学位論文

Ninjurin 1 mediates peripheral nerve regeneration through Schwann cell maturation of $NG2^+$ neural precursor cells

(Ninjurin1 は、NG2 陽性細胞を介して末梢神経再生に関わる)

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Abstract

Ninjurin 1 (Ninj1) is identified as a peripheral nerve injury-induced protein. However, the role of Ninj1 in nerve regeneration is unclear. Schwann cells (SCs) and microvasculature are critical for peripheral nerve regeneration. SCs precursors and microvascular pericytes (PCs), which are nerve/glial antigen 2 (NG2)- positive cells are observed in peripheral nervous system. In this study, we investigated the role of Ninj1 in peripheral nerve regeneration using $NG2^+$cell-specific inducible deletion of Ninj1 mouse model.

The number of $NG2^+$cells, which were associated with and without microvessels was increased after sciatic nerve crush injury. There was a significant increase in the expression of Ninj1 and EphA7 in the injured nerve tissue. This increase was mostly observed in $NG2^+$cells. Genetic tracing of $NG2^+$cells was performed using tamoxifen (Tam) treatment on NG2CreERT:R26R-tdTomato mice. The sciatic nerve was injured following the Tam-treatment, then tdTomato-expressing SCs were mostly observed in regenerated SCs at 21 days after nerve injury. Ninj1 gene knockout (Ninj1 KO) in $NG2^+$cells was induced using NG2CreERT:Ninj1loxp mice. Tam-treated-NG2CreERT or Tam-nontreated NG2CreERT:Ninj1loxp mice were used as controls. Following Tam-treatment, the sciatic nerve in each group was injured. Ninj1KO significantly attenuated the expression of the myelin binding protein (MBP) as well as the number of myelinated axons. The expression of MBP in cultured SCs was significantly reduced by SiRNA-mediated Ninj1 knockdown (KD). Ninj1KD also attenuated the differentiation of SCs by isolated $EphA7^+$multipotent PCs.

The current data indicate that Ninj1 plays a vital role in peripheral nerve regeneration. This is observed particularly in the myelination process of $NG2^+$cells including SCs precursors and multipotent PCs.

1. Introduction

Nerve injury-induced protein, Ninjurin 1 (Ninj1) is originally identified as a transmembrane adhesion molecule expressed in Schwann cells (SCs), which are the primary glial cells in the peripheral nervous system (PNS), in response to nerve injury [1,2]. Ninj1 is now known to express widely in several tissues and cells including inflammatory cells, vasculature and cancer cells and is involved in diverse pathophysiological conditions. In inflammatory cells, Ninj1 acts by inducing neuro-inflammation [3,4], and mediates programmed cell death during ocular development [5]. Ninj1 also mediates vascular functions in penile tissue [6] as well as vascular formation [7].

It has been asserted that Ninj1 plays a role in nerve regeneration due to its expression in neuronal cells following peripheral nerve injury. Ninj1 gene deletion mouse models have been utilized in

previous studies to explore the role of Ninj1. Recently, Le et al. reported that general or neuro-specific deletion of Ninj1 gene induces mental dysfunction although its mechanism is unclear [8]. However, so far, a few studies conducted using Ninj1 gene knockout (KO) mice have shown that Ninj1 contributes to neurogenesis under pathophysiological conditions. A plausible reason for this is that the traditional gene KO model may sometimes mask the actual function of the target gene due to the compensatory response of its deletion. Therefore, little is known about the role of Ninj1 in nerve regeneration.

Nerve/glial antigen 2 (NG2), also known as chondroitin surface proteoglycan 4 (cspg4) expressing cells, are oligodendrocyte precursor cells (OPCs) located in the central nervous system (CNS) [9] as well as immature SCs in the PNS [10,11] and microvascular pericytes (PCs) [12,13]. It was recently asserted by us and other researchers that a specific population of PCs in the CNS and peripheral tissues including the PNS act as neuronal stem cells, differentiating into neuronal cells including SCs [14-16]. Therefore, it is asserted that $NG2^{+}$ cells, including premature Schwann lineage cells and multipotent PCs, may contribute to nerve regeneration in an injured peripheral nerve.

We previously reported that Ninj1 is expressed in $NG2^{+}$PCs of microvasculature [7], and is involved in the formation of functional matured neovessels through PCs and ECs interaction during hind limb ischemia using $NG2^{+}$cell specific- and tamoxifen-inducible Ninj1 KO mice ($NG2^{+}$Ninj1KO) [17]. Timely deletion of the Ninj1 gene in $NG2^{+}$cells is also useful in clarifying the role of Ninj1 in the peripheral nerve system. In this study, we show for the first time that Ninj1 is crucial for peripheral nerve regeneration through the use of a sciatic nerve crush model in an $NG2^{+}$cell-specific Ninj1KO mouse.

2. Material and methods

2.1. Generation of experimental animals

All mice were kept at the Asahikawa Medical University's animal facility under standard housing and feeding conditions. All experimental protocols were approved by the Animals Care and Use Committee of Asahikawa Medical University. C57BL6 and NG2- DsRed mice (male or female 18-29 weeks old) were prepared as described previously [7,17]. NG2CreER:R26R-tdTomato and NG2CreER:Ninj1loxP mice (male or female 16-20 weeks old) were used for genetic lineage tracing and knockout (KO) mice as described in a previous study [17]. For the KO experimental group, tamoxifen (Tam) (Sigma-Aldrich) was intraperitoneally injected into the NG2CreER:Ninj1loxP mice for 5 days. Corn oil (vehicle) treated NG2CreER:Ninj1loxP mice and Tam-treated NG2CreER mice were prepared as controls, control 1 and 2 respectively. All mice were housed individually for 7 days following tam treatment before nerve crush surgery. The sciatic nerves were posthumously removed from the mice at indicated time after surgery.

2.2. Nerve crush surgery

All surgical procedures were performed under 2% isoflurane inhalation anesthesia. The sciatic nerve of the right limb was exposed and compressed with fine forceps at the sciatic notch in two orthogonal directions for 10 s per direction. The crushed sites were labeled by suture to the epineurium using 10-0 nylon. At indicated days following the nerve injury surgery, mice were euthanized with 4% paraformaldehyde (PFA) injected through their heart while anesthetized, then sciatic nerves were subsequently removed.

2.3. Immunocytochemical analyses

The sciatic nerve was removed 2 mm proximally and 10 mm distally from the crush site. It was then immediately placed in a Tissue-Tek OCT before being frozen using liquid nitrogen and stored at 80°C. Short axis cryosections were cut using a cryostat, and an immunostaining experiment was then performed as described previously [17]. The primary antibodies anti-S100β (Leica PA0900), anti-myelin binding protein (MBP) (abcam ab218011), anti- neurofilament H protein (NFH) (GeneTex GTX110065), anti-EphA7 (LSBio, LS-C3329513), and anti-Ninj1(abcam ab213695) were incubated. Immunoreactions were detected by using fluorescent secondary antibodies conjugated with Alexa Fluor 488, 594, or 647 (Invitrogen). Nuclei were counterstained with Hoechst 33258(Lonza). Fluorescent imaging was obtained using a confocal fluorescence microscope (Olympus FV1000D). The total cross-sectional area of each sample was measured along with the S100β, MBP, and NFH positive areas using Image J.

2.4. Whole-mount nerve sample imaging

Mice (NG2-DsRed) were injected with 300 ml phosphate buffered solution (PBS) containing FITC-labeled Griffonia simplicifolia lectin (100 mg/ml, Vector Laboratories) via tail vein in order to stain blood vessels, then the mice were euthanized by cardiac perfusion with 4% PFA, and removed sciatic nerves were fixed in 4% PFA. To observe nerve tissue three-dimensionally, we made fixed tissues transparent using a CUBIC reagent [18]. Clarified nerve samples were imaged using a confocal fluorescence microscope (FV1000D Olympus) and 20 serial slides in 15 mm steps were z-stacked and projected.

2.5. Histological assessment

The sciatic nerves were fixed in 4% PFA for 24 h, followed by fixation in 2% osmium tetroxide for 2 h and embedded in paraffin. Total cross-sectional area of each semithin transverse section (4 mm) was measured using Image J. Osmium-stained myelinated nerves as described previously [19] were manually counted and evaluated as a ratio to the observed area.

2.6. Quantitative reverse-transcription (RT)-PCR analyses

Sciatic nerves were removed at indicated time after the sciatic nerve crush surgery and frozen in liquid nitrogen and stored at 80°C. RT-PCR was performed using a superscript one-step RT- PCR kit (Invitrogen) as described previously [7,17,20]. The relative mRNA expression level was calculated using the comparative threshold cycle method. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control.

2.7. SiRNA-mediated gene knock down in SC cells and EphA7^{+}PCs

An immortalized mouse Schwan cell line (IMS32, Creative Bio- array) was subcultured in an appropriated medium [21]. Multi- potent EphA7^{+}PCs, namely capillary stem cells (CapSCs) were isolated from microvessel fractions of peripheral subcutaneous adipose tissue [16]. For the downregulation of target genes, siRNA against mouse Ninj1 and a control scramble siRNA (Nippon Gene Material) were used. After the transfection of siRNA, the cells were incubated in neurogenic differentiation medium as previously described [20]. Forty-eight hours following treatment, neuronal- differentiated cells were determined by immunostaining [20] or gene expression by quantitative RT-PCR [7,17].

2.8. Statistical analysis

All data are represented as mean values ± SEM. Normal distribution and variance homogeneity of each data set were confirmed using the F-test and Bartlett tests. The student's t-test was also used to compare two groups with each other. For comparisons of more than two groups, one-way ANOVA was employed for normal distributions. A P value of greater than 0.05 was considered statistically significant.

3. Results

3.1. Nerve injury increases NG2$^+$ cells and perineural microvascular formation

To observe the NG2$^+$cells in injured peripheral nerves, a whole- mount sciatic nerve of NG2-DsRed mice was collected at indicated time after nerve crush surgery. As discussed in the methods section, a three-dimensional view of the sciatic nerve was observed after tissue clearing treatment. NG2$^+$cells were observed at the perineurium, the endoneurium, and around the blood vessels in non-injured (control) and injured sciatic nerves (Fig 1A, C, Fig. 2B). The number of NG2$^+$cells present increased in the injured sites with the distal portion peaking 14 days after the nerve injury (Fig. 1A and B). Microvessel growth was seen at the perineurium and epineurium of injured nerves (Fig. 1A, C, Fig. 2B). In parallel with growing microvessels, NG2$^+$cells associated with the microvessels, i.e. pericytes (PCs) were also increased.

3.2. NG2$^+$ cells contribute to the regeneration of myelinated Schwann cells

To examine the contribution of NG2$^+$cells in the regeneration of an injured sciatic nerve, we prepared a genetic lineage tracing mouse model. Prior to nerve injury, NG2$^+$cells were labeled by treatment with Tam using NG2CreERT:Rosa26 tdTomato mice. As shown in Fig. 1C, red fluorescent protein (tdTomato) expressing cells were detected in the non-injured nerve in a similar pattern to that of NG2-DsRed mice (Fig. 1A). Three weeks following the nerve injury, the number of NG2$^+$cells was gradually decreased as detected using NG2-DsRed mice (Fig. 1A and B). By contrast, tdTomato-expressing cells were observed in abundance and were seen to have mostly merged with S100β-stained SCs (Fig. 1C). This data indicates that NG2$^+$cells in non-injured nerves act as an SC precursor or as immature SCs, and once they increase due to nerve damage contribute to the regeneration of the injured nerve in vivo.

3.3. Expression of Ninjurin1 and EphA7 in NG2-positive cells within injured nerves

In parallel with $NG2^+$cells (Fig. 1), expression of NG2 gene was temporally enhanced in sciatic nerve in response to nerve injury (Fig. 2A). As well as NG2 gene, the expression of Ninj1 and EphA7 genes also increased. In the endoneurium of then non-injured control nerves, Ninj1 was mostly stained with $NG2^+$cells, which were located at perivascular sites as PCs (Fig. 2B). In response to the nerve injury, the formation of microvessels and $NG2^+$cells appeared to increase. Ninj1 was well stained with $NG2^+$cells, including PCs and other cells that are not associated with micro- vessels (Fig. 2B).

Recently, the novel marker EphA7 for detecting multipotent PCs was identified, namely CapSCs, which is different from SCs [16]. An immunostaining study demonstrated that EphA7 was well stained with PCs of growing microvessels in the endoneurium and perineurium of injured nerves (Fig. 2C). Therefore, the enhanced expression of Ninj1 and EphA7 in the injured nerve might be mostly due to the growth of $NG2^+$cells including immature SCs and multipotent PCs within the regenerative nervous tissues.

3.4. NG2-specific deletion of Ninj1 attenuates myelination of regenerative nerve

In order to examine the role of Ninj1 in $NG2^+$cells in peripheral nerve regeneration in vivo, the deletion of the Ninj1 gene in $NG2^+$cells (Ninj1KO) was induced using Tam-treatment just before nerve injury using the NG2-CreER:Ninj1loxP mouse. Tam-treatment with this mouse model successfully induced the loss of the Ninj1 expression in $NG2^+$cells, but not other NG2-negative cells [17]. The number of Ninj1-positive cells appeared to have decreased in the sciatic nerve tissue compared to both control groups (Fig. 3A).

Fourteen days after the nerve injury, NFH as a marker for neural axons, and S100β, as a marker for both immature and mature SCs were well stained in injured sciatic nerve of control groups and Ninj1KO (Fig 3AB). Myelination of regenerated nerve was observed in control groups as determined by the expression of MBP (Fig. 3A). Interestingly, the MBP positive area significantly decreased in the Ninj1KO as compared to the control groups (Fig. 3A and B). This finding was consistent with the number of myelinated axons as determined by osmium staining, which was significantly attenuated in Ninj1KO (Fig. 3C and D).

3.5. Effects of Ninj1 in cultured Schwann cells and isolated multipotent EphA7+PCs

NG2 is expressed by several kinds of cells such as PCs, immature SCs and other unknown fibroblast-like cells in peripheral nerves [10,11,22]. To clarify the role of Ninj1 in certain kinds of $NG2^{+}$cells, including SCs and multipotent PCs, we examined the effects of Ninj1 in SCs cell line and also isolated $EphA7^{+}$PCs (CapSCs) in vitro.

When the Ninj1 gene was reduced by transfection of Ninj1 specific siRNA in S100β-positive SCs, the expression of the MBP gene significantly reduced, whereas the S100β gene did not alter (Fig. 4A). CapSCs were isolated as EphA7-and NG2-positive cells from the microvessels of peripheral tissues. CapSCs were differentiated into S100β- and MBP-positive SCs with neurogenic differentiation culture condition [16] (Fig. 4B). SiRNA-mediated Ninj1- knockdown significantly attenuated neural differentiation potency of CapSCs. The ratio of S100β and MBP positive cells to total cells significantly decreased (Fig. 4B).

4. Discussion

SCs are the primary mediators in triggering much of what occurs during Wallerian degeneration [23,24]. In the absence of axonal contact, SCs convert to a non-myelinating behavior and dedifferentiate to immature SCs having extensive proliferation. Non- myelinating SCs proliferate at the injured nerve site and play an essential role in promoting axonal regrowth and the remyelination of regenerated axon [23]. NG2 is expressed by immature SC [10,25]. Indeed, the expansion of $NG2^{+}$cells in the injured nerve was observed in response to the nerve injury (Fig. 1). Regarding the source of SCs in nerve regeneration, it was thought that proliferative $NG2^{+}$ immature SCs are from $NG2^{-}$ matured SCs through dedifferentiation [23,24]. On the other hand, this study as well as previous studies show that $NG2^{+}$cells already exist in the non- injured sciatic nerve (Figs. 1 and 2), although their role in peripheral neural regeneration remain controversial [10,11,26]. Previous studies have estimated the cellular profile within tissues at a certain point using immunostaining methods. However, there studies do not observe the serial cellular conversion of precursor cells to SCs in vivo. In this study, through the use of genetic lineage tracing mouse model, we demonstrated that $NG2^{+}$cells pre- existing in the non-injured nerve are proliferated in response to nerve injury and contribute to nerve regeneration (Fig. 1C). Furthermore, we observed attenuated nerve regeneration in mice in which the Ninj1 gene was deleted in the $NG2^{+}$cells before nerve injury (Fig. 3). These data suggest that $NG2^{+}$cells in non-injured nerve tissue act as a SC precursor and that Ninj1 is critical for the neural regenerative ability of the $NG2^{+}$cells.

Angiogenesis is a common feature of tissue damage and inflammation. It has a role in both the inflammatory response and the subsequent repair processes. The neovascular formation is critical for neural regeneration in CNS diseases such as autoimmune encephalomyelitis [27]. Recently, Cattin et al. reported that formed microvessels in the injured nerve are required to provide a scaffold for the migrating SCs and necessary for the regeneration of peripheral nerve [28]. However, the mechanisms by which microvessels contribute to tissue regeneration are not fully clarified. Recent studies including ours have shown that some perivascular mural cells, namely PCs are multipotent and contribute to tissue regeneration through not only a blood-deriving duct but also a source of regenerative cells [14-16]. In this study, we demonstrated that EphA7^{+} multipotent PCs, CapSCs, increased in the injured sciatic nerves (Fig. 2C). Ninj1 in the CapSCs is critical for their neurogenic ability to differentiate to SCs (Fig. 4B). Therefore, it can be asserted that Ninj1 mediates neural regeneration in part through CapSCs associated with microvessels grown in the injured nerve.

Myelination is a critical step for SC maturation, forming a functional nerve. Several intra- and extra-cellular factors, as well as direct interactions with axons and macrophages, modulate this process [29,30]. Ninj1 plays an essential role as an adhesion molecule in mediating cell to cell interactions [2,17]. Thus, it is postulated that nerve injury-induced expression of Ninj1 contributes to interactions between SCs, axons, and/or other cells to mediate myelination. The mechanism by which Ninj1 regulates SC maturation remains unclear. In this study, using inducible and NG2^{+}cell specific Ninj1 gene deletion as well as a NG2^{+}cell lineage tracing mouse model, we demonstrated for the first time that NG2^{+}cells in non-injured nerve tissue act as a SC precursor. We have also shown that Ninj1 in NG2^{+}cells is critical for the nerve regeneration of an injured sciatic nerve, particularly the SC myelination process in vivo.

Conflicts of interest

None declared.

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Figures and figure legends

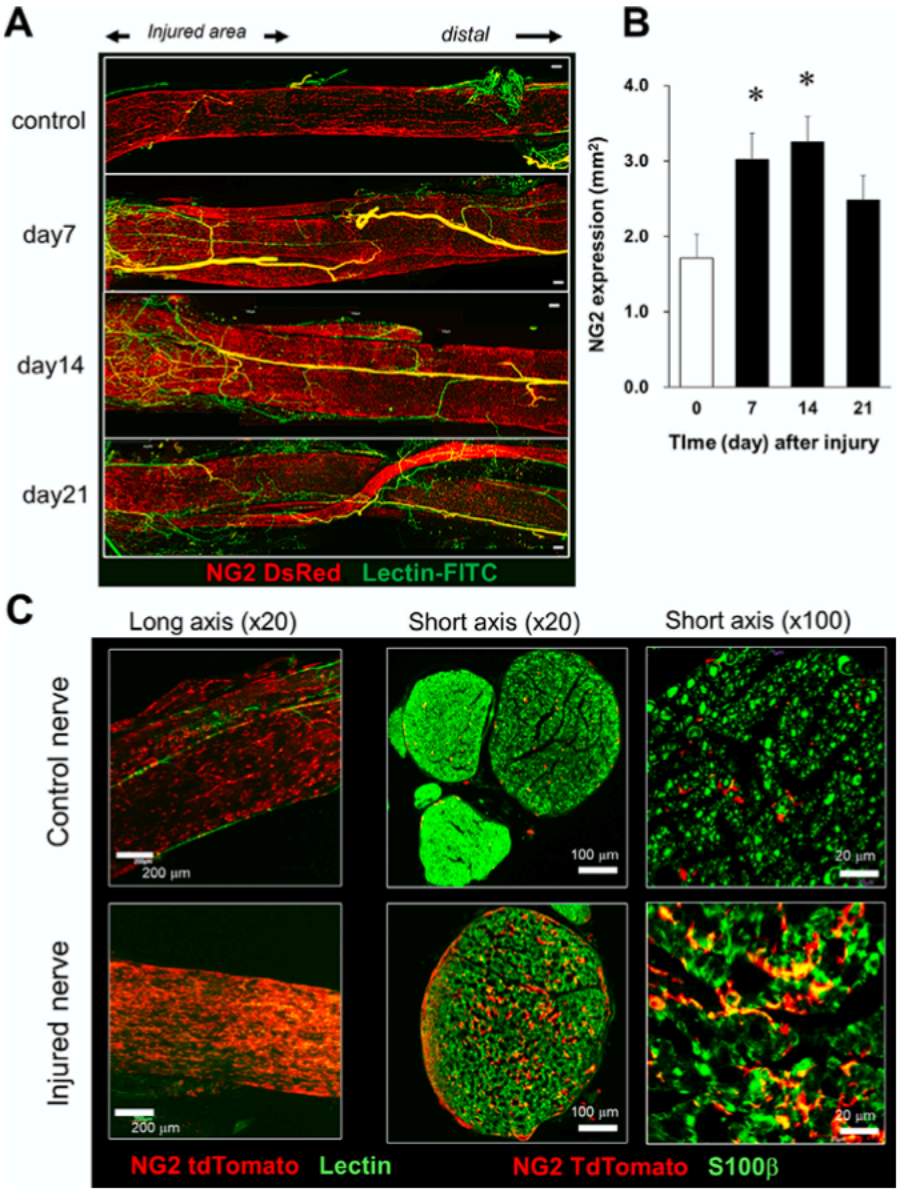

Fig. 1. Expression of NG2$^+$cells and neovascularization in an injured sciatic nerve. (A) At indicated time after sciatic nerve injury of NG2-DsRed mice, the vessels were labeled by lectin-FITC. NG2$^+$cells (red) and microvessels (green) are shown in a representative 3D-view of the sciatic nerve. Scale bars=100 μm. (B) The area of NG2$^+$cells within the observed nerve was measured. The results are shown as the mean ± SEM. *$p<0.05$ compared to control (white bar) (n=5). (C) Genetic lineage tracing of NG2$^+$cells. NG2$^+$cells were labeled by tdTomato (red) before the nerve injury. tdTomato-expressing cells were observed at 21 days following the nerve injury. Schwann cell marker, S100β was immune-stained (green).

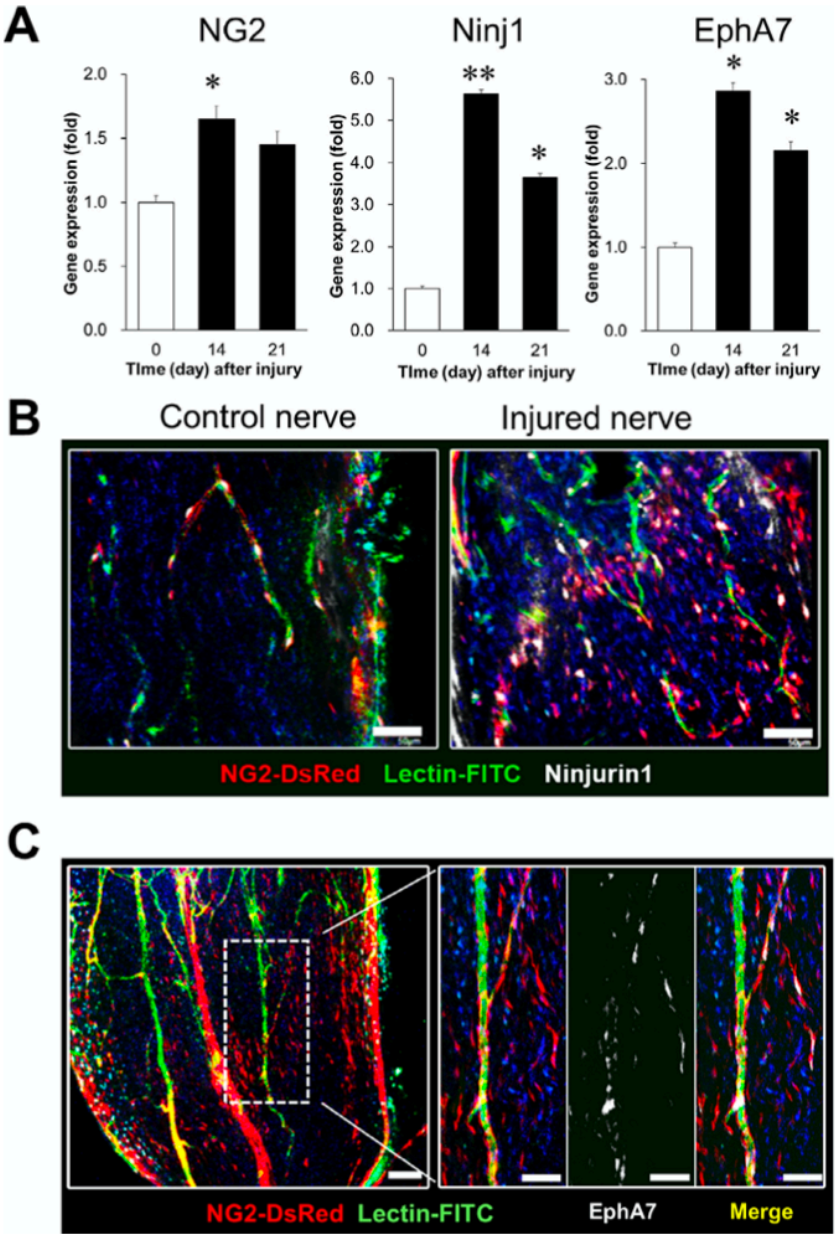

Fig. 2. Expression of Ninjurin1 (Ninj1) and EphA7 in NG2^{+}cells of the sciatic nerve. (A)At indicated time after nerve injury, the mRNA expression of NG2, Ninj1, and EphA7 was determined by qRT-PCR. These results are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$ compared to control (white bar) ($n = 4$). (B, C) At 14 days after nerve injury, a representative z-stacked confocal fluorescence view of cleared nerve tissue was shown. NG2^{+}cells (red) and microvessels (green) were observed in non-injured control and injured nerves. Expression of Ninj1 and EphA7 was immune-stained (white). Nuclei were counterstained by Hoechst 33258 (blue). Scale bars = 20 μm (B), 100 μm (left), 50 μm (right) (C).

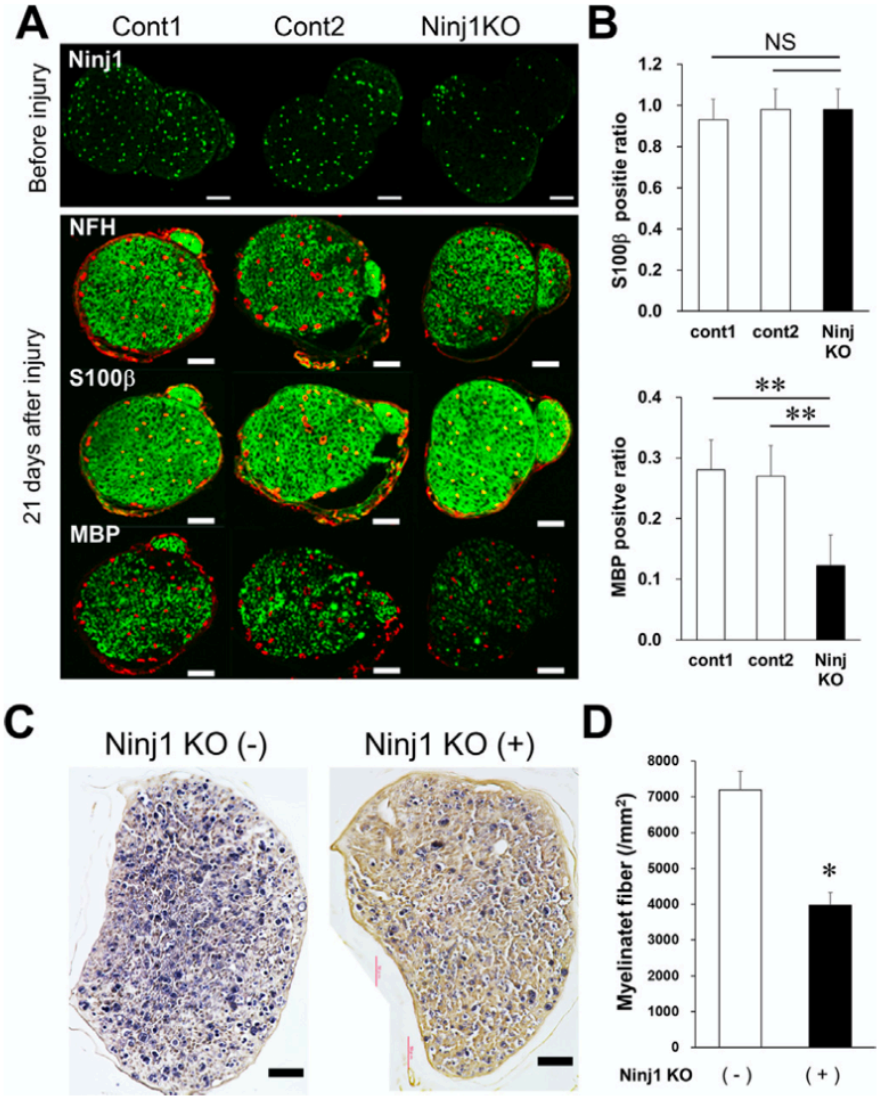

Fig. 3. The expression of the myelin binding protein and myelination of regenerated axon in $NG2^{+}$cells-specific Ninj1 deletion. (A) NG2-specific Ninj1 deletion was induced by tamoxifen treatment on NG2CreER:Ninj1loxp mice (Ninj1KO). Non-tamoxifen- treated mice and tamoxifen-treated NG2CreER were prepared as control 1 and 2 respectively. Representative immuno-fluorescence (green) short axial view of the nerve; Ninj1 at 0, and neurofilament H protein (NFH), S100β, and myelin binding protein (MBP) 14 days post the nerve injury surgery. Vessels were stained using anti- CD31 antibody (red). Scale bars = 100 μm. (B) Expression of NFH, S100β, and MBP was represented as a ratio to the total observed area. (C) Representative Osmium staining view. The myelinated axonal area was estimated, and represented as a ratio to the total area observed. The results are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, NS = non significance (n = 4-6). Scale bars = 100 μm. (A), 50 μm (C).

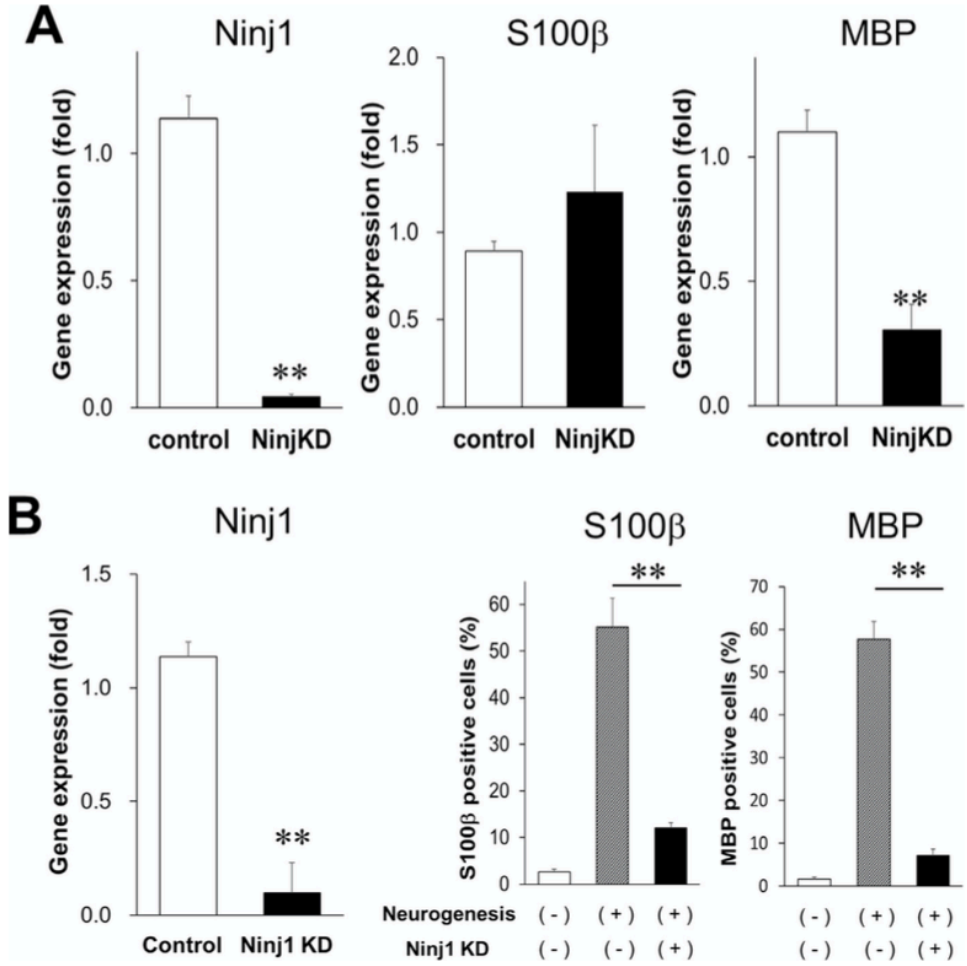

Fig. 4. Effects of Ninj1 deletion in the neurogenesis of Schwann cells and multipotent PCs. (A) SiRNA-mediated Ninj1 knockdown in cultured Schwann cells. Expression of Ninj1, S100β, and MBP was detected using qPCR. (B) After Ninj1 gene in CapSCs was knocked down, CapSCs were incubated in a neural differentiation medium. S100β- and MBP-positive SCs were determined by immunostaining, and the value is represented as a ratio to the total number of cells. Values are expressed as the mean ± SEM. **$p < 0.01$, compared to control (white bar) ($n = 6$).