Perivascular Hedgehog responsive cells play a critical role in peripheral nerve regeneration via controlling angiogenesis

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A B S T R A C T

Hh signaling has been shown to be activated in intact and injured peripheral nerve. However, the role of Hh signaling in peripheral nerve is not fully understood. In the present study, we observed that Hh signaling responsive cells [Gli1(+) cells] in both the perineurium and endoneurium. In the endoneurium, Gli1(+) cells were classified as blood vessel associated or non-associated. After injury, Gli1(+) cells around blood vessels mainly proliferated to then accumulate into the injury site along with endothelial cells. Hh signaling activity was retained in Gli1(+) cells during nerve regeneration. To understand the role of Hedgehog signaling in Gli1(+) cells during nerve regeneration, we examined mice with Gli1(+) cells-specific inactivation of Hh signaling (Smo cKO). After injury, Smo cKO mice showed significantly reduced numbers of accumulated Gli1(+) cells along with disorganized vascularization at an early stage of nerve regeneration, which subsequently led to an abnormal extension of the axon. Thus, Hh signaling in Gli1(+) cells appears to be involved in nerve regeneration through controlling new blood vessel formation at an early stage.

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1. Introduction

Peripheral nerves can be damaged in numerous circumstances including trauma, medical surgery, cancer, abnormal metabolism and/or side effect of medicine. Although the peripheral nerve has the capacity to regenerate after injury, damage often causes refractory motility disturbance or abnormal sensation, which impair a patient’s quality of life and social function. Fundamental treatment for these abnormal peripheral nerves after damages have not established, since their cause of these remains unclear. Therefore, it is critical to understand the molecular mechanisms during peripheral nerve regeneration for both clinical fields and basic science.

Peripheral nerve tissue is unique, as it is able to regenerate following injury. Such regeneration is characterized by complex multicellular responses from Schwann cells (SCs), endothelial cells, and macrophages (Christie and Zochodne, 2013; Cattin and Lloyd, 2016; Dun and Parkinson, 2020). Following injury, endothelial cells migrate into the injury site to form new blood vessels (endothelial phase). Dedifferentiated Schwann cells (SCs) then migrate using the new blood vessels as tracks (SC phase). Subsequently, regenerating axons can extend to distal regions (axon phase).

Hedgehog (Hh) signaling has been shown to be activated in intact peripheral nerve (Bobnarac Dogaru et al., 2018). The Hh signaling pathway is also known to be up-regulated from endothelial through axon phases during peripheral nerve regeneration (Hashimoto et al., 2008), and it is activated in dedifferentiated SCs and regenerating neuron in damaged nerve. However, it remains unclear whether cells showing Hh signaling activity in intact nerves contribute to nerve regeneration when the nerve is injured. To

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address this question, proper reporter system is required to trace cells exhibiting Hh signaling in intact nerve during nerve regeneration. In Hh signaling, the binding of Hh ligands to their receptors allows Smo to activate its downstream targets, Gli1, Gli2 and Gli3. Gli1 is known to act as a critical transcriptional activator of the Hh pathway, and its function is reinforced by a positive feedback loop, since Gli1 is also a target gene in Hh signaling. Therefore, Gli1 is used as a readout of Hh pathway activation. Unlike Gli1, the expression of Gli2 and Gli3 might not be consistent with Hh signaling activity. Gli2 and Gli3 exist as both transcriptional activators and repressors. The function of Gli2 is highly variable and tissue-context dependent, while Gli3 mainly acts as a repressor (Petrova and Joyner, 2014). We have shown that Gli2 is not expressed in either intact or injured peripheral nerve (Yamada et al., 2020). Gli3 is found to function as a repressor in SCs of intact nerve, which is downregulated after injury (Yamada et al., 2020). Thus, Gli1 should be used as an appropriate marker of Hh signaling in peripheral nerve. In Gli1CreER;R26R mice, tamoxifen-inducible Cre activity is under the control of the Gli1 promoter, and LoxP-flanked stop sequence followed by yellow fluorescent protein (YFP) was also inserted in ROSA26 locus. After application of tamoxifen, tamoxifen-inducible Cre recombinase deletes the stop sequence, which lead to the expression of YFP in Gli1-expressing cell. YFP could be detected as Gli1 expression. Mice received tamoxifen treatment for three consecutive days before observation, since it has been shown that tamoxifen treatment for consecutive 3 days could induce sufficient Cre recombinase to delete loxP-flanked gene (Bobarnac Dogaru et al., 2018). Hh signaling responsive cells could be detected in both intact and injured nerve in Gli1CreER;R26R mice. Thus, Gli1CreER;R26R mice is an excellent reporter for tracing cells showing Hh signaling activity.

In the present study, Hh signaling responsive cells [Gli1(+)] cells in Gli1CreER;R26R mice were observed in the endoneurium and perineurium of intact peripheral nerves. Gli1(+) cells in endoneurium were found to be endoneurial fibroblast-like cells (EFLCs). In addition, we found that Gli1(+) cells play a critical role in controlling new blood vessel formation at the endothelial phase of nerve regeneration.

2. Materials and methods

2.1. Animals

All animal experiments were reviewed and approved by the Niigata University Institutional Animal Care and Use Committee prior to the study (approval number SA00236). Mice were housed in a temperature-controlled room under a normal 12 h light/dark cycle with free access to food and water. This experimental study used Male C57BL6 mice (8–12 weeks), male Gli1CreER;R26R mice (8–12 weeks), and male Gli1CreER;SmoA/+/R26R mice (8–12 weeks). These mice were used as heterozygous for Gli1CreER, and they were treated with freshly prepared Tamoxifen 0.2 ml (20 mg/ml) intraperitoneally for 3 consecutive days (Fig. 1A).

2.2. Sciatic nerve transection

Sciatic nerve transection was performed as previously reported (Yamada et al., 2020). Under deep anesthesia by an inhalation of sevoflurane and an intraperitoneal injection of 4 % chloral hydrate (400 mg/kg), the left sciatic nerve was exposed and transected by sharp scissors at a point 1 cm from the sciatic notch. After transection, the wound was sutured.

2.3. Tissue preparation and immunohistochemistry

Under deep anesthesia by an intraperitoneal injection of 4 % chloral hydrate, the mice were transcardially perfused with 20 ml of 4 % paraformaldehyde (PFA) solution. Sciatic nerves including those around the tissue were fixed in 4% PFA overnight at 4 °C before being transferred into 30 % sucrose for cryoprotection. Tissue was embedded in Tissue-Tek (Sakura) and sectioned longitudinally or transversally at 16 μm in a cryostat. Cryostat sections were processed for immunohistochemistry as previously reported (Yamada et al., 2018). After treatment with 0.5 % dry skim milk in 0.3 % Triton X-100 in PBS for 1 h at room temperature, the sections were primarily incubated over night at 4 °C with primary antibodies as follows; Chicken anti-GFP (1/1000, Abcam, Ab13970), Rabbit anti-Gli1 (1/100, Novusbio, NBPI-78259), Rabbit anti-p75 (1/500, Millipore, AB1554), Rabbit anti-PDGFβ (1/2000, Abcam, Ab32570), Rabbit anti-NG2 (1/500, Milipore, Ab5320), Rabbit anti-S100 (1/2000, Abcam, Ab52642), Rabbit anti–α-Ma (1/1000, Abcam, Ab5694), Rat anti–CD31 (1/200, BD Pharmingen, 535370), Rabbit anti–fibronectin (1/2000, Abcam, Ab23750), Rabbit anti–NF200 (1/1000, Sigma Aldrich, NI4142), Rabbit anti–Ki67 (1/200, Abcam, Ab15580), Rat anti–Ki67 (1/200, Invitrogen). After incubation of the primary antibody, that of the secondary antibody was performed for 1 h at room temperature. Secondary antibodies used in experiments follow below; Texas Red goat anti-rabbit IgG (1/500, Vector, TI-1000), Texas Red goat anti-rat IgG (1/500, Vector, TI-9400), FITC goat anti-rabbit IgG (1/500, Vector, FI-1000), Texas Red horse anti-mouse IgG (1/500, Vector, TI-2000), FITC goat anti-chicken IgV (1/500, Abcam, Ab150169), and Alexa Fluor® 647 Goat Anti-Rat IgC H&L (1/500, Abcam, ab150159).

2.4. Gli1(+) cell count

Number of nuclei in each Gli1(+) cells was counted for calculation.

2.5. Cell circularity measurement

Cell circularity was quantified using the following formula as described (Joelcio et al., 2020). Circularity = 4π(area/perimeter)². As the value approaches 0.0, it indicates an increasingly elongated polygon. A circularity value of 1.0 indicates a perfect circle.

2.6. FACS

Intact nerve from Gli1CreER;R26R mice or Gli1CreER;SmoA/+/R26R mice which are treated with tamoxifen were harvested and transferred into HEPES buffered HBSS. Chopped nerves were enzymatically digested in solution containing trypsin (1:250, 3 mg/ml, Gibco, 27250018): collagenase (1.62U/ml, Worthington, CL2): hyaluronidase (1 %, Worthington, LS002594) mix 1:1:0.04, and Pronase (10 %, Sigma-Aldrich, 1016521001) was added to 1 μl/50 μl in the mixed solution. Nerves were broken down for 20 min at 37 °C. The enzymatic reaction was neutralized by 1 ml horse serum (Gibco, 16050130). After centrifugation (10 min, 1000 rpm, 4 °C), the pellet was resuspended in 400 μl PBS, and the cell suspension was filtered through a 40 μm cell strainer ( Falcon, 352340). YFP-expressing cells were sorted by using a cell sorter (FACsAria II, BD Biosciences, San Jose, CA, USA).

2.7. RT-PCR

Total RNA was extracted from the WT intact sciatic nerve and FACS sorted cells by using ISOGEN(NIPPON GENE, 317-02503). RT-PCR was performed using Access
Fig. 1. Distribution of Gli1(+) cells in intact nerve.

(A) Schematic drawing of the protocol of tamoxifen (TMX) injection. Mice were treated with tamoxifen for 3 days before observation. (B) Cross-cut and longitudinal sections showing YFP (green) in an intact nerve. Arrowheads indicating the perineurium Scale bar, 100 μm. (n = 3). (C) Cell composition of an intact sciatic nerve. Pie chart showing the percentage of Gli1(+) cells (n = 3). Number of nuclei in each Gli1(+) cells was counted for calculation. (D, E) Longitudinal sections showing double immunostaining with YFP and CD31, αSMA, NG2, PDGFRβ, p75 or S100. Scale bar, 20 μm. (n = 3). (F) Location and expression pattern of markers in three types of Gli1(+) cells in intact sciatic nerve.

RTPCR kit (Promega, A1260) according to the manufacturer’s instruction. The gene specific PCR primers are Smo (F: CAAAGTTGGAGTGAGGGG, R: GCCAACAGCCAACCTAG, 176bp), YFP (F: TCCATTTGGACACTGGC, R: AGCAGTGTCTTTGAGGTCC, 166bp), GAPDH (F: CCATGGAGAAGGCCGGGG, R: CAAAGTTGCTACGAGGACC).

2.8. Electrophysiology procedure

The sciatic nerve motor nerve conduction velocity (MCV) was measured as described (Yamada et al., 2020). MCV was calculated by dividing the distance between the positions of the stimulating electrode sites (knee and sciatic notch) by the latency differences between the peaks of the muscle action potentials at the two sites.
2.9. Statistical analysis

Graphpad Prism 8 (GraphPad Software, San Diego, CA) was used to perform statistical analysis, which was done using a two-tailed unpaired Student’s t-test and one-way ANOVA with Dunnett’s multiple comparison test. A p-value of less than 0.05 was considered significant.

3. Results

Hh signaling has been shown to be activated in intact peripheral nerves by Dhh (a ligand of Hh signaling) which is secreted from SCs (Parmantier et al., 1999). However, the localization of Hh signaling responsive cells is not fully understood in detail. Therefore, we firstly examined the distribution of Hh signaling responsive cells in intact peripheral nerves using Gli1CreER;R26R mice. In agreement with previous reports, Gli1 positive cells [Gli1(+)] cells were observed in the perineurium (Fig. 1B, Parmantier et al., 1999; Peltonen et al., 2013; Bobarnac Dogaru et al., 2018). In addition to the perineurium, we found that Gli1(+) cells existed in the endoneurium (Fig. 1B). A total of 57.5 % (± 3.8 %) of Gli1(+) cells (5.9 ± 1.1 % of all peripheral nerve cells) were found in the endoneurium (Fig. 1C). We found that there were two types of Gli1(+) cells in endoneurium in terms of their positional relationship with blood vessels. A total of 21.9 % (± 4.1 %) of Gli1(+) cells in the endoneurium were observed in close proximity to blood vessels as confirmed by the expression of an endothelial cell marker, CD31 (Fig. 1D), and we named them blood vessel Gli1(+) cells [bvGli1(+) cells]. On the other hand, the other 35.6 % (± 9.5 %) of Gli1(+) cells in the endoneurium failed to show any positional relationship with blood vessels, and we named them non-bv Gli1(+) cells. Both bvGli1(+) cells and non-bvGli1(+) cells exhibited markers of endoneurial fibroblast-like cells (EFLC), NG2 and PDGFRβ (Fig. 1D, E, Laurence et al., 2014). On the other hand, the expression of a marker of perivascular cells, αSMA, was observed in bvGli1(+) cells, but not in non-bvGli1 (Fig. 1D, E). Conversely, the expression of p75 was found in non-bvGli1(+) cells, but not in bvGli1(+) cells (Fig. 1D, E). Neither bvGli1(+) nor non-bvGli1(+) cells exhibited the expression of a marker of Schwann cells cell, S100 (Fig. 1D, E). Thus, molecular characteristics of bvGli1(+) cells slightly differed from those of non-bvGli1(+) cells, although both cells are likely to be EFLCs (Fig. 1F). On the other hand, Gli1(+) cells in the perineurium showed a similar expression to those of non-bvGli1(+) cells [NG2(+);PDGFRβ(+);p75(+);αSMA(−), data not shown].

Hh signaling activity has been shown to be increased in regenerating nerve (Bobarnac Dogaru et al., 2018). To perform a detailed analysis regarding the timing of the increase in Hh signaling activity during nerve regeneration, chronological fate mapping assay was undertaken following nerve transection in Gli1CreER;R26R mice. Gli1(+) cells could be traced as YFP expressing cells after injury, when the mice were treated with tamoxifen for three consecutive days before injury (Fig. 2A). Numerous Gli1(+) cells were found to accumulate at the transection site from day 3 after injury; this trend was increased at days 5 and 7 after injury (Fig. 2B, C). The Gli1(+) cells were from both the proximal and distal stumps of the injured nerves. These cells came to express Ki67 at the injured stump, showing that they proliferated after injury (Fig. 2D). The peak of proliferation activity was 2days after injury (Fig. 2E). We observed that both perineurial and endoneurial Gli1(+) cells expressed Ki67 (Fig. 2F); however, the ratio of proliferating Gli1(+) cells in the endoneurium was twice that in the perineurium at 2days after injury (Fig. 2G). It has been shown that no significant alteration of perineurial cells can be seen in damaged nerves within 5days after nerve transection (Behrman and Acland, 1981). Taken together, these findings indicated it is reasonable to postulate that Gli1(+) cells in the endoneurium accumulated at the transection site. To confirm which type of Gli1(+) cells proliferated in injured nerves at 2days after injury, we performed a double immunostaining of Gli1 and CD31 in a damaged nerve. bvGli1(+) cells – confirmed by the expression of CD31 in the adjacent cells – were increased in damaged nerve compared to these of intact ones, while no significant changes in non-bvGli1(+) cells could be detected in the damaged nerve (Fig. 2H). In triple immunofluorescence staining (Gli1, Ki67, and αSMA/p75), the bvGli1(+) cells exhibiting Ki67 expression showed higher number than non-bvGli1(+) cells with Ki67 immunoreaction (Fig. 2I). Thus, bvGli1(+) cells are likely to respond mainly to injury in the initial phase. We have already found that Gli1(+) cells showed the expression of EFLC markers, and previous paper described how endoneurial fibroblasts around blood vessels proliferate in damaged nerves (Nesbit and Acland, 1980; Laurence et al., 2014).

Previous reports showed that Hh signaling is activated in dedifferentiated SCs during nerve regeneration (Yamada et al., 2020). Accumulated Gli1(+) cells in the transection site did not differentiate into SCs or endothelial cells (Fig. 3A, B). Upon injury, endothelial cells, SCs and axon are known to migrate to the site of damage at different time points (Parrinello et al., 2010; Cattin et al., 2015). To investigate topographical relationship between Gli1(+) cells and other cells, we compared the localization of Gli1 (+) cells with that of SCs and endothelial cells at the proximal stump of the damaged nerve. An accumulation of Gli1(+) cells into the area of injury preceded that of SCs, and the timing of Gli1(+) cell migration was similar to that of endothelial cells (Fig. 3C). Gli1(+) cells were localized along with endothelial cells at later stage (5 and 7days after injury) when numerous Gli1(+) cells were observed in the transection sites (Fig. 3D). In fact, the percentage of Gli1(+) cells which associated with blood vessels in transection site was significantly larger than that of unassociated (Fig. 3D).

We found that Gli1(+) cells retained Gli1 expression after injury (Fig. 4A). This indicated that activity of Hh signaling was maintained in Gli1(+) cells in injured nerves. Hh signaling is known to be essential for peripheral nerve regeneration (Bobarnac Dogaru et al., 2018; Dobbs et al., 2019). It remains unclear whether Gli1(+) in intact nerves contributes to nerve regeneration when a nerve is damaged. To address this, we examined peripheral nerves following injury in mice with Gli1(+) cells-specific deletion of Hh signaling activity. We used Smo(+/−);Gli1CreER;R26RYFP mice (Smo cKO), since Hh signaling is absent due to Smo (an Hh signaling activator) deletion in Gli1(+) cells by tamoxifen treatment. The mice were treated with tamoxifen for three consecutive days before observation (Fig. 4B). To confirm the loss of Smo in the Gli1(+) cells of Smo cKO mice, the Gli1(+) cells were isolated from the Smo cKO mice by fluorescence-activated cell sorting (Fig. 4C), and RT-PCR for Smo and Yfp was performed. The isolated Gli1(+) cells showed no Smo expression, suggesting that Hh signaling was inactivated in YFP positive cells in peripheral nerve tissues of Smo cKO mice (Fig. 4D). Furthermore, Yfp expression was retained in the isolated Gli1(+) cells, indicating that we can trace Gli1(+) cells following injury in Smo cKO mice, although Hh signaling was deleted by tamoxifen treatment (Fig. 4D). The number and shape of the Gli1(+) cells in the Smo cKO mice without nerve transection was not significantly different from those in the control mice (Fig. 4E). We also examined the nerve functions of Smo cKO mice without nerve transection, but no significant abnormalities could be detected (Fig. 4F). We then examined Smo cKO mice following nerve transection (Fig. 4G). The extension of the axons was impaired in these mice, while a normal extension of axons was seen in the control mice 7days after injury (Fig. 4H). These findings suggested that Gli1(+) cells are essential for nerve regeneration.

To understand how the deletion of Hh signaling in Gli1(+) cells impaired nerve regeneration, we further examined Smo cKO mice.
The number of Gli1(+) cells at the transaction site in Smo cKO mice appeared to be fewer than that in the control mice (Fig. 4L). We demonstrated Ki67 staining in damaged nerves, and percentage of Ki67 positive cells in Gli1(+) cells of Smo cKO mice was significantly lower than that of control mice (Fig. 4K). Reconstruction of blood vessels is known to be one of early essential events in nerve regeneration, and we have already found that in control mice, Gli1(+) cells migrated along with endothelial cells after injury (Fig. 3). We therefore examined blood vessels and Gli1(+) cells in the Smo cKO mice. At the proximal stump of the damaged nerve in the control mice, both endothelial cells and Gli1(+) cells expanded in a spindle shape along the proximal-distal axis (Fig. 4L). However, in Smo cKO mice, the Gli1(+) cells and endothelial cells lost the spindle shape and instead exhibited a round shape and a disorganized pattern of accumulation (Fig. 4L). Subsequent SC phase, SCs migrate into the transaction site after migration of endothelial cells, and also exhibit spindle shape in the control (Fig. 4M). In Smo cKO, SCs exhibited similar abnormal shape and accumulation pattern to those of endothelial cells (Fig. 4M). Therefore, at the control sites, the axons grew toward the distal region of the injured nerve along with spindle-shaped Gli1(+) cells, which is a similar pattern to those of the Gli1(+) cells, endothelial cells and SCs (Fig. 4N). How-
ever, the axons also displayed disorganized growth patterns in Smo cKO mice, alongside the round-shaped Gli1(+) cells that also accumulated in a disorganized manner (Fig. 4N). Thus, abnormal nerve regeneration in Smo cKO mice was occurred from endothelial phase. It is reasonable to consider that abnormal Gli1(+) cells led to aberrant endothelial reconstruction which was likely to result in the failure of nerve regeneration. In fact, a previous paper has noted that the reconstruction of blood vessels is essential for subsequent events including SCs and axon regeneration (Cattin et al., 2015). Thus, it is likely that Gli1(+) cells regulate endothelial cell reconstruction through Hh signaling. To further confirm this, we deleted Hh signaling in Gli1(+) cells at different time points. It is known that blood vessels firstly reconnect only at the core area of nerve (Fig. 5A), which is followed by axonal extension at the same area (Fig. 5B) in a nerve transaction model. The regenerating area then extends to the outer part of nerve afterwards (Fig. 5C, Xiao et al., 2015; Bing et al., 2019). In common with blood vessels, in control mice, the Gli1(+) cells that had accumulated from the proximal and distal stumps of the injured nerve were also found to firstly reconnected at the core area of the nerve (Fig. 2B). Therefore, we deleted Smo from the Gli1(+) cells just after Gli1(+) cells had reconnected only at the core region of the injured nerve to confirm the relationship between blood vessel reconstruction and Gli1(+) cells in nerve regeneration (Fig. 5D). We found that normal blood vessel reconstruction and subsequent axon growth occurred at the core region of the injured nerve in Smo cKO mice, but not in the outer region (Fig. 5E).

4. Discussion

We found Gli1(+) cells in the endoneurium of intact nerve, and they were classified as bvGli1(+) and non-bvGli1(+) cells. Furthermore, both cells showed the expression of markers of EFLC. Thus, Gli1(+) cells in the endoneurium are EFLCs. However, we found that the molecular characteristics differed slightly between bvGli1(+) and non-bvGli1(+) cells. Some 14–31 % of all cells in the wild-type endoneurium have been identified as EFLCs (Schubert and Friede, 1981), which they are classified into several types by the expression of different molecules (Laurence et al., 2014). A total of 5.9 % of cells in the endoneurium were found to be Gli1(+) cells. It is likely that bvGli1(+) and non-bvGli1(+) cells belong to different types of EFLCs.

EFLCs associating with blood vessels have been shown to proliferate in damaged nerves at an early stage following injury (Nesbitt and Acland, 1980). On the other hand, another type of EFLCs – named tactocytes – do not proliferate or accumulate at injured sites at 6 days after injury (Stierli et al., 2018). Thus, each type of EFLCs reacts to injury differentially. We found that bvGli1(+) cells increased in damaged nerves, while non-bvGli1(+) cells showed no significant alteration. Interestingly, non-bvGli1(+) cells displayed similar molecular characteristics [αSMA(-)/NG2(+)/PDGFRβ(+)/p75(+)] to these of tactocytes (Stierli et al., 2018). It is likely that bvGli1(+) cells proliferate and accumulate at injury sites at an early phase of regeneration, but non-bvGli1(+) cells do not.

Fig. 3. Topographic relationship between Gli1(+) cells and other types of cells in injured areas. (A, B) Longitudinal sections showing double immunostaining with YFP and (A) S100 or (B) CD31 at 3 days after nerve injury. Scale bars, 20 μm. (C) Longitudinal sections of proximal stamp of injured nerve showing immunolocalization of NF200, S100 and CD31 with YFP at 3 days after nerve injury. Dotted lines indicate the tips of regenerating axons, and the migration of dedifferentiated SCs and endothelial cells. Scale bars, 200 μm. (n = 3) (D) Left shows high magnified longitudinal sections showing immunostaining with YFP and CD31 at transection site in damaged nerve at 5 and 7 days after injury. Scale bars, 40 μm. Right graph shows percentage of Gli1 cells which associated or unassociated with blood vessels in transection site at 7 days after injury. *p < 0.05.
Blood vessels are known to be reconstructed from both proximal and distal sides of injured nerves (Cattin et al., 2015). Regenerating axons requires guidance in order that they reach the distal region of the injured nerve, and the reconstruction of blood vessels play a part in axon guidance (Cattin et al., 2015). In fact, misdirected blood vessels have been shown to lead to the growth of axons in abnormal directions following injury (Cattin et al., 2015). Thus, the reconstruction of blood vessel is an important event for nerve regeneration; however, it remains unclear how this is controlled. We found that Gli1(+) cells accumulated from both proximal and distal sides of the injured nerve and migrated along with endothelial cells. The dysfunction of Gli1(+) cells due to Smo deletion led to their abnormal accumulation after injury, which also resulted in the failure of blood vessels reconstruction. Genetic ablation of perivascular fibroblasts has been shown to result in dysmorphic blood vessels (Arsheen et al., 2020). Thus, bvGli1(+) cells as perivascular fibroblasts are probably related to the reconstruction of blood vessels following peripheral nerve injury.

Angiogenesis is characterized by endothelial cell migration, chemotaxis, proliferation, and eventually vessel formation, which is highly regulated by signaling molecules. Vascular endothelial growth factor (VEGF) is one of the angiogenic cytokines which modulates the morphology of endothelial cells during angiogenesis. It has been shown that Hh signaling responsive cells secrete VEGF-A in damaged nerves, which is under the control of the Hh signaling activity (Faniku et al., 2020). We found that the deletion of Hh signaling in Gli1(+) cells resulted in abnormally shaped endothelial cells, suggesting that Gli1(+) cells might regulate the morphology of endothelial cell via secreting VEGF in injured nerves.

In wild-type tissues, Gli1(+) cells expanded and showed a spindle shape along the proximal-distal axis along, whereas Smo cKO tissues displayed disorganized round-shaped Gli1(+) cells. Hh sig-
naling is activated in primary cilia, which are organelles that regulate cell polarity. Hence, it is possible that Gli1(+) cells determine their direction of growth through Hh signaling.

Previous studies have shown that Hh responsive cells gathered around injury sites, and that they were derived from the perineurium (Bobarnac Dogaru et al., 2018; Faniku et al., 2020). However, their reported Hh responsive cells seem to be different from bvGli1(+) cells observed at the early stage of nerve regeneration in this study. This is supported by the different timing of occurrence of these cells; the Hh responsive perineurial fibroblasts assembled at more than 2 weeks after injury versus bvGli1(+) cells at postoperative day3. Furthermore, these Hh responsive cells are thought to be derived from perineurium while the bvGli1(+) cells are endoneurial cells. In addition to early stage, we found that Smo cKO showed no perineurium regeneration, and many small fascicles existed in the endoneurium at 30days after injury (data not shown). These fascicles have been described as ‘mini fascicles’ in nerves of wild-type mice (Nesbitt et al., 1980; Popovic et al., 1994); they are formed by endoneurial fibroblasts when the perineurial barrier has been removed. Thus, it is better to mention that the formation of mini fascicles in Smo cKO was induced by the lack of perineurium. Furthermore, the lack of the perineurium in Smo cKO also suggested that Hh signaling in Gli1(+) cells are essential for perineurium regeneration. Further study is required to determine which type of Gli1(+) cells are involved in perineurium regeneration. It is reasonable to consider that the Gli1(+) cells contribute to peripheral nerve regeneration through angiogenesis at early stage and perineurium formation in later stage.

5. Conclusion

Our finding indicates that perivascular Hh responsive cells is involved in nerve regeneration through controlling new blood vessel formation at early stage of regeneration.

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