2003397. doi:10.1183/13993003.03397-2020
23 Kobayashi H, Gieniec KA, Wright JA, et al. The Balance of Stromal BMP Signaling Mediated by GREM1 and ISLR Drives Colorectal Carcinogenesis. *Gastroenterology*. 2021;160(4):1224–1239.e30. doi:10.1053/j.gastro.2020.11.011
24 Minatoguchi S, Saito S, Furuhashi K, et al. A novel renal perivascular mesenchymal cell subset gives rise to fibroblasts distinct from classic myofibroblasts. *Sci Rep*. 2022;12(1):5389. doi:10.1038/s41598-022-09331-5
25 Miyai Y, Esaki N, Takahashi M, Enomoto A. Cancer-associated fibroblasts that restrain cancer progression: Hypotheses and perspectives. *Cancer Sci*. 2020;111(4):1047–1057. doi:10.1111/cas.14346
26 Ohara Y, Enomoto A, Tsuyuki Y, et al. Connective tissue growth factor produced by cancerassociated fibroblasts correlates with poor prognosis in epithelioid malignant pleural mesothelioma. *Oncol Rep*. 2020;44(3):838–848. doi:10.3892/or.2020.7669
27 Takahashi M, Kobayashi H, Mizutani Y, et al. Roles of the Mesenchymal Stromal/Stem Cell Marker Meflin/Islr in Cancer Fibrosis. *Front Cell Dev Biol*. 2021;9:749924. doi:10.3389/fcell.2021.749924

28 Miyai Y, Sugiyama D, Hase T, et al. Meflin-positive cancer-associated fibroblasts enhance tumor response to immune checkpoint blockade. *Life Sci Alliance*. 2022;5(6):e202101230. doi:10.26508/lsa.202101230

29 Iida T, Mizutani Y, Esaki N, et al. Pharmacologic conversion of cancer-associated fibroblasts from a protumor phenotype to an antitumor phenotype improves the sensitivity of pancreatic cancer to chemotherapeutics. *Oncogene*. 2022;41(19):2764–2777. doi:10.1038/s41388-022-02288-9

30 Suzuki A, Sakamoto K, Nakahara Y, et al. BMP3b Is a Novel Antifibrotic Molecule Regulated by Meflin in Lung Fibroblasts. *Am J Respir Cell Mol Biol*. 2022;67(4):446–458. doi:10.1165/rcmb.2021-0484OC

31 Ando R, Sakai A, Iida T, Kataoka K, Mizutani Y, Enomoto A. Good and Bad Stroma in Pancreatic Cancer: Relevance of Functional States of Cancer-Associated Fibroblasts. *Cancers (Basel)*. 2022;14(14):3315. doi:10.3390/cancers14143315

32 Ando R, Shiraki Y, Miyai Y, et al. Meflin is a marker of pancreatic stellate cells involved in fibrosis and epithelial regeneration in the pancreas. *J Pathol*. 2024;262(1):61–75. doi:10.1002/path.6211

33 Owaki T, Iida T, Miyai Y, et al. Synthetic retinoid-mediated preconditioning of cancer-associated fibroblasts and macrophages improves cancer response to immune checkpoint blockade. *Br J Cancer*. 2024;131(2):372–386. doi:10.1038/s41416-024-02734-3

34 Kobayashi H, Gieniec KA, Lannagan TRM, et al. The Origin and Contribution of Cancer-Associated Fibroblasts in Colorectal Carcinogenesis. *Gastroenterology*. 2022;162(3):890–906. doi:10.1053/j.gastro.2021.11.037

35 Kuwano T, Izumi H, Aslam MR, et al. Generation and characterization of a Meflin-CreERT2 transgenic line for lineage tracing in white adipose tissue. *PLoS One*. 2021;16(3):e0248267. doi:10.1371/journal.pone.0248267

36 Zhang K, Zhang Y, Gu L, et al. Islr regulates canonical Wnt signaling-mediated skeletal muscle regeneration by stabilizing Dishevelled-2 and preventing autophagy. *Nat Commun*. 2018;9(1):5129. doi:10.1038/s41467-018-07638-4

37 Xu J, Tang Y, Sheng X, et al. Secreted stromal protein ISLR promotes intestinal regeneration by suppressing epithelial Hippo signaling. *EMBO J*. 2020;39(7):e103255. doi:10.15252/embj.2019103255

38 Liu C, Liu J, Wang T, et al. Immunoglobulin Superfamily Containing Leucine-Rich Repeat (Islr) Participates in IL-6-Mediated Crosstalk between Muscle and Brown Adipose Tissue to Regulate Energy Homeostasis. *Int J Mol Sci*. 2022;23(17):10008. doi:10.3390/ijms231710008

39 Ishihara T, Kato K, Matsumoto K, et al. Meflin/ISLR is a marker of adipose stem and progenitor cells in mice and humans that suppresses white adipose tissue remodeling and fibrosis. *Genes Cells*. 2024;29(10):902–920. doi:10.1111/gtc.13154

40 Kobayashi H, Enomoto A, Woods SL, Burt AD, Takahashi M, Worthley DL. Cancer-associated fibroblasts in gastrointestinal cancer. *Nat Rev Gastroenterol Hepatol*. 2019;16(5):282–295. doi:10.1038/s41575-019-0115-0

41 Fukuoka T, Kato A, Hirano M, et al. *Neurod4* converts endogenous neural stem cells to neurons with synaptic formation after spinal cord injury. *iScience*. 2021;24(2):102074. doi:10.1016/j.isci.2021.102074

42 Harrison M, O'Brien A, Adams L, et al. Vertebral landmarks for the identification of spinal cord segments in the mouse. *Neuroimage*. 2013;68:22–29. doi:10.1016/j.neuroimage.2012.11.048

43 Alizadeh A, Dyck SM, Karimi-Abdolrezaee S. Traumatic Spinal Cord Injury: An Overview of Pathophysiology, Models and Acute Injury Mechanisms. *Front Neurol*. 2019;10:282. doi:10.3389/fneur.2019.00282

44 Ichihara R, Shiraki Y, Mizutani Y, et al. Matrix remodeling-associated protein 8 is a marker of a subset of cancer-associated fibroblasts in pancreatic cancer. *Pathol Int*. 2022;72(3):161–175. doi:10.1111/pin.13198

45 Milich LM, Choi JS, Ryan C, et al. Single-cell analysis of the cellular heterogeneity and interactions in the injured mouse spinal cord. *J Exp Med*. 2021;218(8):e20210040. doi:10.1084/jem.20210040

46 Shiraki Y, Mii S, Enomoto A, et al. Significance of perivascular tumour cells defined by CD109 expression in progression of glioma. *J Pathol*. 2017;243(4):468–480. doi:10.1002/path.4981

47 Chen C, Enomoto A, Weng L, et al. Complex roles of the actin-binding protein Girdin/GIV in DNA damage-induced apoptosis of cancer cells. *Cancer Sci*. 2020;111(11):4303–4317. doi:10.1111/cas.14637

48 Buechler MB, Pradhan RN, Krishnamurty AT, et al. Cross-tissue organization of the fibroblast lineage. *Nature*. 2021;593(7860):575–579. doi:10.1038/s41586-021-03549-5

49 Yin S, Wu H, Huang Y, et al. Structurally and mechanically tuned macroporous hydrogels for scalable mesenchymal stem cell-extracellular matrix spheroid production. *Proc Natl Acad Sci U S A*. 2024;121(28):e2404210121. doi:10.1073/pnas.2404210121

50 Duan L, Yu X. Fibroblasts: New players in the central nervous system? *Fundam Res*. 2023;4(2):262–266. doi:10.1016/j.fmre.2023.01.014

51 Kelly KK, MacPherson AM, Grewal H, et al. Col1a1+ perivascular cells in the brain are a source of retinoic acid following stroke. *BMC Neurosci*. 2016;17(1):49. doi:10.1186/s12868-016-0284-5

52 Vanlandewijck M, He L, Mäe MA, et al. A molecular atlas of cell types and zonation in the brain

vasculature. *Nature*. 2018;554(7693):475–480. doi:10.1038/nature25739
53 Zarodniuk M, Steele A, Lu X, Li J, Datta M. CNS tumor stroma transcriptomics identify perivascular fibroblasts as predictors of immunotherapy resistance in glioblastoma patients. *NPJ Genom Med*. 2023;8(1):35. doi:10.1038/s41525-023-00381-w
54 Mizutani Y, Iida T, Ohno E, et al. Safety and efficacy of MIKE-1 in patients with advanced pancreatic cancer: a study protocol for an open-label phase I/II investigator-initiated clinical trial based on a drug repositioning approach that reprograms the tumour stroma. *BMC Cancer*. 2022;22(1):205. doi:10.1186/s12885-022-09272-2
55 Takenaga M, Ohta Y, Tokura Y, et al. The effect of Am-80, a synthetic retinoid, on spinal cord injury-induced motor dysfunction in rats. *Biol Pharm Bull*. 2009;32(2):225–231. doi:10.1248/bpb.32.225