Synergistic improvements in cell and axonal migration across sciatic nerve lesion gaps using bioresorbable filaments and heregulin-β1

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Received 16 May 2003; revised 20 October 2003; accepted 30 October 2003
Published online 1 March 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.20119

Abstract: The success of entubulation for peripheral nerve regeneration is still limited, especially with long lesion gaps. In this study, we examined if regeneration could be enhanced by constructing implants to both align axonal growth and promote Schwann cell proliferation and migration. Silicone implants were used to bridge a 1.4-cm gap in the rat sciatic nerve. Adult female Sprague–Dawley rats were divided into four groups of tubes containing either 1) Matrigel; 2) Matrigel and heregulin; 3) Matrigel and poly(L-lactic acid) (PLLA) microfilaments; or 4) Matrigel, PLLA microfilaments, and heregulin. Ten weeks postimplantation, the number of axons and Schwann cells were measured at the distal end of implants. Implants with microfilaments displayed better tissue cable formation, increased Schwann cell migration, and regeneration of anti-calcitonin gene-related peptide-positive axons, but not RMDO95-positive axons compared with nonfilament-containing groups. Heregulin treatment caused an increase in Schwann cell number, but it demonstrated no significant improvement in either tissue cable formation or axon number. Extensive regeneration was observed through implants containing Matrigel, microfilaments, and heregulin, which induced significant improvements in the number and longitudinal organization of both Schwann cells and axons. These results indicate that physical guidance of microfilaments and the Schwann cell growth factor, heregulin, act synergistically to improve nerve regeneration across long lesion gaps. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 69A: 247–258, 2004

Key words: peripheral nerve system; guidance channel; PLLA; Matrigel; neuroma

INTRODUCTION

Axons in the adult peripheral nervous system (PNS) have the capacity to undergo spontaneous regeneration after injury. However, if the lesion gap is large (e.g., >10 mm in rat sciatic nerve), axons fail to regenerate across the gap.[1,2] Current techniques and devices available to assist regeneration across large lesion gaps have limited success. Historically, autografts have shown the greatest efficacy for inducing axonal regeneration because they provide appropriate alignment and cellular constituents of the normal nerve. However, even these implants have induced only limited functional recovery. Enhanced regeneration through artificial devices could prove more effective by designing them to mimic the degenerated peripheral nerve to provide growth and guidance factors to enhance functional regeneration. Artificial devices also do not contain the cellular element of the adjacent nerve stumps; thus, it seems necessary to provide an effective intrinsic microenvironment that enhances the migration of endogenous cellular elements into the implant.

Schwann cells are a peripheral nerve glia shown to enhance the regeneration of either peripheral or central nervous system axon.[3–8] Reactive (nonmyelinating) Schwann cells are an excellent growth-promoting substrate, providing regenerating axon with numerous neurotrophic factors, cell adhesion molecules, and other factors involved in axon growth and guidance. Schwann cells are thought to be of great importance in organizing the structure of the peripheral nerve.

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Contract grant sponsor: National Institution of Neurological Disease & Stroke (NINDS); contract grant number: RO1NS40592

Schwann cells produce a basement membrane, containing many extracellular matrix proteins that support robust axonal growth and form the endoneural tubes through which regenerating axons grow. Factors secreted by Schwann cells, such as Desert Hedgehog, signal the surrounding connective tissue cells to organize the perineurium. Schwann cells are also involved in the maturation of the nerve by myelinating axons and increasing their diameter and conductance velocities.[9–11] Perhaps one of the most important considerations required for successful peripheral nerve repair is to enhance Schwann cell migration and proliferation into artificial implants.

The growth factor neuregulin 1 (rat homologue of heregulin) is essential in the proliferation and differentiation of Schwann cells during development, and neuregulin 1 knockout mice show a significant reduction in the number of Schwann cells.[12] During development, neuregulin 1 is produced by motor and dorsal root ganglion (DRG) sensory neurons[13] and is shown to be the bioactive component of the Schwann cell mitogen released by axons.[14] With maturation, the levels of neuregulin expressed by neurons is greatly reduced.[15] After nerve lesion, neuregulin expression quickly increases, but unlike development, regenerating axons within the proximal nerve express very little neuregulin and most likely do not contribute to Schwann cell proliferation.[16] The highest levels of neuregulin are produced by Schwann cells within the distal stump to induce proliferation most probably by an autocrine mechanism.[16] Application of focal or low concentrations of neuregulin promotes Schwann cells migration and the release of trophic factors that support neuronal survival and regeneration.[17] These and other studies indicate a potential role for neuregulins in enhancing nerve regeneration by stimulating Schwann cell proliferation and migration required to reconnect the severed nerve stumps.

To induce axonal regeneration across a peripheral nerve gap, the formation of a tissue bridge or cable is paramount.[18,19] Entubulation procedures are thought to function by concentrating essential matrix materials and growth factors to enhance spontaneous formation of this bridge. Schwann cells are one of the major cells responsible for bridging the lesion and providing the cellular substrate over which axons regenerate. Therefore, a major rate-limiting step in the induction of nerve repair across long lesion gaps is the proliferation and migration of Schwann cells between the nerve stumps.[20,21] Previous experiments in our laboratory and others show that Schwann cell migration, cable formation, and axonal regeneration can be enhanced by adding microfilaments to silicone tubes.[20,22] These microfilaments provide organized guidance channels that direct cell migration and growth longitudinally across nerve stumps.[22] *In vitro*, these microfilaments direct the longitudinal migration of both Schwann cells and dorsal root ganglion neurites.[23] In the present study, we examined if adding human heregulin-β1 and microfilaments to silicone tubes can enhance axonal regeneration and nerve repair across a 1.4-cm gap.

MATERIALS AND METHODS

PLLA filament fabrication

PLLA microfilaments were fabricated by a wet spinning process.[22] Briefly, 10% PLLA polymer solution (MW, 200-kD, pellets from Polysciences, Inc.) in chloroform suspended with 4% BSA was loaded into a glass syringe and placed in a variable speed syringe pump. An 18-gauge dispensing tip that was immersed in a coagulation bath that contained isopropyl alcohol was attached to the syringe by using TYGON® tubing and a Luer adapter. The polymer solution was pumped into the coagulation bath. The polymer fiber, which formed immediately as it left the dispensing tip, was attached to a traversing roller mounted on a lathe. The roller then pulled the fiber through the bath to a final diameter of 50–100 μm. The finished fiber was air-dried and stored in desiccators until use.

Final preparation and sterilization of implants

Silicone tubes (2.5-mm OD; 1.5-mm ID) were purchased from A-M System and autoclaved before use. Before bundled into outer guidance channels, PLLA microfilaments were incubated in 70% alcohol for 1 h and air-dried. Thirty-two PLLA microfilaments were bundled through silicone tube. Implants were washed three times in 0.1*M* phosphate buffer (PB); sterilized by 10× penicillin/streptomycin (Gibco, Invitrogen Corp.) in PB for 2 h at room temperature with constant shaking; and three times washed in 0.1*M* PB again. Finally, the implants were filled with Growth Factor Reduced Matrigel™ Matrix (BD Biosciences) at 1:1.5 ratios in N_2 medium (Gibco, Invitrogen Corp.). For the implants with heregulin, 800-ng human heregulin-β1 (R&D systems) was added for each implant. All the procedures were performed under the tissue culture hood.

Surgical procedure

Adult female Sprague–Dawley rats were anesthetized by intraperitoneal injection of ketamine (66 mg/mL)/xylazine (6.66 mg/mL) at 0.2 mL/100 g. The sciatic nerve was exposed through a posterior thigh muscle-splitting incision and 7 mm of nerve was resected to get a 14-mm nerve lesion gap. Implants were used to bridge the nerve lesions by inserting 1-mm proximal and 1-mm distal nerve stumps into the guidance channels. The nerve stumps were secured inside the channel with Superglue (Quicktite Loctite). Ethicon

TABLE I
Experimental Design and the Number of Successful Tissue Cable Formation

Group Name	Outer Guidance Channel	Inner Guidance Channel	Inner Medium	No. of Surgery Animals (n1)	No. of Animals With Tissue Cable Formation (n2)	n2/n1 *100%
FMH	Silicone	32 PLLA microfilament	Matrigel + Heregulin	8	8	100
FM	Silicone	32 PLLA microfilament	Matrigel	8	8	100
MH	Silicone	no PLLA microfilament	Matrigel + Heregulin	3	3	100
M	Silicone	no PLLA microfilament	Matrigel	8	6	75

and stainless steel wound clips (Autoclips) were used to staple the muscle and skin together. Animals were kept for 10 weeks with full access to food and water according to the University of Kentucky Medical Center animal care policy. National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publication 85-23 Rev. 1985) have been observed.

Experimental groups

Rats were divided into four groups (Table I). Group FMH ($n = 8$) was implanted with silicone tubes containing 32 filaments, Matrigel, and heregulin. Group FM ($n = 8$) was implanted with silicone tubes containing 32 filaments and Matrigel. Group MH ($n = 3$) was implanted with silicone tubes containing Matrigel and heregulin. Group M ($n = 8$) was implanted with silicone tubes with Matrigel only.

Implant retrieval and histological preparation

At 10 weeks postinjury, animals were euthanized and perfused intracardially with 4% paraformaldehyde (PFA) in 0.1*M* PB. The implants were harvested, silicone tubes were removed, and the regenerated nerves were postfixed in 4% PFA for 2 days at 4°C. To prevent tearing of tissue during sectioning, PLLA microfilaments were dissolved by placing in the series of solutions as follows: 50%, 70%, 90%, 100% ethanol, 2 changes of methylene chloride, and ethanol rehydration (above solution reverse order). Once rehydrated, the tissue was cryoprotected by using 30% sucrose in 0.1*M* PB at 4°C for another 2 days. Tissues were then embedded in OCT compound, frozen, and stored at −80°C.

Immunohistochemistry

Transverse sections were taken at the distal end of the regenerated tissue, and the remaining tissue was processed for 30-μm thick longitudinal sections. Sections were fixed in 4% PFA for 5 min and rinsed with 0.1*M* PBS. Endogenous peroxidase activity was neutralized by incubating sections in 0.3% hydrogen peroxide diluted in methanol for 10 min. Sections were rehydrated, and nonspecific antibody binding was blocked in 5% normal goat serum in PBS for 1 h, followed by an overnight incubation in primary antibody at room temperature. Primary antibody binding was visualized by using a biotin-labeled secondary antibody. Biotin-labeled tissues were further processed with the Vectastain Elite ABC reagents (Vector Laboratories, Burlingame, CA) and developed by 3,3-diaminobenzidine (DAB).

Antibodies

Rabbit antiserum anti-calcitonin gene-related peptide (CGRP; Sigma, diluted in 1:20,000) and mouse antiserum RMDO95 (a kind gift from Dr. Virgina Lee, diluted in 1:1000) were used to stain primary nociceptive afferents and phosphorylated neurofilaments (200 kD) in axons, respectively. Rabbit antiserum S100 (DAKO, diluted in 1:1200) was used to stain Schwann cells. Rabbit polyclonal P0 (diluted in 1:3000), a kind gift from Dr. Marie Filbin (Hunter College), was used to identify myelinated axons.

Image analysis

All stained tissue sections were viewed with a Nikon E800 light microscope at 100−400× magnification. Images were captured with Metamorph Imaging System 5.0 (Universal Imaging Corp.). To quantify Schwann cell staining or the number of axons, a standard optical density threshold was applied to each image, and the staining density equal to or greater than the threshold was measured. The whole transverse area of regenerated tissue cable, staining area of Schwann cells, and CGRP- and RMDO95-positive axons at the distal end of regenerated tissues were automatically counted by Metamorph. The number of myelinated axons from P0-positive staining was manually counted at 400× with microscope.

Statistical analysis

For each of the measured variables, one-way analysis of variance (ANOVA) was used to evaluate the overall effect of different treatments. Protected Least Significant Difference (LSD) method[24] was used for *post hoc* comparisons between the control group (M) and any of the other three treatment groups (MH, FH, and FMH). A p value of 0.05 was considered as statistically significant. All analyses were done with the statistical software SAS® version8.2.

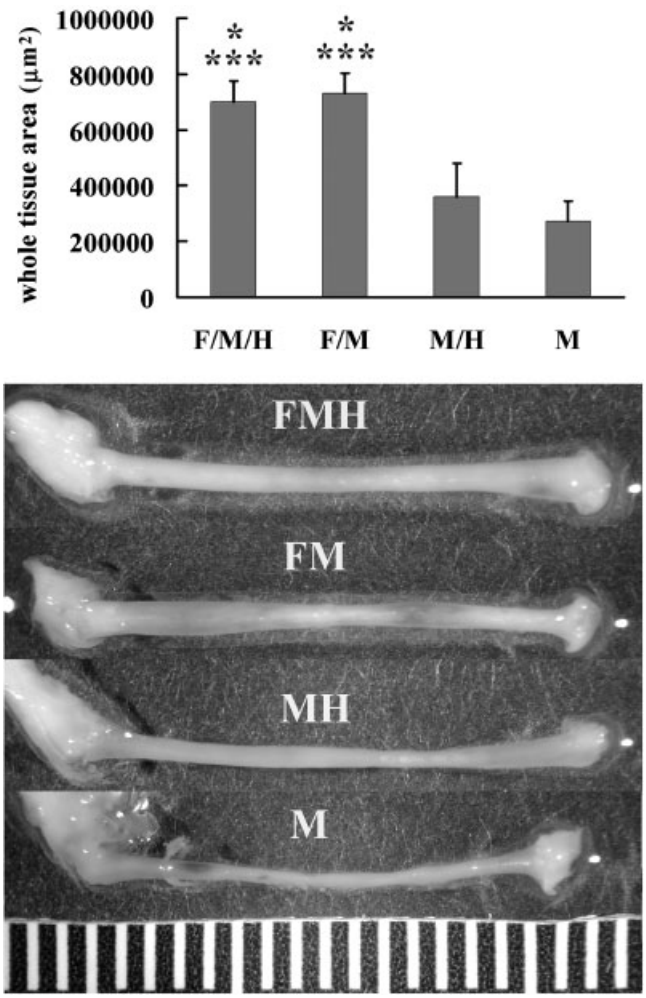

Figure 1. Photographs showing tissue cables regenerated across 14-mm lesion gap from implants filled with Matrigel (M); Matrigel and heregulin (MH); Matrigel and 32 PLLA microfilaments (FM); and Matrigel, heregulin, and 32 PLLA microfilaments (FMH). Implants containing filaments resulted in larger diameter repaired nerve tissues. The whole cross-sectional areas of the regenerated tissue cables from filaments containing groups were twice as large as those from nonfilaments containing groups (*$p < 0.05$ vs MH; ***$p < 0.001$ vs M). Data are shown as means ± SEM. Division scale of the ruler is 1 mm.

RESULTS

Ten weeks postinjury, all but two animals showed tissue cable formations across a 14-mm lesion gap (Table I). Similar to our previous studies, 100% of the tubes containing microfilaments had tissue cable extending between the nerve stumps. The Matrigel group without heregulin was the only group that had <100% cable formation and was consistent with empty silicone implants.[22] Implants containing filaments consistently resulted in larger diameter nerve cables (Fig. 1, FMH and FM). The average cross-sectional tissue area at the distal end of the regenerated nerve cable from implants containing filaments were twice as large as those from implants without filaments (***$p < 0.001$ vs M; *$p < 0.05$ vs MH) (Fig. 1). The cross-sectional area of implants containing both heregulin and Matrigel (group MH) was slightly higher than that containing Matrigel alone (group M). However, the difference between groups MH and M did not reach statistical significance. The whole cross-sectional area in either group FMH or FM included 32 filaments, which occupied about 50,000 μm^2 as measured by Metamorph Imaging software. The inclusion of the filament area made no difference in the statistical comparison of size for regenerated tissue cables between the four groups (data not listed). These results are consistent with previous findings that PLLA filaments might increase cell migration to more efficiently produce nerve cable formation between the nerve stumps.

To investigate the possibility that either filaments or heregulin increased Schwann cell numbers within the nerve cable, the cross-sectional area of the nerve cable containing Schwann cells was compared between the groups. For these experiments, Schwann cells were identified by using S100 protein immunohistological staining (Fig. 2). The average area of the nerve occupied by Schwann cells increased in both heregulin (5272 ± 1991 μm^2) and microfilaments (4716 ± 1219 μm^2) groups compared to the Matrigel group (1142 ± 1219 μm^2). Although the two treatments showed a similar increase in Schwann cell staining, only the filament containing implants were statistically significant compared to the Matrigel alone group. Combining both microfilaments and heregulin within the same tube showed the greatest increase in Schwann cell staining (9707 ± 1219 μm^2), averaging almost

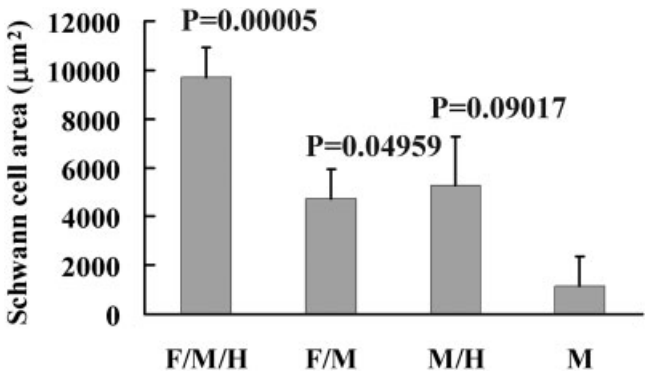

Figure 2. Bar graph shows the area of S100-positive staining for Schwann cells at the distal end of the implants filled with Matrigel (M); Matrigel and heregulin (MH); Matrigel and 32 PLLA microfilaments (FM); and Matrigel, heregulin, and 32 PLLA microfilaments (FMH). Growth enhancement from filaments or heregulin on Schwann cell number was relatively equivalent. However, when combined, the two treatments acted synergistically and extended Schwann cell occupied area up to nine fold compared to M. All the p values are significance levels compared to M. Data are shown as means ± SEM.

twice that of the groups with either heregulin or filaments alone, and more than eightfold greater than the Matrigel group. This synergistic effect is most likely due to directional cues provided by the filaments in the presence of the Schwann cell mitogen. These results show the importance of incorporating both physical guidance structures as well as biochemical growth factors within the implants to enhance proliferation and migration of these glia.

We have previously shown that implants containing microfilaments increase axonal regeneration across long lesion gaps.[22] To investigate whether the addition of heregulin to these implants would further enhance axonal regeneration in the presence or absence of microfilaments, the number of CGRP- and RMDO95-positive axons in cross sections were counted. These antibodies were chosen because CGRP is a neuropeptide that is exclusively found in nociceptive axons that represent most unmyelinated or loosely myelinated axons, whereas RMDO95 identifies a phosphorylated 200-kD neurofilament protein subunit that primarily stains large caliber, myelinated axons.[25,26] In general, all nerve cross sections appeared healthy with many obvious blood vessels through the nerve cable (Fig. 3). Within all of the sections, axons appeared relatively evenly distributed throughout the nerve cable irrespective of the treatment (Fig. 3). One principal difference was that in the nonfilament groups, the nerve cable was small and enveloped by a thick dense epineurium, which was not apparent around the much larger nerve cables in the filament group.[22] In regenerated nerves, the epineurium contains myofibroblasts that can induce wound contracture, leading to chronic constrictions. Thus, the thicker the epineurium, the higher the probability of nerve contraction over time. In general, filaments appeared well integrated into the tissue, were surrounded by fibroblast-like cells, and did not interfere with axonal regeneration, because many axons were observed several cell diameters from the filaments. Within the filament groups, axons appeared evenly distributed between the filaments, as well as around the parameter of the central filament bundle. The number of either CGRP- or RMDO95-positive axons was determined throughout the entire cross section at the distal end of the implant (Fig. 4). In the Matrigel group, very limited axonal regeneration of either CGRP (127 $\pm$ 84) or RMDO95 (633 $\pm$ 325) stained axons was observed. In this group, two of eight animals showed no axonal regeneration, and another two nerve cables only contained CGRP-positive, but no RMDO95-positive axons. Adding heregulin to the Matrigel increased axon regeneration almost twofold for both CGRP (268 $\pm$ 137) and RMDO95 (1138 $\pm$ 530) stained axons; however, this difference was not statistically significant. Implants containing filaments and Matrigel increased regeneration of CGRP-positive axon more than threefold (422 $\pm$ 84), but they had little effect on the regeneration of RMDO95-positive axons (810 $\pm$ 375). Combining filaments, Matrigel, and heregulin had a synergistic effect on axonal regeneration, increasing the number of CGRP-positive axons 5.7-fold (729 $\pm$ 84) and RMDO95-positive axons 2.9-fold (1858 $\pm$ 325). These results corroborate the above results and indicate that the addition of heregulin and microfilaments to the implants improve axonal regeneration in addition to Schwann cell migration and proliferation.

Proper nerve repair is highly dependent on myelination of regenerated axons to ensure their proper physiological function. To examine if filament bundles or heregulin increased myelination within the reconstructed nerve, myelin profiles were identified by using an antibody that recognizes P0 and counted within nerve cross section from the distal most end of the implant (Fig. 5). At 10 weeks postinjury, some nerves treated with Matrigel (four of eight), Matrigel and heregulin (one of three), and Matrigel and filament bundles (three of eight) had no myelin profiles at the distal end of the implant. The average number of P0 stained myelinated axons was very similar between these three treatment groups. All eight nerves reconstructed using filament, Matrigel, and heregulin showed P0-positive myelin profiles at the distal end of the implant. Of samples that showed myelination, the average number of P0-positive myelinated axons was greater than threefold higher in the FMH group (1962 $\pm$ 354) than that in the other three groups [Fig. 5(e)]. At high magnification, the myelin sheath in the FMH, FM, and MH groups all appeared thicker than in group M (Fig. 5). These results were relatively surprising because we expected more myelination in all heregulin-containing groups due to its ability to induce proliferation and maturation of Schwann cells. Therefore, the microfilaments might augment the proliferation effects of heregulin by increasing the longitudinal migration to more quickly generate the nerve cable and support more robust axonal regeneration.

Although cross-sectional staining provides a good means of quantifying nerve repair, it provides little information of whether the microfilaments act to organize longitudinal growth between the nerve stumps. To examine how filament bundles might organize repair, longitudinal sections were prepared for each regenerated nerve. For these experiments, longitudinal organization of both Schwann cells and axons were examined at either the center regions or the distal end of the implant near the nerve interface (Figs. 6 and 7). Schwann cells stained with use of anti-S-100 were identified throughout all nerve cables. In general, the Schwann cells in the filament groups appeared more organized than in nonfilament-containing groups (Fig. 6). The longitudinal organization and density of Schwann cells appeared highest in the FMH-treated groups, which also showed a fairly con-

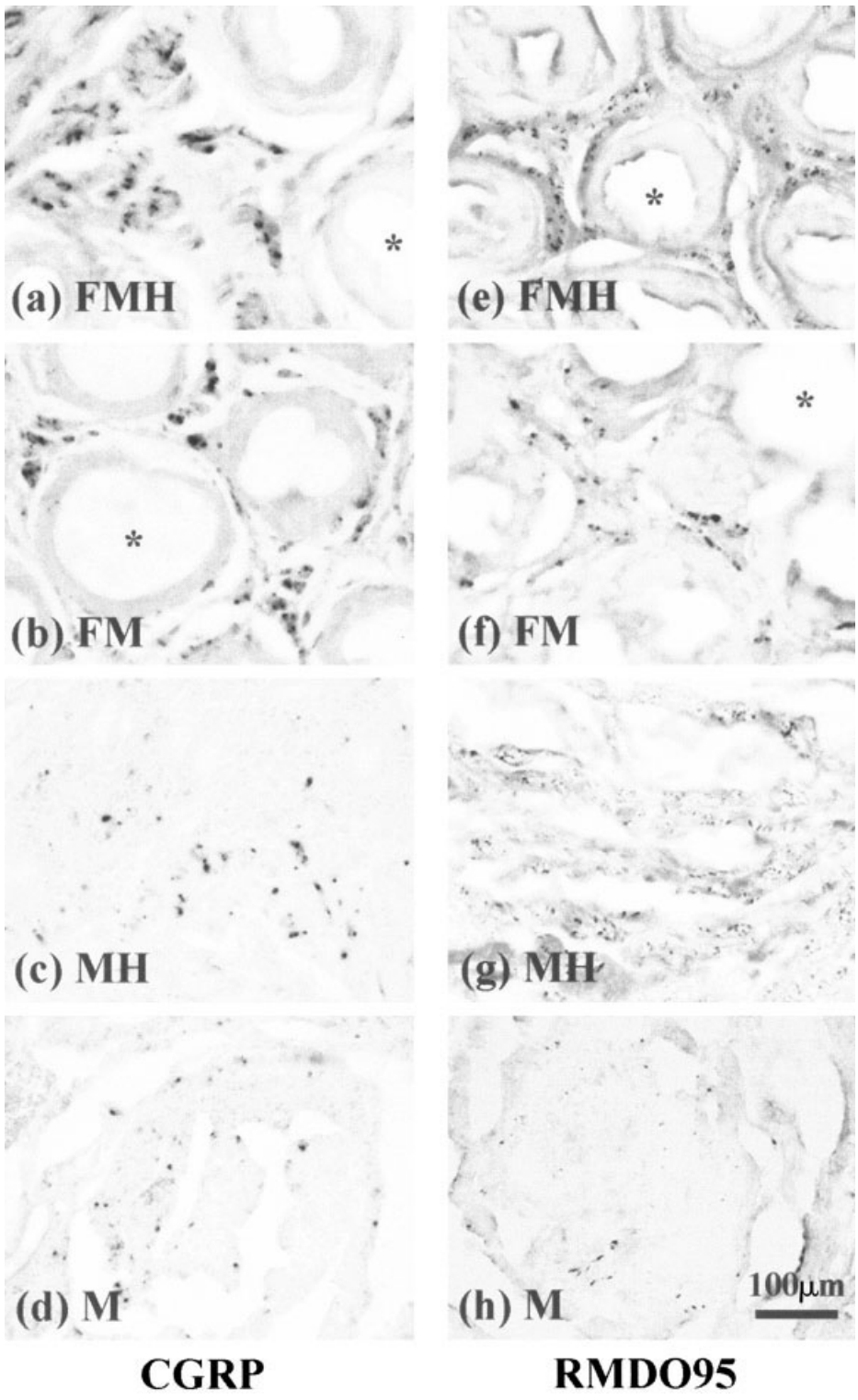

Figure 3. High-magnification photographs of axon staining at the distal end of the implants filled with Matrigel (M); Matrigel and heregulin (MH); Matrigel and 32 PLLA microfilaments (FM); and Matrigel, heregulin, and 32 PLLA microfilaments (FMH). CGRP-positive staining for nociceptive axons is shown in the left column (a–d), whereas RMDO95-positive staining against phosphorylated neurofilament containing axons is in the right column (e–h). *PLLA microfilaments. Axonal migration along filaments was constantly observed in filaments containing conduits. Scale bar shown is 100 μm.

tinuous density of Schwann cells throughout the nerve cable [Fig. 6(a,b)]. This was in direct contrast with nonfilament-containing groups, which often showed discontinuous Schwann cells cables within the repaired nerves, and there was almost no Schwann cells detectable in the center region [Fig. 6(e–h)]. Near the

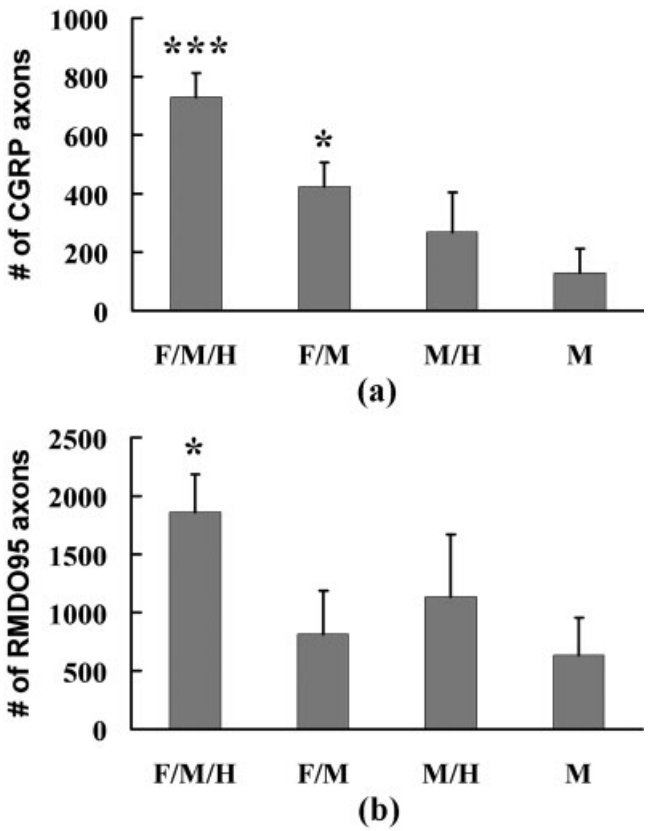

Figure 4. Bar graphs show the number of CGRP- (a) and RMDO95 (b)- positive axons at the distal end of implants filled with Matrigel (M); Matrigel and heregulin (MH); Matrigel and 32 PLLA microfilaments (FM); and Matrigel, heregulin, and 32 PLLA microfilaments (FMH). Very few axons regenerated toward the distal end with Matrigel treatment alone (M). Addition of microfilaments increased the number of CGRP-positive axons that regenerated (FM vs M). A similar increase was observed with the addition of heregulin to Matrigel; however, the increase did not reach significance (MH vs M). Statistical significance was not obtained for RMDO95-positive axonal regeneration observed with either filament or heregulin treatment alone. Combining filament, heregulin, and Matrigel increased regeneration of both axon types and showed synergist improvement (FMH vs M). *$p < 0.05$ versus M; ***$p < 0.001$ versus M. Data are shown as means ± SEM.

distal ends of the nerve cable, Schwann cells appeared to have a more general longitudinal organization in all treatment groups. However, comparison between filament and nonfilament-containing groups showed very limited and less organized cell migration in the latter group (Fig. 6, left column). To determine if microfilaments had an effect on organizing the longitudinal growth of axons, adjacent sections were stained with antibodies to CGRP (Fig. 7). Axons appeared to have a greater longitudinal organization within all implants containing filaments. High density of longitudinally organized axons was observed in the FMH implants [Fig. 7(a)]. At the distal implant/nerve interface, little disruption in the orientation of these axons was observed, and the axons appear to regenerate unimpeded into the distal nerve stump [Fig. 7(b)]. A similar organization pattern was observed for FM implants, although the general axon density in these implants was dramatically less than that observed for the FMH group [Fig. 7(c,d)]. Without filaments to align growth inside the silicone implants or near the implant/nerve interface, the organization of regenerated axons was poor, showing numerous tangles of axons, turning, and end-bulb-like structures reminiscent of abortive axon growth [Fig. 7(e–h)]. In some regions, the disorganization of axon growth was dramatic and sometimes reminiscent of neuromas with numerous abortive regeneration profiles [Fig. 7(i,j)]. These results further validate our hypothesis that the physical support and guidance effects of filaments, combined with increased Schwann cell proliferation and migration by heregulin, enhance nerve repair. Filaments also appear to increase the organizational properties near the implant/nerve interface to better support axonal growth across these junctional interfaces.

DISCUSSION

Previously, we showed that the physical structure of filaments bundled inside silicone tubes improved nerve repair across long lesion gaps (up to 18 mm in the rat sciatic nerve lesion model). These microfilaments were shown to be biocompatible with the nerve tissue and induced minimal immune response.[22] In tissue culture, these microfilaments provide a migratory substrate for Schwann cells out of dorsal root ganglion explants. In those studies, neurite outgrowth was diminished in the absence of Schwann cells, strongly suggesting that Schwann cells play a vital role in nerve repair.[23] In addition, those studies showed that the microfilaments oriented both the migration of Schwann cells and the growth of axons longitudinally along the filaments. In the present study, we set forth to examine the hypothesis that the Schwann cell mitogen, human-heregulin β1, in combination with microfilament bundles, would enhance Schwann cells migration into the implant and, consequentially, improve nerve reconstruction across a sciatic nerve lesion.

Neuregulin 1 (heregulin) plays a vital role in peripheral nerve development by acting as a potent Schwann cell mitogen, chemoattractant factor, and maturation factor. During development, neuregulin is produced by motor and sensory axons to influence Schwann cell migration and maturation associated with nerve myelination. In the adult, neuregulin expression is very low, and after peripheral nerve injury, its upregulation is primarily confined to Schwann cells at the

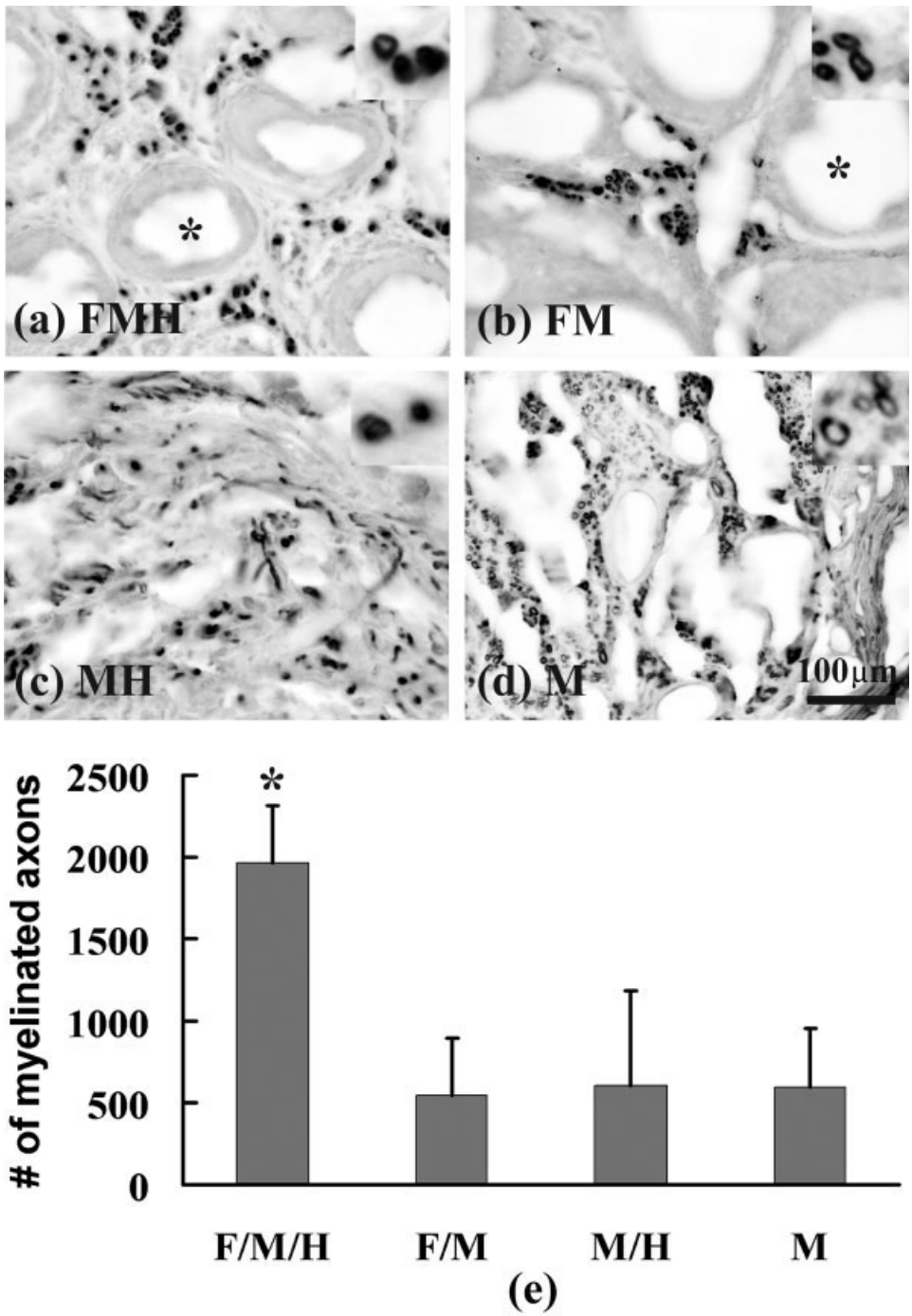

Figure 5. P0-positive myelin was compared at the distal end of the implants filled with Matrigel (M); Matrigel and heregulin (MH); Matrigel and 32 PLLA microfilaments (FM); and Matrigel, heregulin, and 32 PLLA microfilaments (FMH). Images represent P0 staining for each group. *Location of PLLA microfilaments. At higher magnification as shown at the corner of each image, the myelin sheath in the FMH, FM, and MH groups all appeared thicker than in group M. Graph in (e) shows the number of P0-positive axons in each group. Neither filament nor heregulin treatment alone showed improvements in the number of myelinated axons. However, the two treatments acted synergistically on myelination, and the number of myelinated axons almost reached 2000 at 10 weeks postinjury (*$p = 0.01206$ vs M). Data are shown as means ± SEM. Scale bar shown is 100 μm.

nerve stumps and in the entire distal nerve.[16] This expression pattern would support proliferation at the nerve stumps but preclude its role to aid migration across a lesion gap. To enhance the chemoattractive nature of our implants, we inserted Matrigel containing a sufficient amount of heregulin (800 ng/implant) to increase Schwann cell proliferation and migration into the matrix. The addition of heregulin to Matrigel

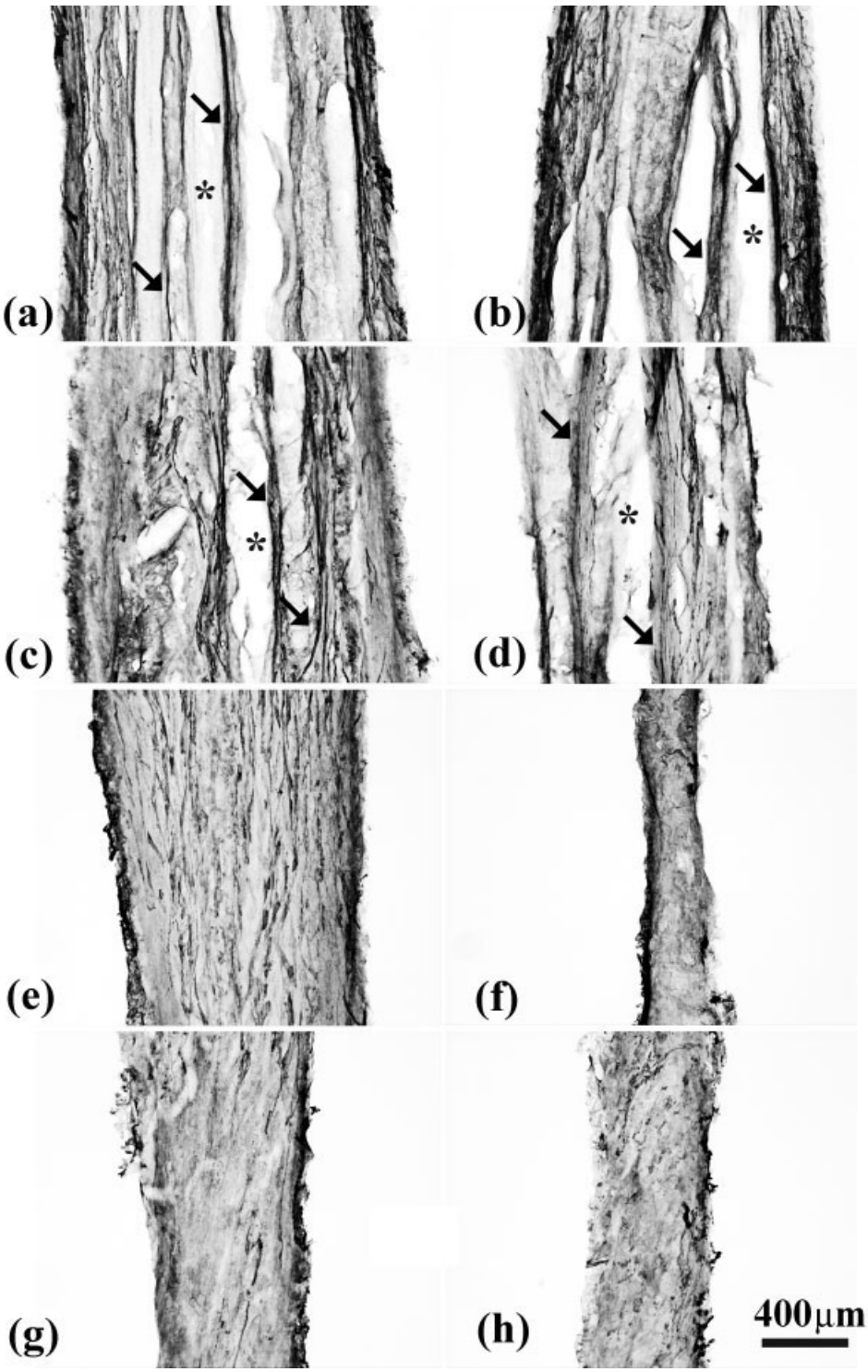

Figure 6. Longitudinal staining showing guidance affect of filaments to align Schwann cell migration. (a, b) Schwann cells staining of implants filled with Matrigel, heregulin, and 32 PLLA microfilaments (FMH). (c, d) Implants filled with Matrigel and 32 PLLA microfilaments (FM). Schwann cell cables (arrows) formed along filaments bundles (*) in these groups. The longitudinal organization and density of Schwann cells appeared highest in the FMH-treated groups. (e, f) Schwann cell staining of implants filled with Matrigel and heregulin (MH). (g, h) Implants filled with Matrigel (M). a, c, e, and g represent section through distal end, whereas b, d, f, and h are of section from the center of the implant. Without filaments bundles, Schwann cell cables formed discontinuously with few cells found in the center region. Even in the distal region of these groups, Schwann cell migration was very limited and disorganized compared with those in filament-containing groups. Scale bar shown is 400 μm.

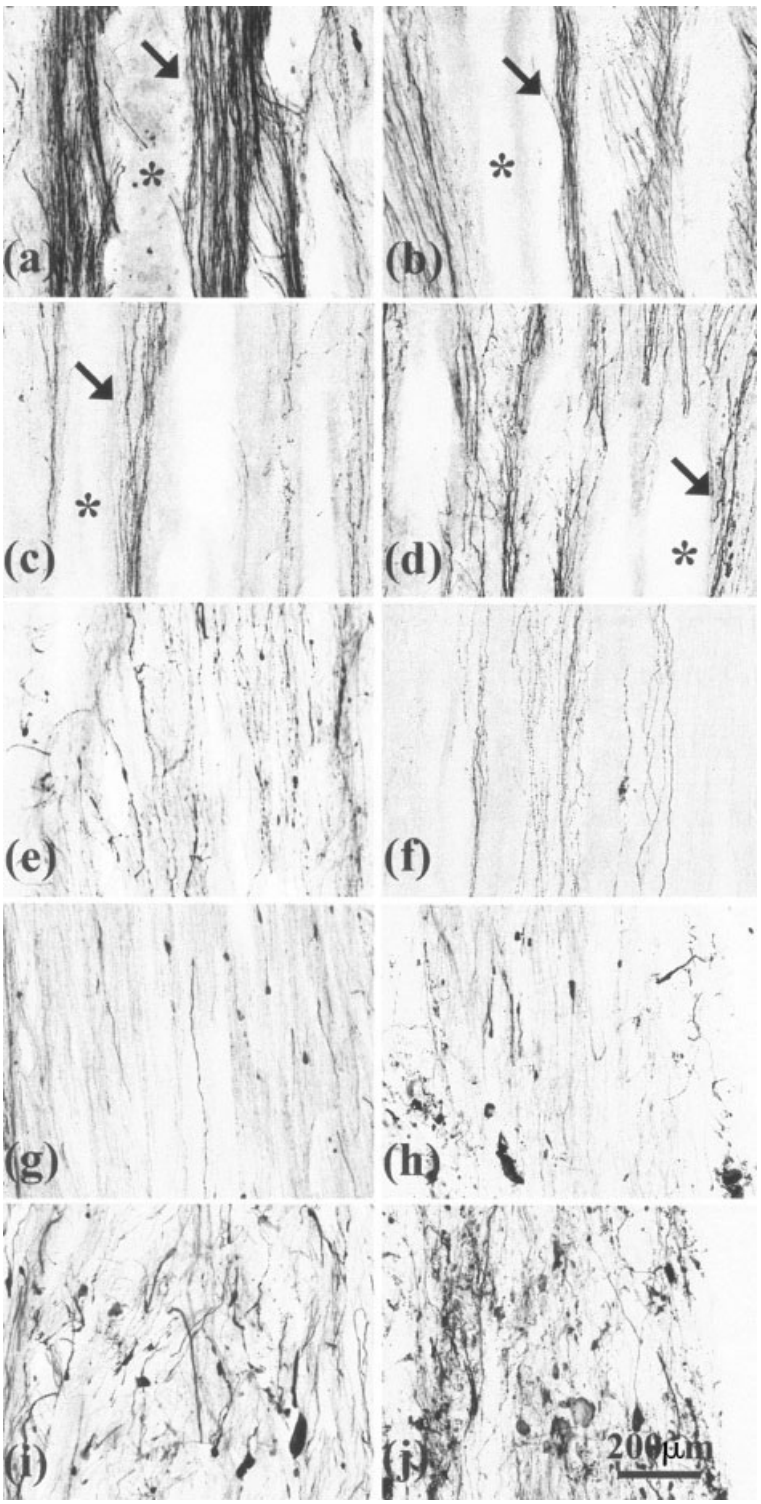

Figure 7. Longitudinal staining showing guidance affect of filaments to align axonal migration. (a, b) Implants filled with Matrigel, heregulin, and 32 PLLA microfilaments (FMH); (c, d) Implants filled with Matrigel and 32 PLLA microfilaments (FM). Axonal migration (arrows) along filaments (*) was observed in all the filament-containing groups. The highest density of longitudinally organized axons was observed in the FMH implants. (e, f) Implants filled with Matrigel and heregulin (MH). (g, h) Implants filled with Matrigel (M). In nonfilament-containing groups, numerous axons tangled, turned, and displayed end-bulblike structures reminiscent of abortive axon growth. a, c, e, and g represent section through the implant center, whereas b, d, f, and h were taken at the distal end. Reminiscent of neuromas, numerous abortive axon terminals were frequently observed in nonfilament-containing groups (**i** for group MH and **j** for group M). Scale bar shown is 200 μm.

increased Schwann cell migration into the implant by more than fourfold but showed only minor increases in axonal regeneration or myelination compared to Matrigel controls. Similar increases in Schwann cell migration and axonal regeneration were observed for implants containing both microfilaments and Matrigel, indicating that both treatments increase Schwann cell migration most likely due to different mechanisms. One potential limitation to the use of Matrigel is that when gelled, it is particularly dense, and migrating cells must digest the material as they progress. Therefore, the chemoattractive ability of heregulin would be limited by the migration rate of the Schwann cells through Matrigel. On the other hand, we previously showed that microfilaments will support axon growth through dense matrix *in vitro,* in which the Schwann cells preferentially migrated along the filaments and not into agarous surrounding the filaments.[23] A fluid space appears to develop at the interface surrounding the filament that supports rapid and directed migration of cells. Longitudinal sections stained for Schwann cells also showed increased Schwann cell density throughout the implant in microfilament-treated implants. In the center of the implants, Schwann cell organization appeared much more linear along the length of the filaments and parallel with axonal growth and nerve cable formation. Longitudinal bands of Schwann cells also appeared directly adjacent to filaments, indicating that the filaments might enhance contact-mediated growth.

The differences in the mechanism of Schwann cell migration and nerve repair using either microfilaments or heregulin is illustrated in their synergistic effect when applied together. In treatment groups containing both filaments and heregulin, there was a twofold increase in the number of Schwann cells within the implant compared to treatment groups containing either heregulin or filaments alone. Increased migration was apparent both at the distal as well as center of the implant, again with Schwann cells maintaining an organization parallel to the orientation of the filaments. With combined treatments, heregulin most likely increased Schwann cell proliferation and chemotaxis toward the implant center, whereas filaments essentially provided the "highway" on which they migrated. Because Schwann cells provide an essential growth substrate for regenerating axons, their organization greatly influences the organization and the rate of axonal migration and maturation. Comparisons of longitudinal sections stained for S-100 and CGRP show similarities in growth between Schwann cells and axons. The higher density of Schwann cells in the FMH group appeared to be associated with earlier maturation of the regenerated axons, where the myelin number was more than fourfold greater than the other groups. It is of interest that filaments not only directed axon growth within the implant itself but also greatly affected the integration and organization of axons at the implant/nerve interface, increasing the efficiency of axon growth out of the proximal stumps and into the distal stump. In groups not containing filaments, the number of retraction bulbs (growth cone that abort regeneration) was dramatically increased.

Neuromas often develop with long lesion gaps, and the mechanism by which they form is not known. Several studies have indicated that neuromas form due to lack of sufficient matrix or neurotrophins within tubes, or by the proliferation of myofibroblasts that encapsulate the nerve stump.[27,28] However, both of these mechanisms probably act to influence whether a neuroma or tissue cable forms. If Schwann cell migration out of the nerve stumps is delayed due to lack of establishment of a migratory substrate or pathway, then myofibroblast can encapsulate the nerve stump and further prevent Schwann cell migration. Conversely, the establishment of a conducive migratory substrate should support Schwann cell migration out of the nerve stump before the formation of a myofibroblastic capsule. We have observed tissue cable formation and no neuroma formation with all treatment groups receiving microfilament implants, except for one animal in which the nerve end detached from the implant.[22] Electromicroscopic examination indicated that myofibroblastic-like cells were observed loosely scattered throughout the implant, along filaments, and surrounding the nerve cable.[22] The density of myofibroblasts surrounding the regenerated nerve was higher than those observed in either normal nerves or with collagen tubes,[28] which show substantial nerve repair.[29] Because a relatively thick myofibroblastic sheath surrounding the repaired nerve might contribute to chronic constriction of the nerve, we are presently examining biodegradable tubes that reduce this potential problem. In addition, the biodegradability of the microfilaments is also being adjusted by using specific blending of PLLA and poly (DL-lactide-*co*-glycolide).[30] In the present experiments, the degradation of the polymer is slow and mostly intact after 10 weeks. For future experiments, filaments will be used that are stable during the initial stage of regeneration, but degrade completely after nerve construction.[22]

In general, implants containing the combination of heregulin and microfilaments act synergistically to improve nerve repair and axonal regeneration in rat sciatic nerve across a 14-mm lesion gap. To further improve the potential efficiency of inner guidance channels, poly(lactide-*co*-glycolide) (PLGA) filament with faster degradation rate designed for slow release of heregulin and specific neurotrophic factors are being tested. Bioresorbable outer guidance channels have also been manufactured in our laboratory to substitute silicone tubes to reduce the risk of nerve

compression in long-term studies. Future studies would include performing long-term implantation periods and behavioral studies to test the functional efficacy of these nerve guidance devices.

Helpful discussion with Teri T. B. Ngo and technical assistance from Lorna Putnam and Raymond Collins are greatly appreciated.

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