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Development of transplantable nervous tissue constructs comprised of stretch-grown axons

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Abstract

Pursuing a new approach to nervous system repair, fasciculated axon tracts grown in vitro were developed into nervous tissue constructs designed to span peripheral nerve or spinal cord lesions. We optimized the newfound process of extreme axon stretch growth to maximize the number and length of axon tracts, reach an unprecedented axon growth-rate of 1 cm/day, and create 5 cm long axon tracts in 8 days to serve as the core component of a living nervous tissue construct. Immunocytochemical analysis confirmed that elongating fibers were axons, and that all major cytoskeletal constituents were present across the stretch-growth regions. We formed a transplantable nervous tissue construct by encasing the elongated cells in an 80% collagen hydrogel, removing them from culture, and inserting them into a synthetic conduit. Alternatively, we induced axon stretch growth directly on a surgical membrane that could be removed from the elongation device, and formed into a cylindrical construct suitable for transplant. The ability to rapidly create living nervous tissue constructs that recapitulates the uniaxial orientations of the original nerve offers an unexplored and potentially complimentary direction in nerve repair. Ideally, bridging nerve damage with living axon tracts may serve to establish or promote new functional connections.

Keywords: Axon growth; Nerve repair; Peripheral nerve injury; Spinal cord injury; Nerve construct; Tissue engineering

1. Introduction

With insufficient techniques to repair peripheral nerve damage and no clinically effective approach to bridge spinal cord lesions, millions of patients endure devastating lifelong disabilities. For peripheral nerve injury (PNI), autologous nerve grafting remains the gold standard for repair. However, this approach is limited by the availability of donor nerve and complications arising from the harvesting surgery (Evans, 2000; Lee and Wolfe, 2000). As an alternative, synthetic conduits made from collagen or polyglycolic acid (PGA) have been increasingly used to reconnect severed nerves in patients (Belkas et al., 2004; Evans, 2001; Meek and Coert, 2002; Schmidt and Leach, 2003). These conduits provide a physical guide for axons sprouting from the proximal nerve stump to reach the disconnected nerve segment. Thereafter, chemical and physical cues from the disconnected nerve form a labeled pathway, which then directs the continued growth of regenerating axons to ultimately reinnervate the tissue (Belkas et al., 2004; Hall, 2001; Lee and Wolfe, 2000). However, synthetic conduits have only been clinically successful for the repair of short nerve lesions and are typically used for gaps less than 1–2 cm (Belkas et al., 2004; Evans, 2000; Hall, 2001; Lee and Wolfe, 2000; Meek and Coert, 2002). Regardless of whether nerves are grafted with donor nerves or synthetic conduits, the axons and many supportive cells of the disconnected portion of the nerve rapidly degenerate, resulting in the loss of the labeled pathway necessary to guide axon outgrowth (Belkas et al., 2004; Evans, 2000; Hall, 2001; Lee and Wolfe, 2000; Meek and Coert, 2002). This degeneration, coupled with the relatively slow growth of sprouting axons (approximately

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1 mm/day), commonly results in poor functional recovery of extremities that are far away from the nerve damage (Belkas et al., 2004; Hall, 2001; Lee and Wolfe, 2000).

Even more daunting than PNI repair is the restoration of axonal pathways transected due to spinal cord injury (SCI). To successfully bridge spinal cord lesions, new intraspinal circuits or 'relays' must be formed (Bareyre et al., 2004; Bunge, 2001; Fawcett, 2002). However, SCI lesions are commonly several centimeters long and therefore require extensive axon growth in an environment that is notoriously non-permissive for axon outgrowth (Bunge, 2001; Fawcett, 2002; Fry, 2001; Hall, 2001; McKerracher, 2001). While many promising preclinical studies have demonstrated techniques that facilitate axon growth in animal models of SCI, producing axons of sufficient length and number for clinical application remains an enormous challenge (Bunge, 2001; Fawcett, 2002; Fry, 2001; McKerracher, 2001).

Recently, we have identified a new mechanism of sustained axon growth that can far exceed the rate of axon growth cone extension (Pfister et al., 2004; Smith et al., 2001). Unlike axon outgrowth in response to chemical cues, established axon tracts can grow under the application of mechanical forces. Since these axons no longer have growth cones, this distinct growth process is driven by mechanical stretch applied to the central portion of the axon cylinder. We have recapitulated this natural axonal "stretch-growth" through the progressive mechanical distraction of axons bridging two populations of cultured neurons. Even at rates of stretch up to 1 cm/day, this process results in axon expansion in both length and caliber, producing uniaxially oriented tracts.

Here we exploited this axon stretch growth process to develop nervous tissue constructs comprised of living axons. Rather than enticing axons to regenerate in vivo, axons are rapidly stretch grown ex vivo to a length sufficient to span the nerve damage. These stretch-grown axon tracts serve as the core element in a three-dimensional transplantable nervous tissue construct that recapitulates the uniaxial orientations of the original nerve. This approach may establish or promote functional connections necessary for nervous system repair that have not been achieved by other methods.

2. Methods

2.1. Neuronal cultures

Dorsal root ganglia (DRG) were isolated from E15 rat embryos (Charles River, Wilmington, MA) as described elsewhere (Kleitman et al., 1998). DRG explants from 8 to 10 pups were plated immediately following dissection along the elongator substrate interface. Cultures were maintained in NeuroBasal Medium (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen), 1% FBS (Hyclone, Logan, UT), 0.4 mM L-glutamine (Invitrogen), 2.5 g/L glucose, and 10 ng/mL 2.5S nerve growth factor (Becton Dickinson, Bedford, MA). Cultures were treated with the mitotic inhibitors (MI#1, 5 μM cytosine β-D-arabinofuranoside (Ara-C, Sigma, St. Louis, MO), 20 μM 5-fluoro-2′-deoxyuridine (Sigma) and 20 μM uridine (Sigma)) on the day of plating. For stretch grown cultures described below, a very high density of neurons is required, which inhibits the elimination of non-neuronal cells. We found that the addition of Ara-C to the first mitotic inhibitor application hasted non-neuronal elimination without compromising culture viability. After 2 days, the medium was exchanged including the mitotic inhibitors (MI#2, 20 μM 5-fluoro-2′-deoxyuridine (Sigma) and 20 μM uridine (Sigma)) for 3 days. Thereafter the media was changed every 2–3 days and the mitotic inhibitors MI#2 were applied once a week to prevent non-neuronal proliferation.

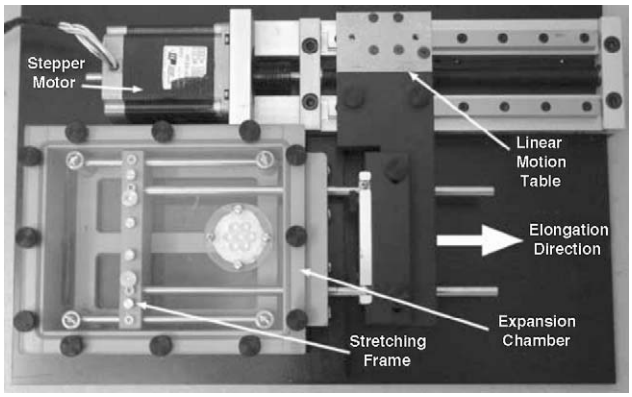

Fig. 1. Axon stretch-growth system. The expansion chamber serves as a sealed tissue culture housing with a port for CO_2 exchange, removable axon stretching frame and connecting rods. Axons are mechanically stretched by a stepper motor and linear motion table, which is fixed to the connecting rods. In turn, the rods displace the stretching frame with 0.5 μm accuracy. System is computer controlled by a programmable stepper motor indexer (not shown).

2.2. Axon stretch-growth system

The axon stretch-growth bioreactor is composed of a custom designed axon expansion chamber, linear motion table, stepper motor and controller (Fig. 1). The expansion chamber serves as tissue culture support and housing consisting of a sealed enclosure with a port for CO_2 exchange, removable axon stretching frame, and connecting rods to apply displacements. The axon stretching frame was designed to slowly divide two populations of neuronal somas thereby stretching their interconnecting axons. The stretching frame arranges two adjoining substrates in an overlapping fashion on which neural cells are cultured (Fig. 2). The bottom substrate (198 μm thick), made of optically transparent Aclar 33C film (Structure Probe, West Chester, PA), covers the entire bottom of the stretching frame on which a stationary population of neurons is cultured. An overlapping movable substrate (10 μm thick) was placed on top of the bottom Aclar substrate and serves as the moving population of cells. Using an automated microstepper motor and controller system (Servo Systems, Montville, NJ), the moveable substrate was strategically displaced across the bottom stationary culture substrate.

Axon stretch growth was evaluated on poly-L-lysine (PLL), collagen and laminin culture surface preparations. Elongator Aclar substrates were washed with laboratory soap, treated in 1 M NaOH for 24 h, sterilized in 100% ethanol for 10 min and then attached to the stretching frame using medical grade RTV

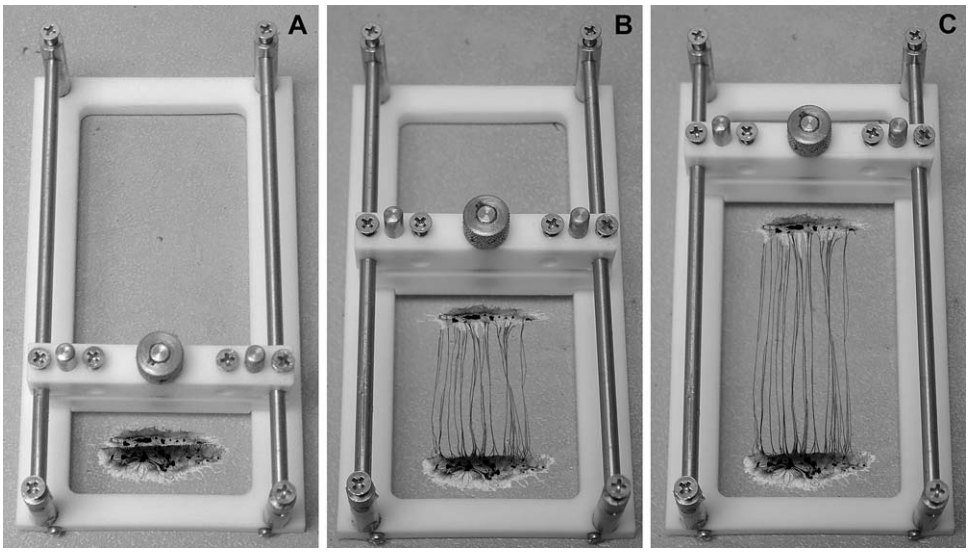

Fig. 2. A schematic of axon stretch-growth. (A) Neurons are plated on two adjoining substrates and are given sufficient time for axons to bridge the two substrates and integrate with neurons on both sides. (B) The stretching frame displaces one population of neurons away from the other, thereby elongating the interconnecting axons. (C) Axon stretch-growth is a process that can be gradually induced to achieve a rate of 1 cm/day of growth and to lengths of at least 10 cm. Here, Adobe PhotoShop was used to overlay modified images of stretch grown cultures (similar to Fig. 3) onto an axon stretching frame.

silicone (NuSil, Carpenteria, CA). All culture surfaces were first treated with 10 μg/mL PLL and then followed by collagen or laminin coating. Type 1 rat-tail collagen (Becton Dickinson) as supplied was spread over the surface, polymerized by exposure to ammonia vapors for 2 min and then allowed to dry completely before plating cells. Alternatively, culture surfaces were treated with 20 μg/mL laminin (Sigma) in NeuroBasal medium for 2 h at 37 °C.

2.3. Stretch-growth of transplantable axons

As illustrated in Fig. 2, rat DRG explants were plated on the two adjoining substrates and axons were allowed to develop for 5 days in culture. Within this time period, axons readily grew across the interface between the two adjoining substrates and into the population of neurons on either side. Axons bridging the two populations of neurons were stretch-grown by displacing the two adjoining substrates in a stepwise fashion. Each elongation program started at 1 mm/day (2 μm displacements every 172 s) for the first 24 h and then a gradually increasing growth rate was strategically applied to achieve extreme stretch-growth rates (Pfister et al., 2004).

For this study, two paradigms were used to create nerve constructs: a 1 cm construct in 6 days and a 5 cm construct in 14 days. One centimeter constructs were created by elongating axons at a rate of 1 mm/day for 1 day, 2 mm/day for 4 days, and then slowed to 1 mm/day over the final day. Five centimeter constructs were created by escalating the elongation rate over 3 days to 4 mm/day and held constant for 11 days, as shown in Table 1. These complex paradigms were automated using a stepper motor indexer and computer software (SiProgrammer, Applied Motion Products, Bloomington, MN).

While spinal cord injuries may require a nerve construct of a few centimeters, peripheral nerve injuries are often longer, requiring constructs several centimeters in length. Variations of our axon stretch-growth system have been designed to optimize the creation of nervous tissue constructs up to 15 cm in length. Since stretch-growing axons for long constructs can take several weeks in culture, a port was created to remove and inject culture medium with the use of a syringe to facilitate media change without disturbing the expansion chamber. In consideration of

Table 1
Axon stretch-growth paradigms used to escalate to 1 cm/day and for 1 and 5 cm long nervous tissue constructs

Time	Displacement step (μm)	Resting period (s)	Net growth rate (mm/day)	Total length (mm)
Escalation to 10 mm/day				
24 h	0.002	172.0	1	1
12 h	0.002	86.4	2	2
12 h	0.002	57.6	3	3.5
24 h	0.004	86.4	4	7.5
12 h	0.004	69.1	5	10
24 h	0.004	57.6	6	16
12 h	0.004	49.4	7	19.5
24 h	0.004	43.2	8	27.5
24 h	0.005	48.0	9	36.5
24 h	0.005	43.2	10	46.5
1 cm in 6 days				
1 day	0.002	172.0	1	1
4 days	0.002	86.4	2	9
1 day	0.002	172.0	1	10
5 cm in 14 days				
1 day	0.002	172.0	1	1
1 day	0.002	86.4	2	3
1 day	0.002	57.6	3	6
11 days	0.004	86.4	4	50

The net rate of elongation was applied through a displacement and resting time in a stepwise manner. As shown here, 1 mm/day was delivered with 2 μm displacements every 172 s over the first 24 h. Escalation of the stretch-growth rate required 12–24 h of acclimation time to each 1 mm/day increase in growth rate.

making extremely long constructs rapidly, we also investigated escalating the axon stretch growth up to a rate of 1 cm/day. To accomplish this, the rate of stretch was increased in a stepwise manner by 1 mm/day every 24 h over 10 days to reach a growth rate of 1 cm/day.

Once axons were grown to the desired length, their removal for nervous tissue construct assembly required an unobstructed access to the stretch-grown axons. A removable stretching frame was designed such that the frame and entire tissue culture could be separated from the expansion chamber and placed on a workspace. Microsurgical instruments could be easily manipulated to detach the Aclar substrates from the frame without damaging the delicate nervous tissue.

2.4. Immunocytochemistry and electron microscopy

Stretch-grown axons were fixed with 4% paraformaldehyde for 1 h. Following three rinses in phosphate buffered saline (PBS), elongated axons were treated with 0.1% Triton X and 4% normal goat serum (NGS) in PBS at room temperature for 1 h and then rinsed three times with PBS. The primary antibodies were applied in 4% NGS, 0.1% Triton X in PBS for 1 h and then rinsed three times in PBS. Secondary antibodies diluted in PBS were applied for 1 h. Primary antibodies targeted the phosphorylated state of the 200 kDa neurofilament fragment (SMI-31, Sternberger Monoclonals, Lutherville, MD, 1:400 dilution), the assembled form of β-tubulin (SMI-62, Sternberger Monoclonals, dilution of 1:400), the phosphorylated state of Tau (Tau5, Dako, Carpenteria, CA, #A0024, 1:400 dilution), and MAP2 (AP-20, Sigma, 1:500 dilution). Fluorescent secondary antibodies were obtained from Molecular Probes, Eugene, OR.

For transmission electron microscopy (TEM), elongated axons were fixed in 4% paraformaldehyde, 2% glutaraldehyde, in 0.1 M sodium cacodylate buffer overnight at 4 °C, post-fixed in 1% osmium tetroxide for 1 h at 4 °C, and dehydrated in a graded ethanol series before infiltration and embedding in epoxy resin (EMbed-812, Electron Microscopy Sciences, Fort Washington, PA). After staining with uranyl acetate and lead citrate, sections were examined with a JEOL 100CX transmission electron microscope. For scanning electron microscopy (SEM), elongated axons were fixed in 4% paraformaldehyde, 2% glutaraldehyde, in 0.1 M sodium cacodylate buffer overnight at 4 °C, post-fixed in 1% osmium tetroxide for 30 min and dehydrated in a graded ethanol series followed by a drying step of two 10 min applications of hexamethyldisilazane (Electron Microscopy Sciences). Sample was then sputter coated with aluminum prior to visualization and examined on a JEOL 6300F FEG HRSEM electron microscope.

2.5. Nervous tissue construct

Nervous tissue constructs consisted of three components: an outer implantable sheath to form a conduit, an extracellular matrix within the lumen, and *living* stretch-grown axons embedded within the extracellular matrix. Axons, grown to the desired nerve construct length, were supported in collagen hydrogels to protect axons from damage and preserve their uniaxial orientation during construct assembly. To determine the optimal gel stiffness, we tested hydrogels consisting of 40, 60 and 80% (v/v) type 1 rat tail collagen (Becton Dickinson). Working on ice, collagen, 10X DMEM medium (Sigma) and sterile water were mixed and the pH was adjusted to 7.4 with sodium hydroxide. Hydrogel solution was gently pipetted over the elongated axons and incubated at 37 °C for 1 h.

The elongated axon-hydrogel complex was removed from the axon stretching frame by cutting away the Aclar substrates with a scalpel. The elongated cultures, embedded in a hydrogel, were then rolled into a cylindrical shape and transferred into the lumen of a PGA conduit (Neurotube®, Neuroregen, LLC, Bel Aire, MD) to form a transplantable construct. Alternatively, a nylon membrane was secured to the bottom of an elongation chamber using RTV silicone. Axons were stretch-grown on top of the nylon membrane and then supported in collagen gel as described above. In this case, nervous tissue constructs were formed by removing the membrane, hydrogel and stretch-grown axon combination from the elongation device and folded to form a tubular conduit.

3. Results

3.1. Axon stretch growth for transplantation

As a natural progression of our previous identification of stretch growth of integrated axon tracts (Pfister et al., 2004), we identified several key optimal conditions to rapidly produce long, numerous and viable axon tracts that could be used for transplantation and repair. We found that axon tracts can be mechanically elongated at the unprecedented growth rate of 1 cm/day without axon disconnection. This was accomplished by applying a more conservative growth rate escalation scheme in comparison to our previous findings (Pfister et al., 2004). Rather than push the limits of the neuron's ability to adapt to an aggressive rate escalation scheme, the stretch-growth rate was increased by 1 mm/day every 24 h, thereby allowing ample time for axons to acclimate to each step in growth rate. As a result, our first attempt allowed the axon growth rate to be escalated to 1 cm/day and maintained for at least 48 h without evidence of axon rupture. Over the following four consecutive experiments, the acclimation time was reduced from 24 to 12 h, one by one, in four of the rate increase steps, Table 1. Each culture was successfully escalated to 1 cm/day and maintained for at least 48 h without axon disconnection. This reduced the total rate escalation time to reach 1 cm/day from 10 days to 8 and resulted in axon tracts almost 5 cm in length in a repeatable manner ($n = 3$). No further increases in the rate escalation scheme were investigated.

In addition to establishing a higher rate of axon stretch growth, we optimized the elongator substrate preparation to increase the density of elongating axons in each culture, thereby increasing the total number of axons available for incorporation into a nervous tissue construct. We evaluated axon stretch growth from neurons plated on the elongator substrates coated with PLL ($n = 3$), laminin ($n = 3$), or a collagen gel ($n = 10$), to determine which preparation would allow for the greatest number of stretch growing axons. After neurons were plated, cultures

Fig. 3. Stretch-grown DRG axons. DRG explants from eight rat pups were plated within an elongation chamber and allowed 5 days for axons to integrate with neurons on both adjoining substrates. Axon stretch-growth was escalated to and sustained at a growth rate of 8 mm/day for 48 h producing 2.2 cm of axon tracts (shown) in 7 days. Sample was stained with 1% osmium tetroxide and urinal acetate followed by embedding in an epoxy resin. Tissue was digitally scanned and contrast adjusted for clarity. The aberration through the center of the image was an artifact of the fixation process.

were maintained for five days in vitro to allow developing axons to grow across the interface between the two adjoining elongator substrates and into the population of neurons on either side. Very few axons would grow across the substrate interface using PLL or laminin. After the initiation of stretch growth, these cultures consisted of individual and small caliber fascicles that could only be observed microscopically (not shown). In contrast, coating with a collagen gel allowed a high density of axons to cross the two adjoining substrates. In these cultures, stretch growing axons fasciculated into larger caliber tracts as they grew in length. Unlike PLL and laminin cultures, axon tracts produced from collagen gel preparations could be easily seen with the naked eye, as seen in Fig. 3.

The collagen gel coating provided two advantages over PLL and laminin. First, after the collagen gel dried, the culture surface became hydrophobic and allowed neurons to be densely concentrated and plated within 2 mm on either side of the interface between the elongation substrates. Second, the collagen coating rehydrated with the addition of culture medium which provided an unobstructed coating over the interface between the two elongation substrates. These improvements resulted in a dramatic increase in the number of axons projecting to the neuron populations on each culture substrate. Thus, the total number of isolated axon tracts undergoing stretch growth was substantially enhanced.

3.2. Structural characteristics of stretch-grown tracts

Confirmation that the processes in the stretch-grown tracts were purely axonal was demonstrated through staining with MAP2 and tau antibodies. Typical MAP2 immunoreactivity was found in the cell bodies and dendrites while no MAP2 staining was observed along the elongated tracts. In contrast, tau labeling was present throughout the cell including the elongated tracts (Fig. 4A and B).

Extreme stretch growth of axons at high rates and over sustained time periods inevitably puts a substantial demand on the soma and the axon synthesis process. To examine the cytoskeletal assembly of axons stretch-grown at 1 cm/day, we labeled axon tracts with antibodies against phosphorylated neurofilament (NF-H), tau, and the polymerized state of microtubules (Fig. 4C–E). Labeled axon tracts were evaluated along their entire length and abundant labeling was found for all three cytoskeletal proteins in all axons elongated at 1 cm/day and up to 5 cm in length. Immunoreactivity was consistent along the entire axon tract, even in portions very distant from the soma.

SEM of the longitudinal aspect of stretch-grown axons was also examined (Fig. 5A). Elongating axons begin as a web of small interconnecting fascicles that coalesce into larger axon tracts as they grow in length. Close examination of individual tracts showed uniform axon caliber and no signs of thinning due to stretch. In addition, TEM cross-sections of elongated axons were examined, which demonstrated a normal cytoskeletal structure including neurofilaments, microtubules and mitochondria (Fig. 5B). Furthermore, phase microscopy, immunocytochemistry, SEM and TEM studies revealed no alterations or pathology induced in the neuronal somas at each end of stretch grown axons.

3.3. The nervous tissue construct

Isolated axon tracts were stretch-grown from DRG explant cultures to create a 1 cm nervous tissue construct in 6 days and a 5 cm nervous tissue construct in 14 days (Table 1). Due to the remarkable potential of rapid axon stretch growth, reaching 1 cm/day within a few days from the start of mechanical elongation, it is conceivable that much longer nerve constructs could be produced rapidly. Following stretch growth to 1 cm, collagen hydrogels were used to support and protect the axon tracts from damage while Aclar substrates were cut away from the stretching frame, and to maintain their orientation during assembly of the construct. Collagen hydrogels required a minimum of 80% (v/v) ($n = 3$) collagen solution (approximately 2.8 mg/mL) to produce

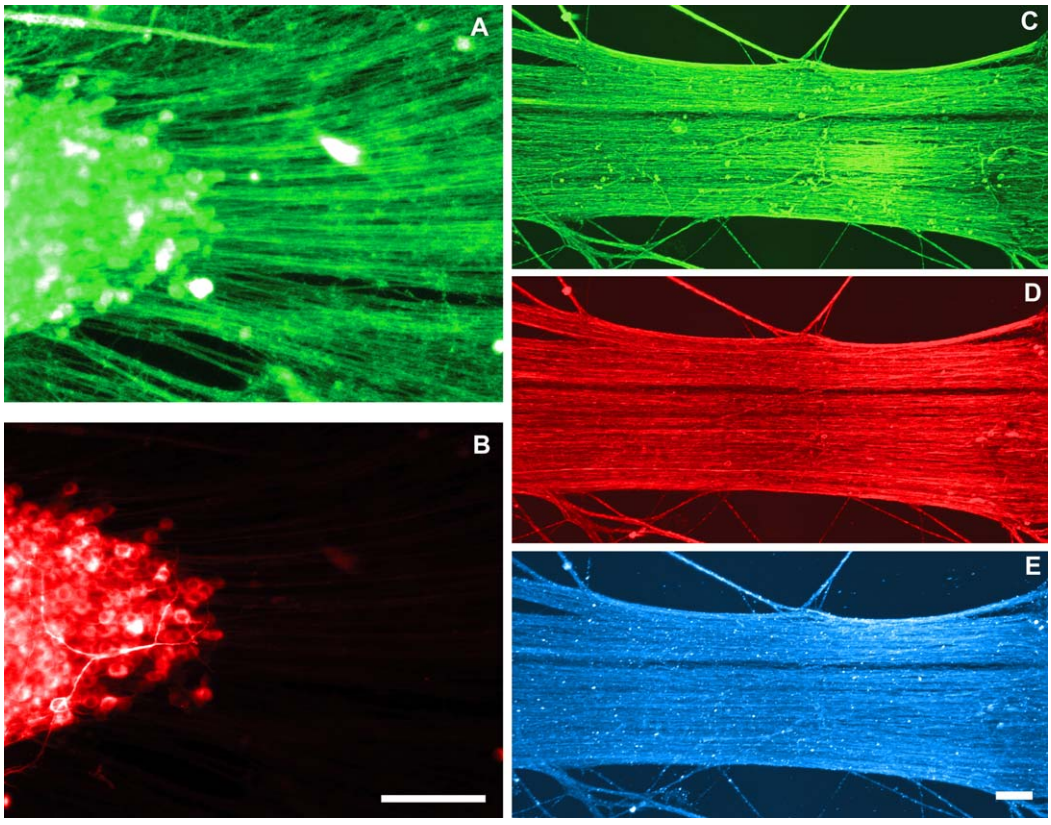

Fig. 4. Immunocytochemical analysis of axon stretch-growth. (A) and (B) Antibodies against tau and MAP2 were utilized to determine that elongating processes were axons. (A) The entire length of stretch-growing axons labeled positive for tau protein. (B) MAP2 was labeled within the cell bodies and was void along elongating processes indicating that these processes are axons. (C)–(E) Confocal microscopic images of axons elongated to 5 cm in length. Antibodies against (C) β-tubulin (SMI-61), (D) 200 kDa phosphorylated neurofilament (SMI-31), and (E) tau strongly labeled axons along their entire 5 cm of length. Scale bars: (B) 50 μm; (E) 10 μm.

a gel of sufficient strength to remove axons without damage. In contrast, 40% ($n = 2$) and 60% ($n = 2$) gels would tear easily and were too compliant to maintain the uniaxial alignment of the stretch-grown axons during the removal process. The 80% collagen hydrogel was also of sufficient strength to remove axons 5 cm in length ($n = 3$).

To create a nervous tissue construct for use in peripheral nerve repair, the axon-hydrogel complex was lifted from the Aclar substrate and transferred to the lumen of a PGA tube using microsurgical instruments. Despite the protection provided by the 80% collagen hydrogel, we found that the excessive handling involved in this transfer process could potentially lead to accidental axon damage or alteration in the alignment of axon tracts. To eliminate the need to physically transfer the axon-hydrogel complex, an alternate procedure was developed to stretch-grow axon tracts on top of a biocompatible membrane that could be directly formed into a cylinder ($n = 2$), Fig. 6. A nylon membrane was fixed to the bottom of the elongation chamber and the movable substrate was positioned in the same manner by overlapping it onto the nylon membrane. A collagen gel was applied to fill in the pores of the nylon membrane and over the movable substrate so unobstructed axon growth would occur across their interface. Axons readily grew across the interface and could be elongated using our established strategies. The membrane, hydrogel, and elongated axon combination was easily cut away from the axon stretching frame and folded into a tubular structure for transplantation.

4. Discussion

Harnessing the process of extreme stretch growth of integrated axons, we developed transplantable nervous tissue construct that are suitable to span extensive lengths of peripheral nerve damage or spinal cord lesions. In this study we enhanced the axon stretch growth paradigm to induce growth of the central portions of integrated axons in culture at the unprecedented peak rate of 1 cm/day after only 8 days of acclimation to mechanical elongation. In addition, we found that coating elongator substrates with a collagen gel allowed for the greatest number of elongating axons in each culture, thereby maximizing the total amount of tissue available to form the core element of a living nerve construct. Immunocytochemical analysis confirmed that elongating fibers were axons and that all major cytoskeletal constituents were present along axon lengths. Nervous tissue constructs were constructed by supporting stretch-grown axons in a collagen hydrogel and then transferring this complex into a PGA conduit. In an alternative procedure, axons were grown on a surgical membrane that was formed into a cylindrical construct. We have recently demonstrated, in a successive study, that these nervous tissue constructs can survive for at least

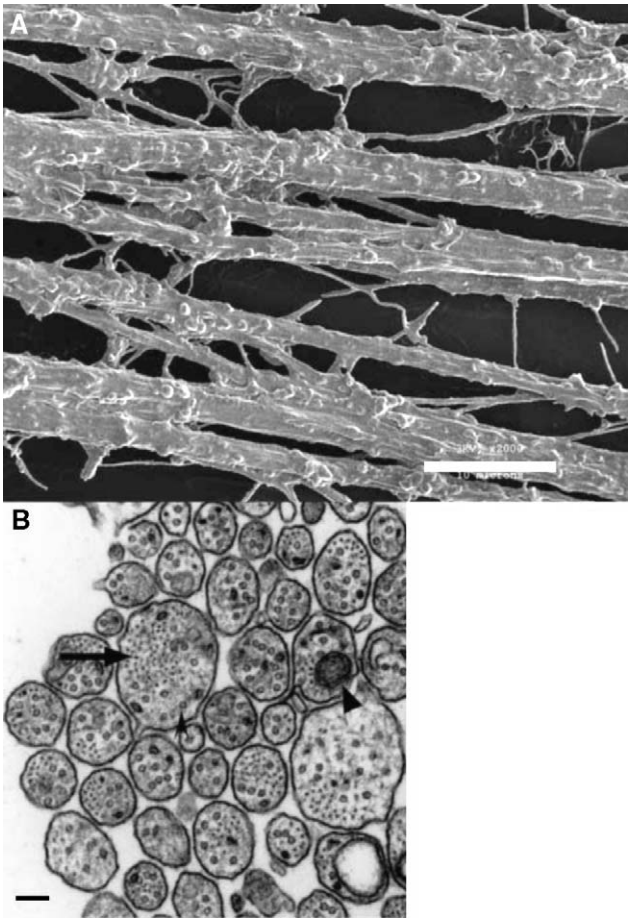

Fig. 5. Electron microscopic images of stretch-grown axons. (A) Scanning electron micrograph showing a 50 μm long section of stretch-grown axon tracts. Stretch-growing axons begin as a web of intertwined fascicles (shown) that coalesce as they are elongated in length. Axons appear uniform in caliber and there was no evidence of thinning due to stretch. (B) Transmission electron micrograph showing a cross-sectional image of stretch-grown axons. Arrow points to neurofilaments, arrowhead points to a mitochondrion, and concaved arrowhead points to a microtubule. Scale bars: (A) 10 μm; (B) 100 nm.

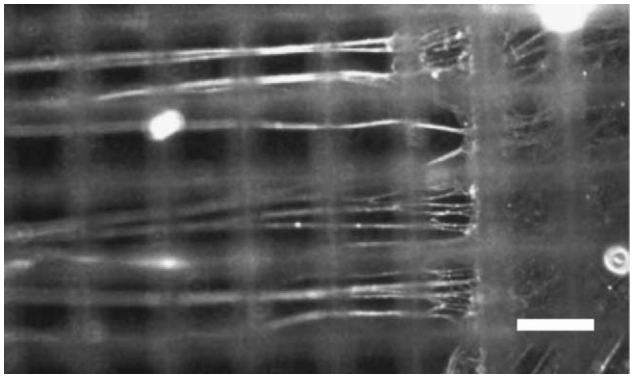

Fig. 6. Axon tracts elongating on top of a surgical membrane. Axons stretch-grown on transplantable membranes can be supported in a hydrogel prior to removal from the elongation chamber and folded into a cylindrical conduit without the need to manipulate the axon-hydrogel combination into a tubular conduit. Subsequently, the construct can be transferred directly to the transplant site and ensure that the uniaxial alignment of the axons remains intact. Scale bar, 200 μm.

1 month in the injured spinal cord of the rat (Iwata et al., 2006).

It is remarkable that axon stretch growth can proceed at a rate of 1 cm/day and sustain a normal cytoskeletal structure despite being maintained with tissue culture medium formulated for static neuronal cultures without modification. While axons clearly have the physiological capacity to continuously expand at a rate of at least 1 cm/day, extreme axon stretch growth defies the conventional understanding of the limitations of axon growth. In particular, stretch growth is not consistent with the approximately 1 mm/day maximal growth rate measured for growth cone extension or the average transport speed of 1 mm/day for structural elements such as neurofilaments. Accordingly, mechanical stretch may induce unknown cellular processes that initiates and maintains stretch growth of the axon cylinder, which is not limited by protein synthesis, transport rates or the availability of structural elements.

Nerve grafts and synthetic conduits are often successful for bridging peripheral nerve damage of less than 1–2 cm (Evans, 2000; Lee and Wolfe, 2000; Hall, 2001; Meek and Coert, 2002; Belkas et al., 2004). However, only nerve grafts are currently used to repair extensive lengths of damage, which is severely limited by donor nerve availability. Here we demonstrate that axons can undergo stretch-growth of 1 cm/day without damage which can provide 5 cm of axons in only 8 days. Considering that DRG neurons survive for months in culture (Kleitman et al., 1998), it is conceivable that DRG axon tracts stretch-grown at rate of 1 cm/day could reach the length of an entire human limb. Thus, DRG nerve constructs may also be useful for extensive nerve repair in the chronic state, even after the distal transected nerve has degenerated. In general, DRG neurons are clinically expedient since they have been shown to survive following transplantation in both the CNS and PNS (Bauchet et al., 2001; Davies et al., 1999; Kuhlengel et al., 1990; Rosario et al., 1993). Furthermore, a unique advantage of DRG neurons for clinical application is their availability. DRGs can be harvested from organ donors for allografts or from the patient themselves to create autografts (Maddox et al., 2004; Sosa et al., 1998).

The nervous tissue construct developed in the present study combines stretch-grown axons within a collagen hydrogel and entubulated within an implantable sheath. The hydrogel serves to: (1) support and protect axons from damage during their removal from the elongation device, (2) preserve their orientation within the construct (uniaxially aligned axons with DRG neurons at each end), and (3) serve as an extracellular matrix to support host regeneration within the nerve lesion. This requires that the matrix be formed around axons in a liquid phase and polymerized in a non-toxic fashion. We found that a biocompatible hydrogel made from 80% (v/v) collagen solution provided sufficient support to preserve axon orientation and integrity during removal of the stretch-grown cultures from the elongation device and insertion into a tubular PGA conduit. As an alternative means to protect the stretch-grown cultures during manipulation, we developed a method to grow axons on a biocompatible membrane that can be folded into a cylindrical nerve construct.

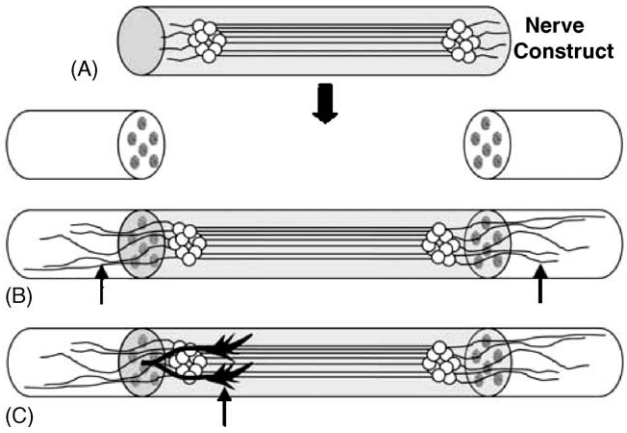

Fig. 7. Illustration of the nervous tissue construct design and two potential repair processes that may occur following transplantation. (A) The nervous tissue construct is formed from three components. (1) The stretch-grown axon tracts and associated neural cell populations at each end form the core and functional component of the construct. (2) A hydrogel is formed around the elongated tissue for support during surgical manipulation and serves as a substrate for further regeneration. (3) For PNS repair, the construct is wrapped in a biocompatible sheath that can be sutured to hold the graft within the lesion. (B) In one possible repair process, the nervous tissue construct itself may restore function. Following transplantation, the neurons associated with the stretch-grown axons may serve to integrate into host tissue restoring conduction of signals across the lesion. (C) In a second possible repair process, regenerating axons from the host tissue could be chemically and physically enticed to grow through the lesion by following the transplanted axon pathway.

For PNI repair, most strategies have been based entirely on promoting axon outgrowth along the previous nerve route by grafting donor nerve or spanning the damage with synthetic conduits. However, neither technique can overcome the rapid degeneration of the disconnected portion of the nerve and loss of a labeled pathway for axon guidance (Belkas et al., 2004; Evans, 2000; Hall, 2001; Lee and Wolfe, 2000). In addition, current synthetic conduits do not supply chemical cues for axon growth, thereby limiting their effective length for clinical use. We propose that nervous tissue constructs composed of living axon tracts may overcome these limitations. Rather than promoting axon outgrowth in vivo, axon stretch growth is performed in vitro, rapidly creating living axon tracts while allowing complete control of the number and length of the elongated axons created for each nerve construct.

Transplantation of nerve constructs containing elongated axons for PNI repair may provide two potential therapeutic benefits. The neurons associated at each end of stretch-grown axons may integrate into host tissue and establish a relay across the lesion, Fig. 7A and B. In addition, transplanted axons might enhance the regeneration of damaged axons across the lesion by providing a physical and chemically labeled pathway, Fig. 7C (Bak and Fraser, 2003; Hall, 2001; Raper et al., 1984). In the case of nerve grafts, conventional thought considers that the Schwann cells contribute to the pathfinding of nerve regeneration (Evans, 2000; Hall, 2001) and has shown some improvements when used in nerve conduits (Evans, 2001; Evans et al., 2002; Schmidt and Leach, 2003; Son and Thompson, 1995). However, axons themselves may also provide a labeled pathway, which has not yet been investigated. Accordingly, nerve constructs containing viable axons could be used to bypass the degenerating distal nerves, providing a living and persistent pathway for nerve regeneration from the lesion site to the end of the limb. Furthermore, with axon stretch growth at 1 cm/day, creating nervous tissue construct the length of an entire human limb could be possible.

Although there are currently no clinical methods to bridge SCI lesions, many preclinical approaches in animal models are based on promotion of axon growth at the margins of the lesion. Several techniques have shown promise, including implanting peripheral nerve or biomaterials to act as physical guides, support cell transplantation to promote axon growth, and administration of agents that either counteract the natural inhibitors of axon growth or enhance growth, for review see (Bunge, 2001; Fawcett, 2002; Hulsebosch, 2002; McKerracher, 2001; Stichel and Muller, 1998). In contrast to these approaches, axon tracts can be stretch-grown in vitro to match the length of the lesion, supported in a hydrogel and transplanted within the damaged cord. There are two potential means by which this axon-hydrogel construct may create new intraspinal circuits. Bridging SCI lesions with living PNS axons would provide a persistent chemical and physical pathway, which may promote outgrowth of host axons from one end of the lesion to integrate with viable tissue on the other side (Bak and Fraser, 2003; Hulsebosch, 2002; Raper et al., 1984; Stichel and Muller, 1998). In addition, the neurons associated with the transplanted axons (at each end of the nerve construct) may integrate with viable spinal cord tissue proximal and distal to the lesion to form new intraspinal circuits (Bareyre et al., 2004; Bauchet et al., 2001; Blight, 2004; Cheng et al., 1996; David and Aguayo, 1981; Davies et al., 1999; Fawcett, 2002; Topka et al., 1991; Weidner et al., 2001).

Bridging nerve lesions with nervous tissue construct consisting of stretch-grown living axons that recapitulate the uniaxial orientations of the original axonal tracts offers a new direction in nerve repair, especially for extensive nerve lesions. Importantly, this approach may be complimentary to alternative strategies, such as therapies that enhance axon growth. The ultimate benefit of nerve tissue construct used either alone or as part of a combinational therapy may be to facilitate the natural regenerative capacity of the nervous system.

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