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Integration of engrafted Schwann cells into injured peripheral nerve: Axonal association and nodal formation on regenerated axons

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Abstract

Transplantation of myelin-forming cells can remyelinate axons, but little is known of the sodium channel organization of axons myelinated by donor cells. Sciatic nerve axons of female wild type mice were transected by a crush injury and Schwann cells (SCs) from green fluorescence protein (GFP)-expressing male mice were transplanted adjacent to the crush site. The male donor cells were identified by GFP fluorescence and fluorescence in situ hybridization (FISH) for Y chromosome. In nerves of GFP-expressing mice, GFP was observed in the axoplasm and in the cytoplasmic compartments of the Schwann cells, but not in the myelin. Following transplantation of GFP-SCs into crushed nerve of wild type mice, immuno-electron microscopic analysis indicated that GFP was observed in the cytoplasmic compartments of engrafted Schwann cells which formed myelin. Nodal and paranodal regions of the axons myelinated by the GFP-SCs were identified by Na_v 1.6 sodium channel and Caspr immunostaining, respectively. Nuclear identification of the Y chromosome by FISH confirmed the donor origin of the myelin-forming cells. These results indicate that engrafted GFP-SCs participate in myelination of regenerated peripheral nerve fibers and that Na_v 1.6 sodium channel, which is the dominant sodium channel at normal nodes, is reconstituted on the regenerated axons.

Keywords

Schwann cells; Transplantation; Peripheral nerve

Following peripheral nerve transection, axons in the proximal nerve region sprout and can regenerate toward peripheral targets often leading to target reconnection [13]. Schwann cells derived from the distal nerve segment subsequently myelinate these regenerated axons leading to rapid impulse conduction. Transplantation of mitogen-expanded Schwann cells into transected nerve can compete with endogenous Schwann cells and contribute to the myelination of the regenerated axons [6], and transplantation of human Schwann cells can remyelinate

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rodent regenerated peripheral nerve fibers [11]. Demyelinated spinal cord axons are also capable of being remyelinated by engraftment of Schwann cells [1,3,5,8,9].

A prerequisite for proper impulse conduction of regenerated myelinated axons is the construction of nodes of Ranvier with appropriate sodium channel organization to achieve rapid and secure impulse conduction. While it has been known for some time that sodium channel density is high at the node of Ranvier (see [16] for a review), recent work indicates that normal mature nodes of Ranvier express Na_v 1.6, which corresponds to a TTX-sensitive, kinetically fast sodium channel subtype [4]. Here, we report that transplanted Schwann cells derived from GFP-expressing transgenic rodents align with the regenerated axons forming internodes and nodes of Ranvier. The nodal regions of the host axons associated with the GFP-Schwann cells showed Na_v 1.6 sodium channel immunostaining flanked by paranodal Caspr immunostaining. Thus, an appropriate sodium channel subtype is expressed on new nodes of Ranvier on regenerated axons that have been myelinated by identified transplanted Schwann cells. These results indicate that engraftment of exogenous donor Schwann cells are capable of signaling the regenerated host axons for appropriate sodium channel deposition at nascent nodes of Ranvier, which is compatible with normal impulse conduction.

Schwann cells were isolated from sciatic nerves of GFP-expressing mice ([C57BL/6-TgN (ACtbEGFP)]; chicken β-actin promoter; Jackson Laboratories Inc.) isolated as described previously in Lankford et al. [10]. Briefly, sciatic nerves were desheathed, minced, incubated for 45 min in DMEM (Gibco BRL, Grand Island, NY, USA) with 10 mg/ml each collagenase A and collagenase D (Boehringer Mannheim, Mannheim, Germany), pelleted, incubated an additional 15 min in complete saline solution CSS with 2.5 mg/ml trypsin, and triturated though a fire-polished siliconized pasture pipette and washed twice with DMEM containing 10% fetal bovine serum (FBS). The cells were washed three times in DMEM without FBS before transplantation.

The mice were anesthetized with ketamine (75 mg/kg i.p.) and xylazine (10 mg/kg i.p.). Sciatic nerves of wild type mice ($n = 10$) were exposed near the tendon of the piriformis muscle and crushed for 20 s with fine forceps. This procedure transects all of the axons in the nerve, but because the epineurium is intact, the axons can regenerate into the distal nerve segment. Immediately after the nerve crush, 0.5 μl of a GFP-Schwann cell suspension (3.0×10^4 cells/μl) was injected just distal to the crush site through a glass pipette (40 μm tip diameter) attached to a 1 μl Hamilton syringe. Ten to 14 days after cell injection, the animals were perfused with 4% paraformaldehyde and the nerves removed and desheathed. Half of the nerves were placed in buffer on a slide and gently teased with fine forceps to identify individual sciatic nerve fibers and the remainder prepared for frozen sections.

FISH for Y chromosome was performed as previously described [15]. The Y-chromosome probe was generated by PCR and labeled with digoxigenin by Nick Translation.

Tissue on glass slides (Lab Tek, Miles Scientific) was rinsed once with phosphate-buffered saline (PBS) and incubated for 30 min in blocking solution. Tissue was then incubated overnight at 4 °C with polyclonal antibodies to Na_v 1.6 (1:100; Alomone, Jerusalem) with and without mouse monoclonal anti-Caspr (1:200; generous gift from Matt Rasband, University of Connecticut). Following extensive washes, tissue was incubated in secondary antibody(ies) (goat anti-rabbit IgG-Cy3, 1:3000, Amersham; goat anti-mouse IgG-Cy5, 1:250, Rockland). Tissue sections or teased nerve fibers were cover-slipped with Aqua-poly-mount (Polysciences). Tissues were examined with a Nikon E800 microscope equipped with bright field, differential interference and epifluorescent optics and fitted with a Dage DC 330 cooled CCD color camera or a Nikon PCM-2000 laser scanning confocal microscope. Images were

captured with IPLab (Nikon E800) or simple PCI (Nikon PCM-2000). Images were processed and composed in Adobe Photoshop version 5.5.

Animals were perfused transcardially with PBS followed by 4% paraformaldehyde/0.02% glutaraldehyde in PBS. Sciatic nerves were removed, post-fixed overnight in 4% paraformaldehyde, and embedded in 3% agar for vibratome sectioning. Hundred and fifty micrometers thick free-floating sections were preincubated with 2% normal goat serum for 30 min. GFP^+ cells were identified by fluorescence microscopy. For immunoperoxidase staining, the sections were incubated with rabbit anti-GFP antibody (1:2000, Chemicon) overnight at 4 °C. The sections were incubated in Vectastain anti-rabbit ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature according to the manufacturer's instruction. The sections were post-fixed with 1% osmium tetroxide for 4 h, dehydrated in graded ethanol solutions, and embedded in Epox-812 (Ernest Fullam, Latham, NY, USA). Thin plastic sections were cut on an ultramicrotome and counterstained with uranyl and lead salts and examined with a Zeiss (Oberkochen, Germany) EM902A electron microscope operating at 80 kV.

Nodal and paranodal regions of dissociated normal non-transected sciatic nerve fibers derived from a GFP transgenic mouse can be seen with both bright field and fluorescence microscopy (Fig. 1A and B, respectively). The green fluorescence associated with myelinated axons of GFP transgenic mice has a distinct distribution in the myelinated axons reflecting the cytoplasmic distribution of GFP. Moreover, GFP is observed within the axoplasm of the axon and in a thin slip surrounding the axon (Fig. 1C). A thin rim of Schwann cell cytoplasm surrounds myelin (outer mesaxon, [12]), and the thin band of outer green fluorescence corresponds to this region [14]. Between these two regions of intense fluorescence, a region of low fluorescence is observed (Fig. 1C and D), probably corresponding to the position of myelin. Intense sodium channel immunostaining was observed at the putative nodes of Ranvier (Fig. 1C and D). Thus, in the normal GFP transgenic rat, GFP was observed in the cytoplasmic compartments of axons and Schwann cells.

Fluorescence in situ hybridization (FISH) for the Y chromosome was carried out on dissociated sciatic nerves from male GFP transgenic mice and nuclei were counterstained with DAPI (Fig. 1E). A clear association of Y chromosome (pink) can be seen within the large elongated nuclei (blue) of the Schwann cells. These results indicate that the regional distribution of GFP from GFP transgenic male mice can be observed in cytoplasmic compartments of axons in normal peripheral nerve fibers and their myelinating partners, Schwann cells, and that FISH analysis for the Y chromosome is associated with the Schwann cell nuclei.

A comparable analysis was carried out on regenerating peripheral nerve fibers from non-transgenic female mice into which Schwann cells from GFP transgenic male mice were transplanted into the injured nerves. The sciatic nerve fibers were completely transected by fine forceps crush and GFP Schwann cells were microinjected into the nerve region just distal to the injury site. Fig. 2A shows a single isolated teased fiber from the regenerated nerve segment 2 weeks after transplantation. Note the periodic regions of high-intensity green fluorescence in bulbous enlargements along the course of the axon. These regions correspond to the large cytoplasmic compartment around the nuclei of the remyelinating GFP Schwann cells. A higher power micrograph (Fig. 2B) shows a node-like region (arrow) between two Schwann cells. Note that the green fluorescence intensity is low at the putative node.

Unlike axons from the GFP-expressing mice (Fig. 1) the regenerating wild type axons do not express GFP, and thus, GFP should only be observed in the transplanted Schwann cells. Sodium channel immunohistochemistry revealed nodal regions and short internodal regions of these regenerated axons (Fig. 2C), which is characteristic of remyelinated axons [2]. Sodium channel

$Na_v1.6$ and paranodal immunostaining (Caspr) of the regenerated axons associated with the engrafted GFP-SCs indicates nodal and paranodal regions have formed on the regenerated axons (Fig. 2E). Note the green fluorescence in the Schwann cell cytoplasm adjacent to the node.

FISH analysis indicated that the nuclei of the transplanted cells were associated with the Y chromosome (Fig. 2D). Thus, the donor cells could be identified by both Schwann cell GFP expression and by the presence of a Y chromosome. It is important to note that not all regions of the sciatic nerve exhibited GFP remyelination profiles; variable regions of nerve distal to the transection site showed non-GFP myelin profiles. It is known that without cell transplantation endogenous myelination will occur in this model system. It is likely that the extent of GFP Schwann cell-associated myelination was dependent on the proximity of the injected cells to regenerating axons and the extent of competition between endogenous Schwann cell and GFP-donor cell availability.

Electron microscopic examination of anti-GFP immunoperoxidase reacted nerve sections revealed that GFP^+ cells were in direct contact with host axons. Reaction product was clearly evident in the cytoplasm of some cells that formed well-defined multi-laminate structures characteristic of myelin (Fig. 2F and G). The immunoreaction product was clearly observed in the cytoplasmic domain in the outside of the compact myelin.

In the present study, we report that transplantation of Schwann cells from GFP transgenic rats into transected sciatic nerve results myelination of regenerated axons and that the newly formed nodal regions express $Na_v1.6$ sodium channel. The donor cells were identified by GFP observed in their cytoplasm and by FISH for the Y chromosome in the Schwann cell nuclei. This combination of markers provides a powerful method to identify donor myelin-forming cells in injured nerve and white matter. The GFP fluorescence, while limited to cytoplasmic regions, readily allowed characterization of internodal and nodal regions, thus allowing internodal lengths of the regenerated axons to be characterized. $Na_v1.6$ corresponds to a TTX-sensitive, kinetically fast sodium channel subtype which is expressed in normal mature nodes of Ranvier [4]. The intense $Na_v1.6$ immunostaining at the nodes of Ranvier of the regenerated axons suggests that nodal sodium channel organization for appropriate impulse conduction at the nascent nodes of the regenerated axons. Moreover, these data also suggest that appropriate signaling by the transplanted Schwann cells occurs for nodal formation of the regenerated axons.

Nerve fibers of non-injured GFP transgenic mice showed structural details of the myelinated fiber, although GFP was observed only in cytoplasmic compartments. The axoplasm, the paranodal region, and the thin cytoplasmic regions outside of the myelin showed intense green fluorescence. The myelin domain immediately surrounding the axon did not express green fluorescence reflecting the lack of cytoplasm in the myelin. In agreement with this characterization, sodium channel immunostaining was intense on the nodal membrane and sparse at the internodal axon region, and not present in the myelin. In the axons myelinated by GFP donor cells, the GFP fluorescence was intense in donor cell cytoplasm including the large cytoplasmic region around the Schwann cell nuclei and the outer cytoplasmic slip (mesaxon) surrounding the myelin, but not in myelin or axoplasm. This compartmentalization of GFP was confirmed by immunoelectron microscopy. These results indicate that donor Schwann cells can integrate into regenerating peripheral nerve and provide appropriate signaling mechanisms for appropriate sodium channel expression at nascent nodes and myelination of regenerated axons.

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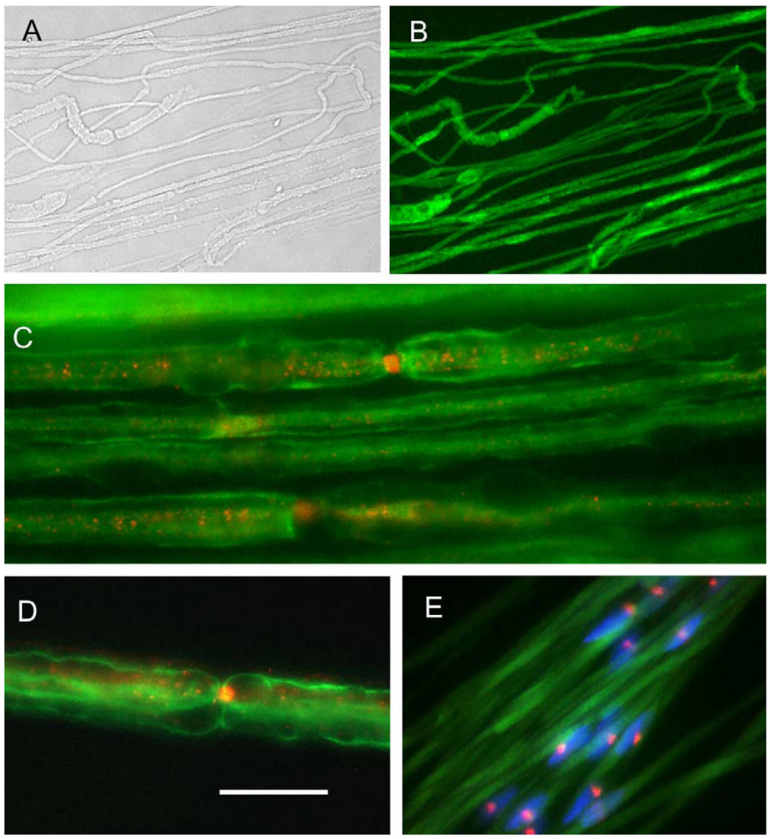

Fig. 1.

Morphology of dissociated GFP-expressing mouse nerve. Bright field image (A) and fluorescence (B) images of same teased axon field. (C) Nodal regions were studied by Na^+ channel immunohistochemistry on normal GFP-expressing mouse nerve. (D) Higher magnification of immunostained sodium channel at a node of Ranvier showing more detailed nodal and internodal structure. (E) Y chromosome identification in Schwann cells of mouse peripheral nerve in a normal sciatic nerve of a male GFP mouse. The Schwann cell nuclei were stained with DAPI (blue) and the Y chromosome (pink) was visualized with FISH. Calibrations: (A and B) 40 μm; (C and D) 10 μm; (E) 20 μm.

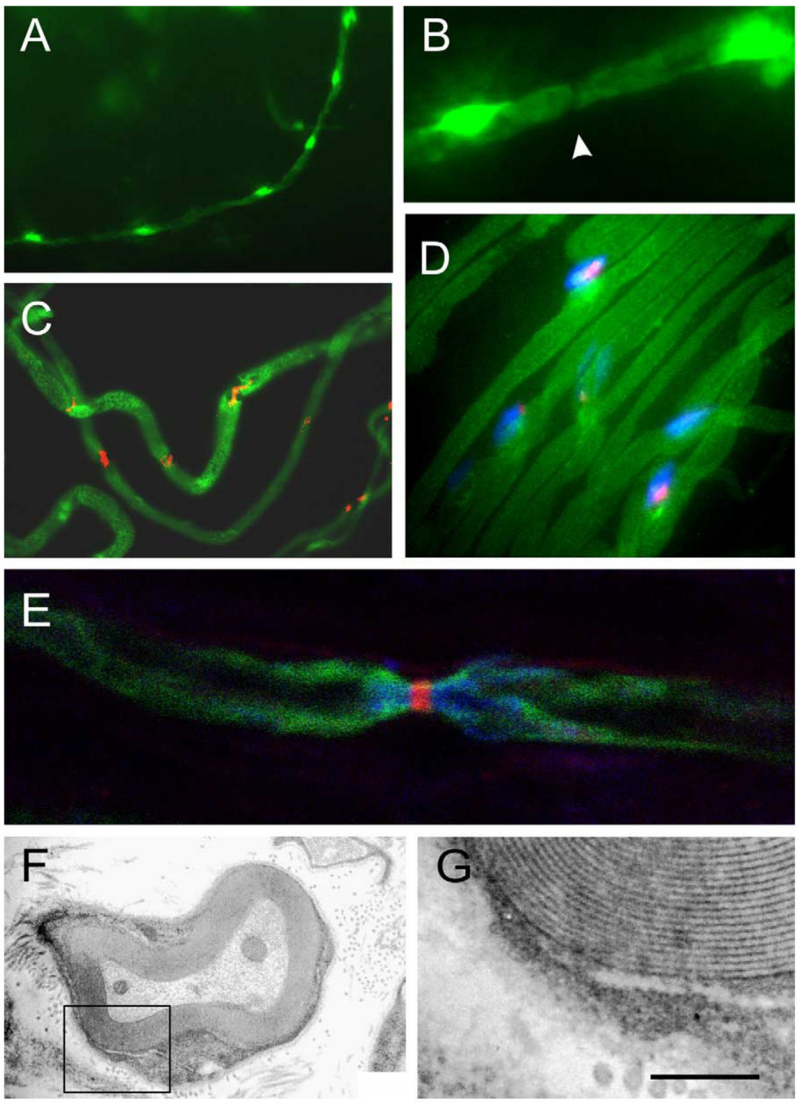

Fig. 2.

Node of Ranvier in regenerated nerve fibers following transplantation of GFP-expressing Schwann cells into crushed non-GFP-expressing mouse nerve. (A) Note the sequential myelinated segments by the transplanted GFP-expressing cells. (B) Higher power image showing node-like structure (arrow) and two adjacent regions with intense green fluorescence near nuclei. (C) Immunostaining for Na^+ channel shows three nodal regions with short internodes. (D) FISH (pink) for Y chromosome on transplanted male Schwann cells into crushed female sciatic nerve. Nuclei are stained with DAPI (blue). (E) Sodium channel (Na_v 1.6; red) and paranodal (Caspr; blue) immunostaining showing node formation of regenerated axons myelinated by engrafted Schwann cells (confocalimage). (F) Immunoreaction product for GFP can be seen in the cytoplasm of cells forming myelin. (G) Boxed area in (F). Calibrations: (A) 60 μm; (B and D) 20 μm; (C) 30 μm; (E) 10 μm; (F) 0.3 μm: (G) 0.1 μm.