Side-To-Side Nerve Bridges Support Donor Axon Regeneration Into Chronically Denervated Nerves and Are Associated With Characteristic Changes in Schwann Cell Phenotype

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BACKGROUND: Chronic denervation resulting from long nerve regeneration times and distances contributes greatly to suboptimal outcomes following nerve injuries. Recent studies showed that multiple nerve grafts inserted between an intact donor nerve and a denervated distal recipient nerve stump (termed "side-to-side nerve bridges") enhanced regeneration after delayed nerve repair.

OBJECTIVE: To examine the cellular aspects of axon growth across these bridges to explore the "protective" mechanism of donor axons on chronically denervated Schwann cells.

METHODS: In Sprague Dawley rats, 3 side-to-side nerve bridges were placed over a 10-mm distance between an intact donor tibial (TIB) nerve and a recipient denervated common peroneal (CP) distal nerve stump. Green fluorescent protein-expressing TIB axons grew across the bridges and were counted in cross section after 4 weeks. Immunofluorescent axons and Schwann cells were imaged over a 4-month period.

RESULTS: Denervated Schwann cells dedifferentiated to a proliferative, nonmyelinating phenotype within the bridges and the recipient denervated CP nerve stump. As donor TIB axons grew across the 3 side-to-side nerve bridges and into the denervated CP nerve, the Schwann cells redifferentiated to the myelinating phenotype. Bridge placement led to an increased mass of hind limb anterior compartment muscles after 4 months of denervation compared with muscles whose CP nerve was not "protected" by bridges.

CONCLUSION: This study describes patterns of donor axon regeneration and myelination in the denervated recipient nerve stump and supports a mechanism where these donor axons sustain a proregenerative state to prevent deterioration in the face of chronic denervation.

KEY WORDS: Axon regeneration, Chronic denervation, Peripheral nerve, Schwann cell dedifferentiation, Schwann cell remyelination, Side-to-side nerve bridges

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Peripheral nerve regeneration following injury often involves long regeneration times and distances to reestablish connection with peripheral targets. These long denervation times result in the prolonged loss of contact between neurons and Schwann cells in the distal nerve, a state referred to as chronic denervation.[1,2] Factors that contribute to this limited functional recovery include the fragmentation and collagenization of endoneurial channels[3-5] and the denervation atrophy of target muscle.[2,6,7] Primarily, Schwann cell atrophy and loss of neurotrophic support account for the progressive loss of the nerve capacity to support outgrowth of regenerating axons.[1,8-14]

The growth-supportive environment within denervated nerve is composed of neurotrophic factors, contact guidance cues, and cell adhesion molecules.[15-17] Schwann cells play a critical role in creating this environment.[18,19] Following the loss of axonal contact, Schwann cells dedifferentiate into a nonmyelinating, proliferative phenotype that is identified by the upregulation of several markers

ABBREVIATIONS: CP, common peroneal; **TA,** tibialis anterior; **TIB,** tibial; **PBS,** phosphate-buffered saline; **PBST,** PBS with0.1% Triton X-100

that include p75 neurotrophin receptor, neural cell adhesion molecule, and glial fibrillary acid protein.[18] The mitogens responsible for the proliferation of these nonmyelinating Schwann cells include soluble neuregulins that bind to their endogenous ErbB2 receptors.[20,21] As Schwann cells proliferate, they line basal lamina tubes and create an environment rich in soluble growth factors, cell adhesion molecules, and contact guidance cues. These regeneration "tracts" are known as the "Bands of Bungner" and serve as the organizing structures within which all degeneration, Schwann cell proliferation, and nerve regeneration take place.[3,16,22] However, this growth-permissive microenvironment is transient.[8-10,17,23,24] In the absence of contact with axons, denervated Schwann cells progressively lose the ability to support regeneration and thus contribute to the poor functional outcomes associated with chronic denervation.

Currently, there are no reliable medical or surgical options to oppose these changes that account for poor functional recovery following chronic denervation.[25,26] However, we have recently shown that a novel surgical technique protects against the deleterious effects of chronic denervation over a 4-month period in a rat hind limb model.[27] In this technique, several short nerve grafts were inserted between the side of an intact donor nerve and the side of a denervated recipient nerve. These side-to-side nerve "bridges" serve as a conduit for donor axons to regenerate into the recipient nerve. After delayed repair and a 5-month period of axon regeneration, nerves treated with side-to-side bridges demonstrated a 2-fold increase in the number of motoneurons that regenerated their axons, a 3.6-fold increase in the number of regenerated myelinated fibers, and a 1.6-fold increase in wet muscle weight.[27] We hypothesize that the protective effects of side-to-side nerve bridges resulted from the neurotrophic support of donor axons regenerating into the recipient nerve. In this study, we asked whether the morphological features of donor axon regeneration into the recipient stump and changes in the Schwann cell population add insight into the protective mechanism of side-to-side nerve bridges.

We show that over a 16-week period of denervation, axon regeneration across the 2 coaptation sites of the 3 inserted bridges leads to robust donor axon regeneration into the denervated recipient nerve. Further, we observe Schwann cell dedifferentiation and redifferentiation instead of the attenuation normally associated with chronic denervation. This observation indicates that the factor(s) responsible for protection preserve the Schwann cell permissive state in the presence of donor axons.

METHODS

Animals

Two cohorts of rats were used in this study. The first cohort was used for the imaging analyses and involved creating side-to-side bridges in the bilateral hind limbs of two 250- to 300-g female *Thy-1* green fluorescent protein (GFP) transgenic Sprague Dawley rats. To reduce the potential for background GFP signal, these side-to-side bridges were derived from the bilateral common peroneal (CP) nerves of one wild type, non-GFP-expressing littermate. The second cohort of 6 rats was used for histological analysis of axon growth across the bridges. All protocols used in this study were approved by the Hospital for Sick Children's Laboratory Animal Services Committee (Toronto, Canada), and adhered strictly to the Canadian Council on Animal Care guidelines.

Surgical Procedure

All surgical procedures were performed under isoflurane anesthesia and performed in an aseptic manner by using standard microsurgical techniques with an operating microscope. The bilateral hind limbs of all rats were shaved, and a lateral skin incision was made. The intermuscular septum between the biceps femoris and the vastus lateralis muscles was divided and the sural, tibial (TIB), and CP nerves were identified. In all cases, the CP nerve was separated from TIB nerve and transected proximal to the trifurcation. To induce chronic denervation of the distal CP nerve stump, the proximal and distal stumps of the transected CP nerve were sutured in opposite directions, away from one another, thus preventing any nerve regeneration from the proximal to the distal nerve stumps.

In the first cohort of rats used for imaging, each experimental hind limb underwent placement of 3 side-to-side bridges between the donor TIB nerve and the transected and ligated distal CP nerve stump. The bridges were placed over a distance of 10 mm with equal spacing between them. The nerve segments to be used as bridges were created by harvesting the bilateral CP nerves from an isogeneic wild-type rat littermate. Each of these donor nerves were cut into 3.5-mm lengths and stored in saline-moistened gauze within a Petri dish until needed for bridge isograft insertion. Placement of these bridges in situ involved the opening of 1- to 1.5-mm epineurial windows on the TIB and CP nerves and tacking the bridges into their relative positions along the nerves with a single epineurial suture (Figure 1A and 1B). Once all bridges were sutured in place and positioned orthogonally to the TIB and the distal denervated CP nerve stump, each of the 6 coaptation sites were reinforced with Tisseel fibrin glue (Tiseel, Baxter Healthcare). A few moments were allowed for the Tisseel to cure, after which the biceps femoris muscle was repaired. Animals received subcutaneous meloxicam (~0.5 mg/kg) for analgesia after each surgical procedure. After 4 or 12 weeks, the right hind limb was opened aseptically to visualize and remove the bridges and the CP and TIB nerves for immunohistochemical analysis. This wound was then closed in layers. In a final procedure at 8 and 16 weeks, the left leg was opened and the bridges and CP and TIB nerves were removed.

In the second rat cohort for histomorphometry of nerve cross sections, the same procedures for placing nerve bridges were used except that the CP nerve was denervated 2 weeks before bridge placement, and the nerve segments used as bridges were 6 mm long. This was to allow for clearance of degenerative debris and artefact within the bridges and denervated CP nerve stump.

Bridge Construct Harvesting for Imaging

After 4, 8, 12, or 16 weeks, the bridge constructs were harvested and fixed in a flat plane to incorporate all 3 bridges into the same cryostat section for imaging. All repaired structures were encased in fibrotic scar tissue that was removed carefully with microdissection around the entirety of all bridges and nerves to be imaged (Figure 1C). Once removed from the animal, the construct was laid flat on a thick piece of precut filter paper and arranged so that all bridges were orthogonal to the TIB and CP nerves (Figure 1D). This filter paper sandwich was arranged between 2 sponges and fixed in 4% paraformaldehyde and 30% sucrose w/v (Figure 1E and 1F). Supporting the tissue in this way allowed the nerves to be fixed and sectioned in a single plane.

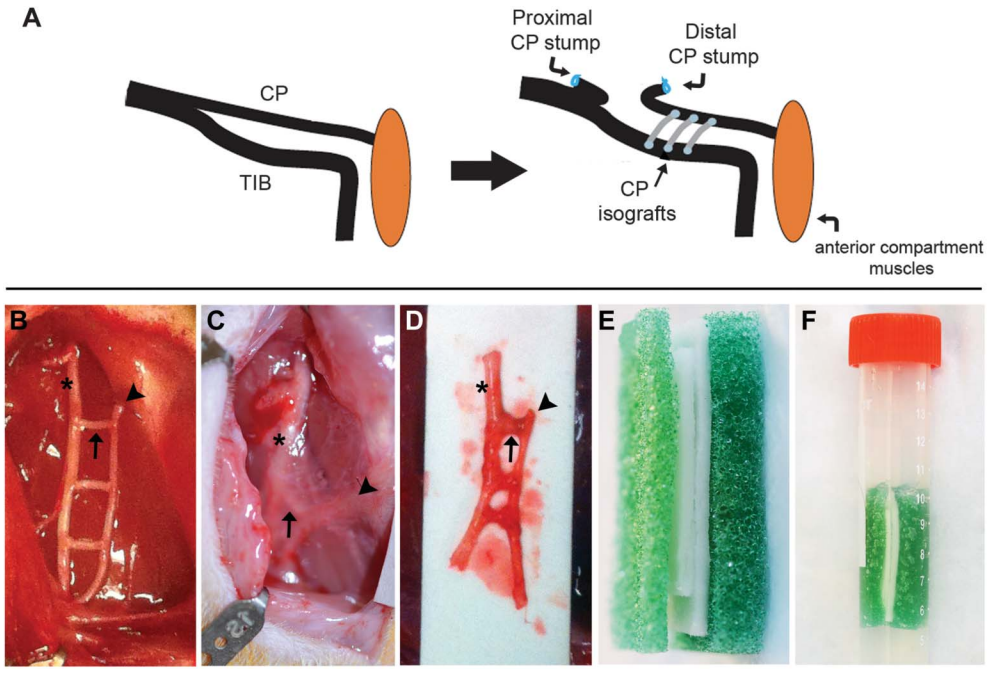

FIGURE 1. *Surgical placement of bridges in situ and removal for immunohistochemical imaging of the entire construct in a single plane.* **A**, *three 3.5-mm common peroneal (CP) nerve isografts were used to bridge opposing epineurial windows on an uninjured donor tibial (TIB) nerve and denervated CP distal nerves. The flexor muscles in the anterior muscle compartment include the tibialis anterior and extensor digitorum longus.* **B**, *bridge "constructs" at the completion of surgery. Proximal TIB nerve (star), first bridge (arrow), and the distal stump of CP nerve (arrowhead) are provided for orientation.* **C-E**, *appearance of bridges at the time of surgical excision of the CP/TIB/bridge construct followed by meticulous clearing of soft tissue around the nerves for placement on a piece of thick filter paper. Once positioned properly, the "construct" was overlaid with another piece of filter paper and sandwiched between 2 pieces of soft green sponge.* **F**, *this filter paper sandwich was gently inserted into a conical tube filled with 4% PFA with 30% sucrose.*

Immunohistochemistry

The flattened nerve constructs were incubated for at least 48 h in 30% sucrose (w/v) in 4% PFA at 4°C. The specimens were embedded in OCT medium, and 20-μm-thick longitudinal sections were cut on a cryostat and mounted on glass slides. Specimens were postfixed with ice-cold 100% ethanol, rinsed with phosphate-buffered saline (PBS; pH 7.6), and washed with PBS with0.1% Triton X-100 (PBST) (Sigma). Nonspecific staining was blocked using PBST w/1% bovine serum albumin (Fisher Scientific) and 10% donkey serum (Sigma). Primary antibodies were applied and incubated overnight at 4°C. The specimens were then washed in PBST, and secondary antibodies were applied for 1 hour at room temperature. The slides were rinsed and mounted with a cover slip. The fluorescent secondary antibodies (Alexa Fluor series, Invitrogen, 1:200) were imaged on a fluorescence microscope (Leica DM2000, Leica Biosystems). Primary antibodies included anti p75 neurotrophin receptor (Abcam, Inc, 1:300) and anti-myelin basic protein (MBP) (Abcam, Inc, 1:200).

Staining intensity was evaluated using ImageJ software (ImageJ, National Institutes of Health, Bethesda, Maryland), which measures the mean gray values of the regions of interest. In brief, the mean gray value of the area between the proximal and distal bridges was evaluated in the TIB nerve and the recipient denervated distal CP nerve stump. In each sample evaluated, the intensity of TIB axons within the CP nerve stump was normalized to the intensity of the uninjured TIB nerve and averaged over triplicate measurements for each time point.

Histomorphometry

After 4 weeks, the denervated CP nerve and the 3 side-to-side bridges intended for histomorpometry (as described above) were dissected free and fixed in 2% glutaraldehyde, postfixed with 1% osmium tetroxide, ethanol dehydrated, and embedded in Araldite 502 (Polyscience Inc., Warrington, Pennsylvania). Axons were examined and counted within the center of the nerve bridge specimens including the proximal, middle, and distal bridges in addition to the proximal and distal terminal segments of the denervated CP nerve. Thin sections (0.6 μm) were prepared on an ultramicrotome (LKB-Produckter A.B., Broma, Sweden) and examined under light microscopy using a 1% toluidine blue stain. Tiled composite images of the entire nerve section were imaged at 100× overall using Image-Pro Analyzer version 9.0 (Media Cybernetics, Rockville, Maryland). ImageJ (National Institutes of Health) was used to count all myelinated axons.

Muscle Mass

After 16 weeks, the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were harvested bilaterally and weighed. The right hind limb muscles were denervated for 4 weeks, having had the TIB nerve,

denervated CP nerve, and bridges removed aseptically at 12 weeks for imaging. The 4-week denervated TA and EDL muscles from this hind limb were compared with muscles that were reinnervated by TIB axons that had crossed through the bridges and denervated CP stump over the full 16-week period. Muscle masses were compared with a historical control data set generated by weighing freshly harvested TA and EDL muscles from 4 previously uninjured Sprague Dawley rats weighing approximately 250 to 300 g.

Statistics

Mean axon counts in each of the 3 bridge groups were compared using a 1-way analysis of variance. A post hoc Bonferroni pairwise comparison was applied to compare the means between individual groups and reported as a *P* value. Significance was taken as a *P* value less than 0.05. The estimates of axon counts are reported as mean ± standard error of the mean.

RESULTS

Tibial Donor Axons Regenerate Across Bridges and into Denervated Common Peroneal Nerve Stumps

The nerve isografts (bridges) remained in continuity with the donor TIB nerve and the recipient denervated CP nerve stump during the 16-week study period (none became inadvertently disconnected). The bridges supported the growth of donor TIB axons into the denervated CP nerve stump (Figure 2), in confirmation of earlier studies.[27] At 4 weeks, there were very few GFP-expressing axons that had grown into the recipient denervated CP nerve stump compared with the number of robust GFP axons in the uninjured donor TIB nerve. At this time, donor TIB axon outgrowth was well established within the proximal and middle bridge, and the axons had reached the interface with the recipient denervated CP nerve stump. However, there were few donor axons within the third distal bridge at this early time point. Progressively more TIB nerve fibers grew into the distal bridge and recipient denervated CP nerve stump at 8 and 12 weeks. By 16 weeks there was a confluence of donor TIB axons occupying the full length of the previously denervated CP nerve stump.

Counts of donor nerves that had regenerated through the bridges at 4 weeks revealed that most of the nerves crossed the first bridge, with there being a significant difference in the numbers of myelinated axons crossing each of the 3 bridges ($P = .02$; 1-way analysis of variance). The greatest number of axons crossed through the first bridge (101 ± 32; mean ± standard error of the mean). This was greater than the number found in the second bridge (31 ± 12) and significantly greater than the numbers found in the third bridge (12 ± 2) (Figure 3A; $P = .02$). Axons grew among degenerative debris (Figure 3B). Within the proximal and distal regions of the denervated CP nerve stump, Wallerian degeneration was complete at 4 weeks postinjury, and few, if any, myelinated axons were seen (data not shown).

Schwann Cells Dedifferentiate Following Injury and Reestablish a Myelinating Phenotype as Donor Axon Regeneration Proceeds Across Side-to-Side Nerve Bridges

Four weeks following transection injury of the CP nerve, all axonal and myelin debris had been cleared (Figures 2 and 4).

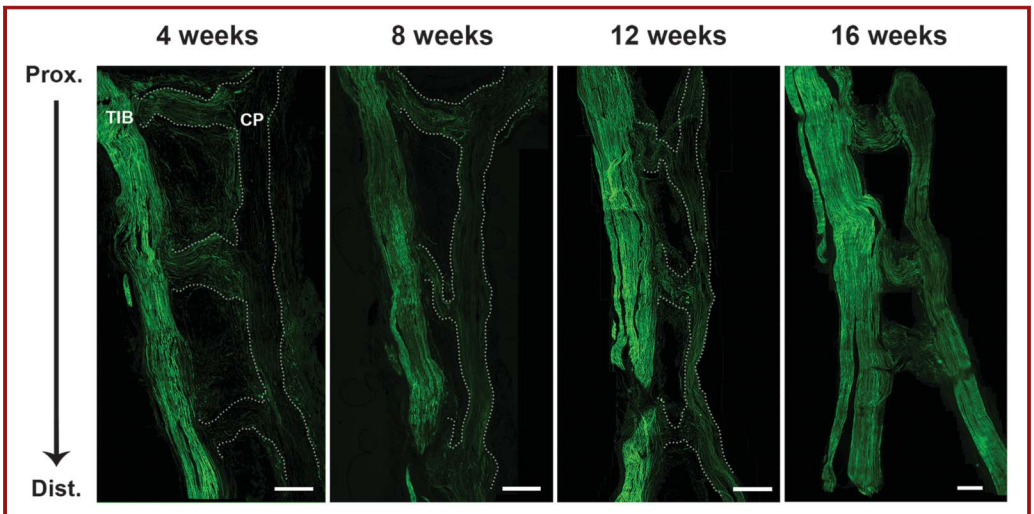

FIGURE 2. *Side-to-side nerve bridges support donor axon regeneration from an uninjured TIB nerve into a denervated CP nerve stump. Following Wallerian degeneration of native axons in the denervated CP nerve and bridges, donor GFP-expressing TIB axons grew across the bridges into the denervated CP nerve stump entering the proximal bridge first, followed sequentially by regeneration across the middle and distal bridges. By 4 weeks, donor axons had reached the interface with the CP nerve and completely occupied the full length of the CP nerve gradually over the subsequent 8, 12, and 16 weeks. Scale bars represent 800 μm. CP, common peroneal; GFP, green fluorescent protein; TIB, tibial.*

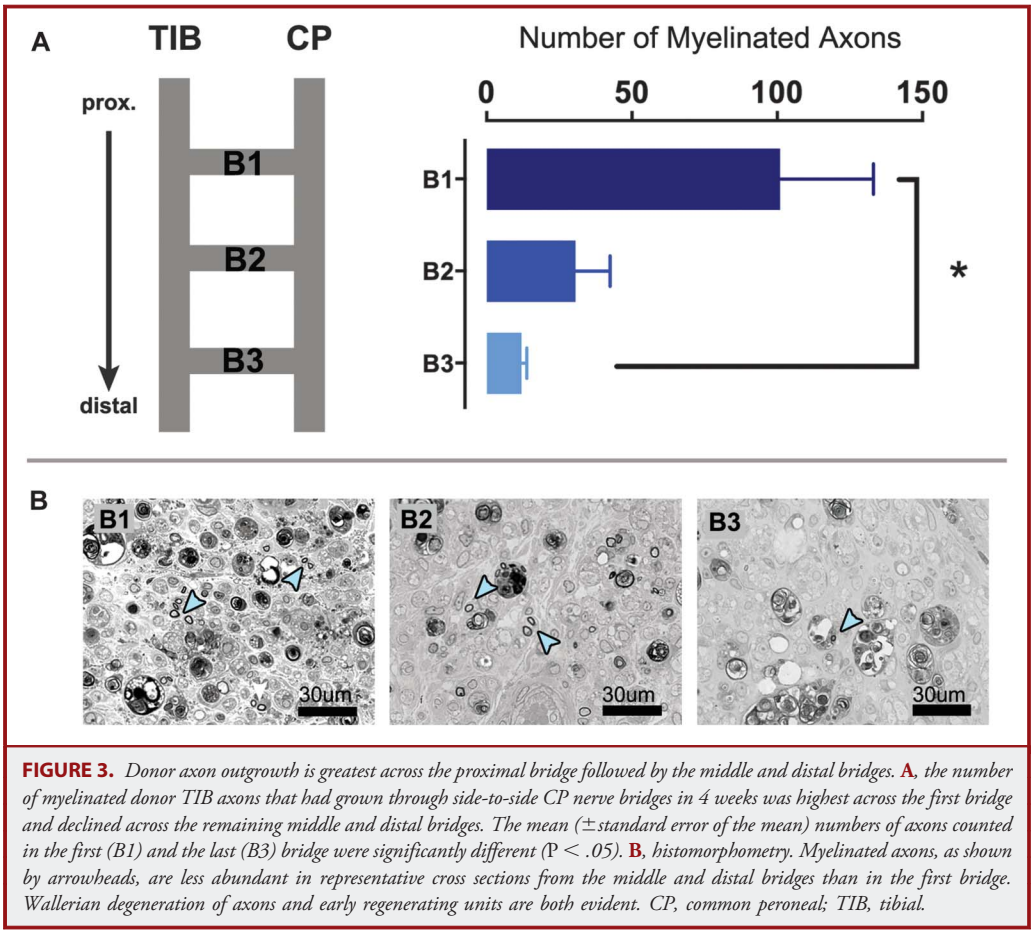

FIGURE 3. *Donor axon outgrowth is greatest across the proximal bridge followed by the middle and distal bridges.* **A**, *the number of myelinated donor TIB axons that had grown through side-to-side CP nerve bridges in 4 weeks was highest across the first bridge and declined across the remaining middle and distal bridges. The mean (±standard error of the mean) numbers of axons counted in the first (B1) and the last (B3) bridge were significantly different* ($P < .05$). **B**, *histomorphometry. Myelinated axons, as shown by arrowheads, are less abundant in representative cross sections from the middle and distal bridges than in the first bridge. Wallerian degeneration of axons and early regenerating units are both evident. CP, common peroneal; TIB, tibial.*

Donor GFP-positive TIB axons had grown across the bridges but not yet into the denervated CP nerve stump (Figures 2 and 4, A1-A4). The denervated Schwann cells in the bridges and distal denervated nerve stump dedifferentiated into an immature, proliferating phenotype expressing p75 (Figure 4, B1-B4), but not MBP (Figure 4, C1-C4). At this time, dedifferentiated Schwann cells were densely packed within the bridges and the denervated CP nerve stump (Figures 4 and 5). There were a few p75 immunoreactive Schwann cells found at the site of insertion of a side-to-side bridge. These reflect the damage to the donor TIB axons that proceed to regenerate across the bridges and into the recipient denervated CP nerve stump (Figure 5). As progressively more donor TIB axons grew across the bridges, the p75 immunoreactivity of the Schwann cells within the bridges (Figures 5 and 6) declined progressively over 16 weeks. This was concurrent with a progressive and parallel increase in MBP immunoreactivity as these axons become myelinated (Figure 5). The time course of TIB axon growth into the denervated CP nerve stump is shown and compared with the expression of p75 and MBP in the histograms in Figure 6. The intensity of the immunoreactivities of GFP, MBP, and p75 within the denervated CP nerve stump over the 10-mm distance between the proximal and distal bridge was determined and plotted relative to the immunoreactivity within the same length of the intact donor TIB nerve. Entry of TIB axons into the denervated CP nerve stump was evident by the decline of p75 immunoreactivity and a reciprocal increase in MBP immunoreactivity in the recipient denervated CP nerve stump. The more gradual increase in GFP immunoreactivity represents the progressive remyelination of the ingrowing TIB axons within the denervated CP nerve stump. The progressive axon growth across the 2 interfaces for each bridge is consistent with the asynchronous, staggered pattern of axon growth across suture sites that was described by Brushart et al.[28]

Donor Axons are Myelinated as They Cross the Side-to-Side Nerve Bridges

The progressive myelination of ingrowing TIB axons is seen in the longitudinal sections of Figure 4 (C1-C4) and the relative fluorescence intensity of MBP in Figure 6. It was also evident in the bridges depicted in Figure 7. The MBP reactivity of some axons, but not others at 4 weeks, becomes robust after 8 weeks, when the majority of the TIB nerve fibers within the recipient denervated CP nerve stump were myelinated (Figure 7).

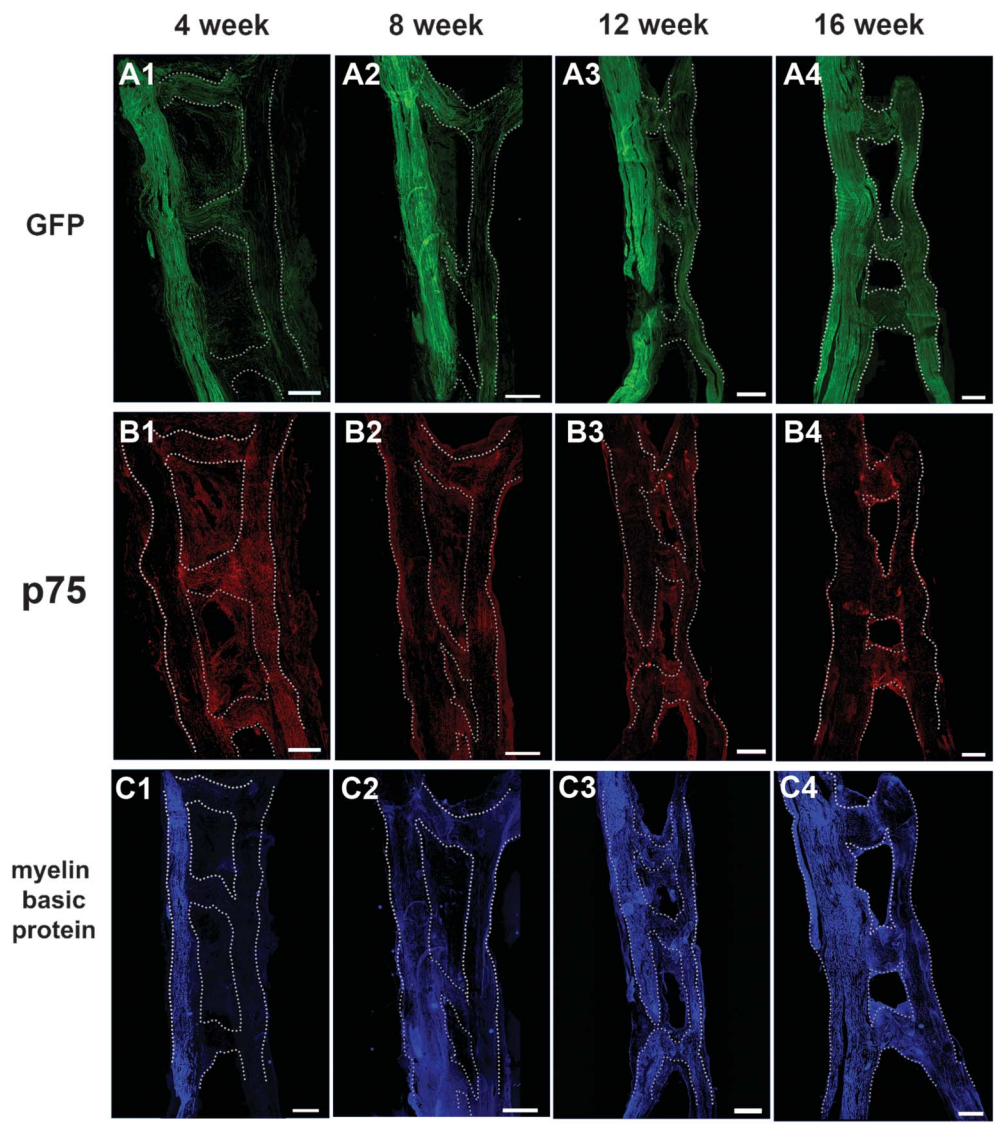

FIGURE 4. *Schwann cells within the denervated nerve dedifferentiate initially and, as axons regenerate adjacent to them, they gradually redifferentiate and myelinate the axons.* **A1**-**A4**, *GFP donor TIB axons regenerate across the bridges and into the recipient denervated CP nerve stump following injury. By 16 weeks (***A4***), donor TIB axons are densely packed within the denervated CP nerve stump.* **B1**-**B4**, *red p75-labeled immature Schwann cells are densely clustered within the bridges and the denervated CP nerve stump after 4 weeks (***B1***), but this staining attenuates considerably by 8 weeks (***B2***) as TIB axons grow through the bridges and into the CP nerve stump. At 16 weeks (***B4***), the p75 immunoreactivity in the Schwann cells had returned to the baseline levels of the myelinating Schwann cells in the adjacent, uninjured TIB nerve.* **C1**-**C4**, *following Wallerian degeneration, there was no evidence of myelination as seen by the lack of myelin basic protein signal (blue) within the bridges or denervated CP nerve at 4 weeks (***C1***) in comparison with the uninjured TIB nerve. Over subsequent weeks, donor TIB axons regenerating into the denervated recipient CP nerve stump gradually became remyelinated with restored myelin basic protein signal. Scale bars represent 800 μm. CP, common peroneal; GFP, green fluorescent protein; TIB, tibial.*

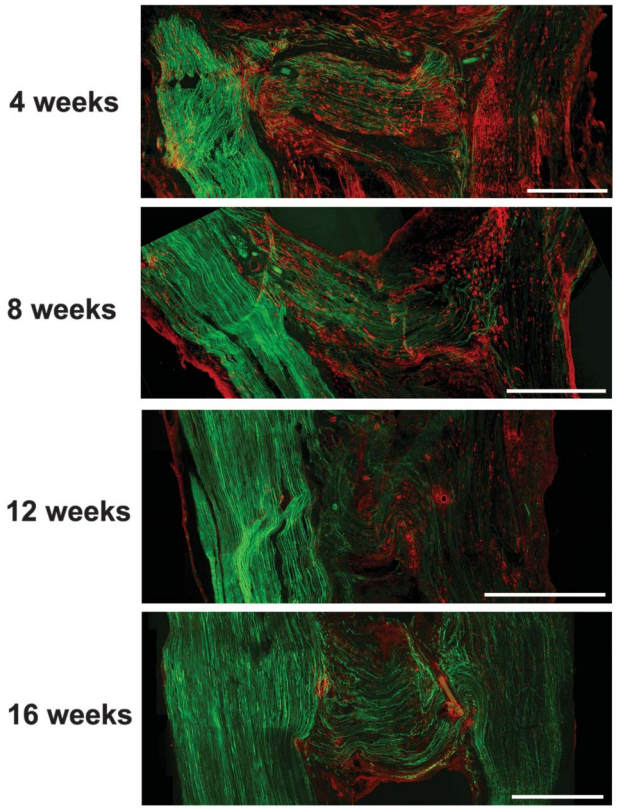

FIGURE 5. *Dedifferentiated Schwann cells densely populate the "cross-bridges" early on at 4 weeks, but decline in number as donor axons regenerate into the bridges and denervated CP nerve. The dedifferentiated p75-labeled Schwann cells (red) can been seen uniformly and densely clustered with the first cross-bridge and CP nerve 4 weeks postinjury. GFP-labeled donor axons can be seen to progressively regenerate across the bridges and into the denervated CP nerve. Dedifferentiated p75 positive Schwann cells decrease concurrently with increasing donor axon regeneration. Scale bars represent 800 μm. CP, common peroneal; GFP, green fluorescent protein.*

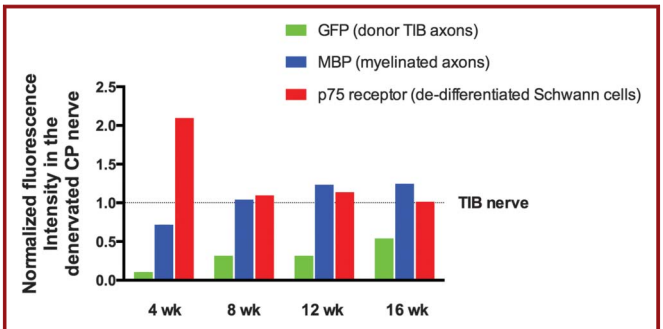

FIGURE 6. *Regeneration of GFP positive donor axons coincides with expression of the myelination marker, myelin basic protein (MBP), and a sharp decline in the expression of marker for dedifferentiated denervated Schwan cells, p75. Fluorescence intensities of the 3 markers were normalized to the TIB nerve fluorescence intensity from the same sections. The intensities were determined in triplicate in sections taken from 1 rat. CP, common peroneal; TIB, tibial.*

Neuronal-Glial Relationships are Restored by the Progressive Regeneration of Donor Axons into the Recipient Denervated CP Nerve Stump

The cellular components of the in-growing TIB nerves included axons, Schwann cells, and myelin. Normal relationships between axons, Schwann cells, and myelin in an uninjured TIB nerve were visualized as close alignment of robust, myelinated axons (Figure 8 top row). Few, if any, GFP axons were seen initially at 4 weeks on a homogenous background of high p75 immunoreactivity from dedifferentiated Schwann cells within the denervated recipient CP nerve stump (Figure 8). There was a corresponding absence of MBP immunoreactivity at this time. By 8 and 12 weeks, a decline in p75-immunoreactive Schwann cells at the same location of the denervated CP nerve stump accompanied a progressive increase in axonal infiltration and intensifying MBP immunoreactivity. By 16 weeks, many of the gross structural relationships between axons, Schwann cells, and myelin were restored to levels approaching preinjury status (Figure 8 lower panel). This was marked by the levels of dedifferentiated Schwann cells declining to baseline (Figure 6) and axons became myelinated (Figure 7). Of note, the caliber of the regenerated axons was visibly smaller than normal.

Side-to-Side Nerve Bridges Preserve Wet Muscle Weight in the Target Muscles of the Denervated CP Nerve

After 16 weeks, the TA and EDL muscles were harvested bilaterally for analysis. The TA and EDL muscles in the right hind limb were denervated at 12 weeks by removing the CP nerves. These muscles weighed ~80% less than innervated muscle from uninjured control rats of equal size (Figure 9A). The same muscles in the contralateral left hind limb were significantly larger (Figure 9B). In these limbs, the bridges remained intact for 16 weeks and demonstrated clear reinnervation of target muscle by TIB axons via the denervated recipient CP nerve stump. These muscles weighed ~50% less than uninjured control muscles.

DISCUSSION

This immunohistochemical study qualitatively and quantitatively analyzed the outgrowth of donor TIB axons across side-to-side nerve bridges in a rat model of chronic denervation (Figures 2 and 3). Donor axons asynchronously crossed the 2 suture sites for each bridge first occupying the proximal bridge, followed in sequence by the middle then distal bridges (Figure 3). Axon regeneration after the denervation of the CP nerve stump and the insertion of the 3 cross-bridges progressed to the point at which the axons completely occupied the denervated recipient CP nerve stump over a 16-week period. Denervated Schwann cells dedifferentiated to a proliferating phenotype and then redifferentiated toward a myelinating phenotype as donor TIB axons grew across the bridges and into the

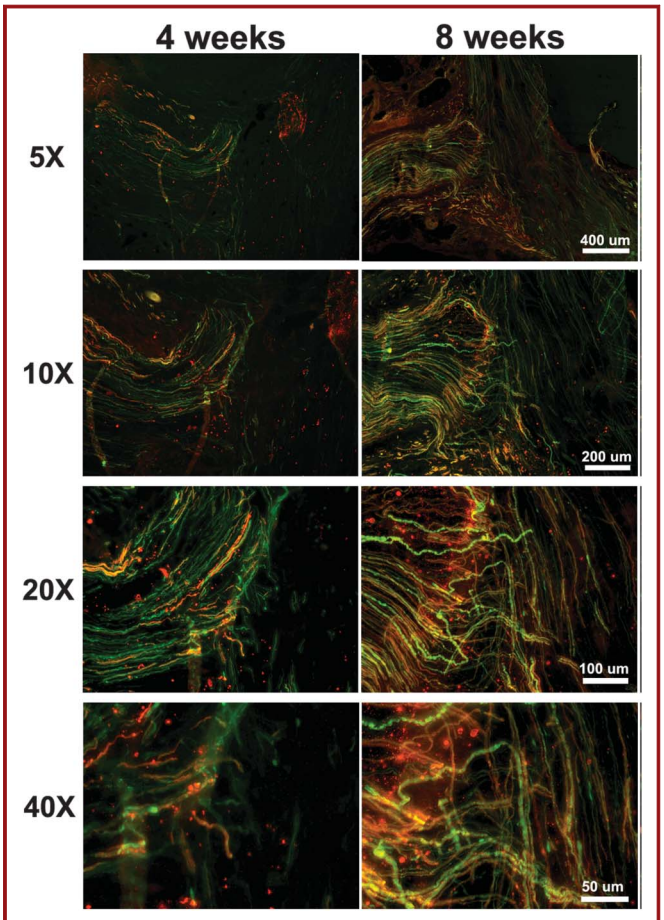

FIGURE 7. *Donor axons are remyelinated as they regenerate across side-to-side bridges between the donor nerve and recipient denervated nerve stump. Immunostaining of longitudinal sections showed that after 4 weeks, many donor TIB axons had reached the intersection of the first bridge and the recipient denervated CP nerve stump. Myelination was incomplete at this time point: several GFP axons colabeled with the red anti-myelin basic protein stain (thus producing yellow) and many did not. After 8 weeks postinjury, the majority of fibers were myelinated. The combined yellow appearance of the colabeling stains of axons and myelin seen at low magnification (5× and 10×) demonstrate the widescale myelination of regenerating axons. CP, common peroneal; GFP, green fluorescent protein; TIB, tibial.*

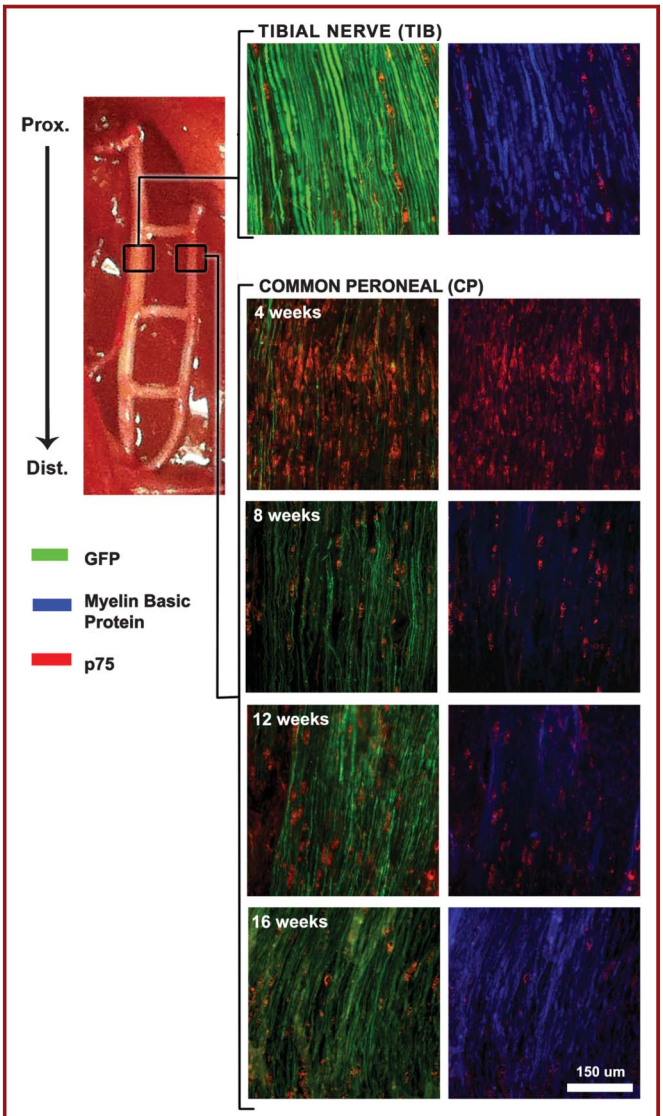

FIGURE 8. *The presence of side-to-side nerve bridges permits a transition from the denervated state toward near-normal cellular relationships within the reinnervated CP nerve stump after 16 weeks. The uninjured donor TIB nerve and denervated CP nerve stump were compared at the same level (between the first and second "bridge"). Four weeks after placing the cross-bridges, the denervated CP nerve contained dedifferentiated Schwann cells almost exclusively, very few axons, and very little myelin. However, there is a gradual transition toward robust axonal ingrowth, few dedifferentiated Schwann cells, and restoration of the myelin basic protein immunofluorescent signal. CP, common peroneal; GFP, green fluorescent protein; TIB, tibial.*

recipient denervated nerve stump (Figures 4-8). Over the period of 16 weeks, the wet muscle mass of the target musculature for the CP nerve increased, indicating that these myelinated donor TIB axons seen within the recipient denervated nerve stump had reinnervated the muscles (Figure 9).

The ingrowth of donor TIB axons into the denervated recipient CP nerve stump through 3 cross-bridges is consistent with the findings of Ladak et al.[25] These investigators used retrograde backlabeling of motoneurons to demonstrate that the majority of the TIB neurons regenerated their axons across the bridges, with only a small minority sprouting axons. Nonetheless, the functional implications of the resulting partial denervation of the TIB innervated extensor muscles are unlikely to be serious because the remaining intact TIB motor axons sprout to reinnervate denervated muscle fibers. This capacity for distal sprouting can compensate for losses of up to 80% to 85% of motor neurons within a nerve.[29] That the TIB axon reinnervated

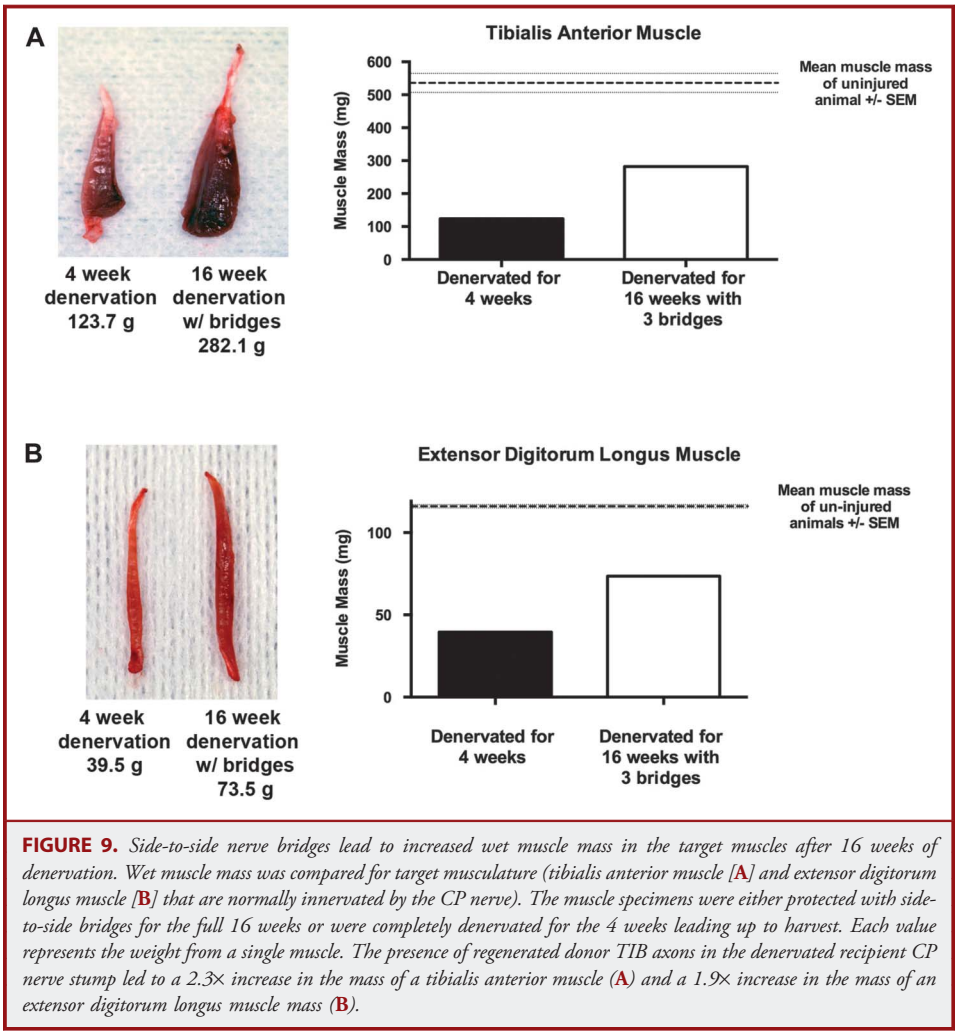

FIGURE 9. *Side-to-side nerve bridges lead to increased wet muscle mass in the target muscles after 16 weeks of denervation. Wet muscle mass was compared for target musculature (tibialis anterior muscle [A] and extensor digitorum longus muscle [B] that are normally innervated by the CP nerve). The muscle specimens were either protected with side-to-side bridges for the full 16 weeks or were completely denervated for the 4 weeks leading up to harvest. Each value represents the weight from a single muscle. The presence of regenerated donor TIB axons in the denervated recipient CP nerve stump led to a 2.3× increase in the mass of a tibialis anterior muscle (A) and a 1.9× increase in the mass of an extensor digitorum longus muscle mass (B).*

the flexor muscles via the recipient CP nerve is supported by findings that the size of the TIB axons was larger than when the axons were prevented from reaching the denervated muscles by ligation of the CP nerve distal to the placement of the 3 bridges.[30] Donor TIB axons crossed into the first cross-bridge with progressively fewer axons crossing through successive bridges (Figures 2 and 3) 4 weeks postinjury. It is unlikely that each neuron regenerated many axons through the bridges, because the ratio of the numbers of axons to the number of motor and sensory neurons that grew these axons was 2:1.[30] The decline in numbers of axons regenerating through the 3 successive cross-bridges may be related to the insertion of the cross-bridges in the same plane. In larger nerves where it would be possible to rotate the insertion of the cross-bridges could be inserted around the donor nerve with the possibility of more equal nerve crossing through the successive cross-bridges.

The initial phase of Schwann cell dedifferentiation and proliferation is a critical response to nerve injury leading to the formation of the growth-supportive environment.[14,16,19,22,31,32] In the current study, each feature of this Schwann cell response occurred within the denervated recipient CP nerve stump. Following the loss of axonal contact, dedifferentiated Schwann cells express several cell surface markers that include p75, neural cell adhesion molecule, glial fibrillary acid protein, and many others.[18] Among these markers, the p75 receptor is a robust marker of a mature Schwann cell's transition to the dedifferentiated phenotype following denervation.[12,18,22,33] Soon after the injury, p75 immunoreactivity of the denervated Schwann cells within the denervated bridges and recipient CP nerve rose dramatically, indicating that a proregenerative environment had been established (Figures 5 and 6). As donor TIB axons regenerated across the bridges and into the denervated recipient CP nerve, restoration of axonal contact with Schwann cells triggered the redifferentiation of the Schwann cells back to the myelinating phenotype with a dramatic decline in p75 immunoreactivity. The process of conversion of Schwann cells from a dedifferentiated to

a differentiated myelinated phenotype involves the upregulation of several markers of peripheral myelination including MBP, P-zero (P0), peripheral myelin protein PMP22, and myelin association glycoprotein .[18] As would be expected, the immunoreactivity of p75 at the later time points diminished, along with a reciprocal increase in the immunoreactivity of the MBP associated with newly myelinated axons. The major transition point for the relative levels of p75 and MBP was seen at 8 weeks. Interestingly, the decline in the p75 immunoreactivity to baseline levels preceded the increase in the MBP reactivity to normal levels (Figure 6).

The timing of remyelination was consistent with the staggered pattern of axon regeneration across a repair site.[28,34] Many of the *thy-1* GFP-labeled axons did not colabel with MBP 4 weeks after insertion of the bridges across an intact nerve and denervated nerve stump. One interpretation would be that only "early" TIB axons crossing the repair site at this time point would have been present in the bridge sufficiently long to undergo myelination. However, by 8 weeks, nearly all donor axons present in the distal aspect of the first bridge were myelinated (Figure 7), suggesting that both the early and late crossing axons had sufficient time to myelinate.

Ladak et al[27] demonstrated that side-to-side nerve bridges "protected" chronically denervated nerve stumps in a rat hind limb model. Their placement promoted the increased number of motoneurons regenerating axons through these chronically denervated nerve stumps, increased numbers of myelinated regenerated nerve fibers, and enhanced muscle reinnervation. The current finding of robust donor axon infiltration of the denervated recipient nerve that promoted recovery of wet muscle weight (Figure 9) is consistent with the previous findings of Ladak et al,[27] the recovery of the weight being a sufficient representation of muscle reinnervation.[13] Since the introduction of the nerve "babysitter" technique by Terzis,[35] support for the clinical use of end-to-side repair has been growing and based on encouraging functional muscle recovery in animal models and sensory return in humans.[27,36-43] This study in combination with that of Ladak et al[27] provide additional preclinical evidence to support the use of various end-to-side repair techniques in surgically managing peripheral nerve injury. The side-to-side bridge technique would be indicated where donor axon infiltration (babysitting) of the recipient nerve is desirable at multiple points along the nerve with no injury to the donor nerve. Of note, there are reports that mild degrees of donor axon injury and muscle fiber denervation occurs with the surgical placement of the epineurial windows, but that this does not translate into a functional deficit in the donor muscle over time.[37,44-46] Reassuringly, no histological evidence of donor axon injury was observed in this study.

The mechanism underlying the protective effects of side-to-side nerve bridges observed by Ladak et al[27] may be supported by evidence of maintained axon-glial interactions seen in this study. Specifically, the processes that regulate a change in Schwann cell phenotype and also remyelination are directed, at least in part, by neurotrophic factor signaling. For example, the process of myelination has been shown in mutant knockout models to be regulated by axonally membrane-bound Neuregulin-1 type III.[47,48] The finding of progressively increasing MBP immunoreactivity beyond 12 weeks within the distal recipient nerve suggests that neurotrophic factor levels, including Neuregulin-1, are sustained beyond the time period when they would normally drop off.[8,21] In this way, donor axon infiltration into the recipient nerve may have a direct influence in maintaining the regenerative environment well into the period of chronic denervation.

Future work will expand the number of immunohistochemical markers used in analyzing peripheral nerve regeneration across side-to-side nerve bridges. Another important component of future work will be to focus on electrophysiological and histological analysis of the denervated muscle tissue that is reinnervated by donor axons that migrate across the bridges. Further, determining the motor and sensory neuron composition of the protected, denervated recipient nerve will help to provide important information about the expected recovery of sensory and/or motor function.

CONCLUSION

Currently, there are no medical or surgical therapies that specifically address the harmful consequences of chronic denervation following peripheral nerve injury. However, Ladak et al[27] reported in 2011 that side-to-side nerve bridges can directly enhance nerve regeneration and denervated muscle mass in the setting of chronic denervation. Therefore, understanding the mechanisms responsible for these effects may have substantial clinical impact in the future. Consistent with this, we examined the cellular aspects of regeneration and showed: (1) donor axons readily regenerate across the side-to-side nerve bridges in a staggered fashion; (2) extensive Schwann cell dedifferentiation occurs within the denervated recipient nerve following injury as a result of losing axonal contact secondary to Wallerian degeneration; and (3) donor axons become remyelinated as Schwann cells redifferentiate over the 16-week period. Many aspects of axon regeneration, Schwann cell dedifferentiation, redifferentiation, and remyelination are regulated in part by soluble or contact-based neurotrophic signaling. These processes are also known to become dysregulated over the course of chronic denervation in the absence of restored axonal contact.[1,8,9,13,33] Taken together, the patterns of regeneration observed in this study illustrate the axon-glial interactions that may promote a regenerative environment within the chronically denervated nerve stump.

Disclosures

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