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Differentiation of Bone Marrow Stem Cells into Schwann Cells for the Promotion of Neurite Outgrowth on Electrospun Fibers

Jiajia Xue[†], Junyu Yang[†], Deirdre M. O'Connor[‡], Chunlei Zhu[†], Da Huo[†], Nicholas M. Boulis[‡], and Younan Xia^{*,†,§}

[†]The Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia 30332, United States

[‡]Department of Neurosurgery, Emory University, Atlanta, Georgia 30322, United States

[§]School of Chemistry and Biochemistry, School of Chemical and Biological Engineering, Georgia Institute of Technology, Atlanta, Georgia 30332, United States

Abstract

Seeding nerve guidance conduits with Schwann cells can improve the outcome of peripheral nerve injury repair. Bone marrow stem cells (BMSCs) represent a good choice of cell source as they can differentiate into Schwann cells under appropriate conditions. In this work, we systematically investigated the differentiation of BMSCs into Schwann cells on scaffolds comprising electrospun fibers. We changed the alignment, diameter, and surface properties of the fibers to optimize the differentiation efficiency. The uniaxial alignment of fibers not only promoted the differentiation of BMSCs into Schwann cells but also dictated the morphology and alignment of the derived cells. Coating the surface of aligned fibers with laminin further enhanced the differentiation and thus increased the secretion of neurotrophins. When co-cultured with PC12 cells or chick dorsal root ganglion, the as-derived Schwann cells were able to promote the outgrowth of neurites from cell bodies and direct their extension along the fibers, demonstrating the positive impacts of both the neurotrophic effect and the morphological contact guidance. This work offers a promising strategy for integrating fiber guidance with stem cell therapy to augment peripheral nerve injury repair.

Graphical Abstract

*Corresponding Author: younan.xia@bme.gatech.edu.

ORCID

Jiajia Xue: 0000-0002-7209-7935

Chunlei Zhu: 0000-0003-2477-306X

Younan Xia: 0000-0003-2431-7048

Notes

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Supporting Information

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Primer sequences of the genes for real-time PCR analysis; the distributions of fiber diameters for different types of scaffolds; quantification analysis of the alignment of the electrospun fibers; immunofluorescence micrographs to confirm the isolated as BMSCs; viabilities of the BMSCs after seeding for one day on different types of scaffolds; representative F-actin immunofluorescence micrographs of the BMSCs cultured on different types of scaffolds for 3 days; quantification of the numbers and viabilities of BMSCs after culturing for 3 days on different types of scaffolds; quantification of the average lengths of the actin filaments in the derived cells; neurite extension from PC12 cells cultured on scaffolds in the presence and absence of the derived Schwann cells; analysis of the average and the longest neurite lengths of the neurites outgrowth from the PC12 cells; additional experimental details (PDF)

Keywords

electrospun fibers; bone marrow stem cells; Schwann cells; differentiation; neural tissue engineering

1. INTRODUCTION

Peripheral nerve injury is a large-scale problem that affects more than 1 million people per year.^{1–4} During the repair, a nerve graft is usually interposed between the proximal and distal stumps to guide the regeneration of the axon.⁵ Autologous nerve grafts are the most successful clinical materials because they can supply intact extracellular matrices (ECMs) and abundant glial cells for nerve extension without triggering an immune response.^{6,7} However, in addition to the sophistication associated with surgical procedure, the use of autograft is a self-destructive process.^{5,8,9} As an alternative, most of the current research efforts are directed toward the development of nerve guidance conduits (NGCs) from natural or synthetic polymers.^{10–12} An NGC is supposed to maintain the nerve regeneration environment, guide axon regeneration, keep the nerve growth factors (NGFs) in the wound healing space, and prevent the invasion of cells that could form scars.^{12–14} Among the reported systems, NGCs based on electrospun fibers have received the most attention owing to their highly porous walls and their ability to present the fibers as a uniaxially aligned array.¹⁵ The aligned fibers can provide a topographical cue to direct and enhance axonal extension during regeneration.^{16–18} Additionally, bioactive molecules such as growth factors can also be readily incorporated in the conduit. Sustained release of these effectors from the conduit has resulted in a better outcome for peripheral nerve regeneration.¹⁹

The use of acellular NGCs has encountered limited success as cellular components are also needed to provide a neurotrophic support for axonal regeneration. For example, Schwann cells are known to play a pivotal role in the repair of an injured nerve. Following nerve injury, Schwann cells clear the myelin debris while proliferating and forming bands of Büngner that provide longitudinal guidance channels to direct axon regeneration from the proximal nerve stump.^{20,21} In addition, Schwann cells secrete neurotrophins and other types of growth factors to promote neural regeneration both in vitro and in vivo.^{22,23} In an attempt to promote the regeneration process, autologous Schwann cells were pre-seeded in the NGCs and further demonstrated significant enhancement in terms of neurite extension and re-myelination.²⁴ In the native structure of a peripheral nerve, the axon is wrapped by a myelin sheath formed by Schwann cells, so it is critical to be able to supply an adequate

number of Schwann cells to the site of repair. In practice, the supply of donor Schwann cells has been limited by the low proliferative capacity of the cells harvested from adult peripheral nerves and high donor site morbidity. As such, the availability of an alternative source of Schwann cells has emerged as an important prerequisite for the successful development of future NGCs.

Schwann cells can be transdifferentiated from various types of stem cells, including embryonic stem cells,²⁵ neural stem cells,²⁶ adipose-derived stem cells,^{27–29} ectomesenchymal stem cells,³⁰ and bone marrow stem cells (BMSCs).^{31,32} In particular, BMSCs provide a viable means for the majority of clinical nerve injury repairs because they can be harvested using an autologous and relatively noninvasive method, followed by proliferation rapidly in vitro to attain an adequate number for in vivo implantation. In previous studies,^{33,34} BMSCs were either differentiated into Schwann cells in Petri dishes and then injected into NGCs or directly differentiated into Schwann cells in the NGCs in vivo. When compared with acellular NGCs, cellular NGCs were able to substantially improve the repair of a sciatic nerve injury.³⁵ However, the conduits used in all prior studies were based on casting tubes or gel sponge scaffolds rather than those constructed from electrospun fibers.^{36–38} The additional impact of electrospun fibers on the differentiation of BMSCs into Schwann cells is yet to be investigated. It is well known that both micro- and nano-scale topographical cues play an important role in regulating stem cell differentiation, Schwann cell behavior, and axon extrusion from a neuron.^{26,39–41} The presence of proteins on their surface can also modulate the interactions between the fibers and the cells, thereby affecting the differentiation of stem cells.^{42,43}

In the present work, we systematically investigated the differentiation of BMSCs into Schwann cells when seeded in different densities on electrospun fibers with distinct diameters, alignments, and surface properties. We quantitatively examined the differentiation efficiency through immunofluorescence microscopy and quantitative real-time polymerase chain reaction (PCR) analysis. We further investigated the maturation and function of the as-derived Schwann cells by examining their impact on the extension of neurites from PC12 cells and chick dorsal root ganglion (DRG).

2. EXPERIMENTAL SECTION

2.1. Fabrication and Characterization of Electrospun Fibers

The fibers were fabricated using the traditional electrospinning process with a setup similar to what our group has used previously.⁴⁴ Polycaprolactone (PCL) ($M_w \approx 80\,000$ g/mol; Sigma-Aldrich, St. Louis, MO) was dissolved in a mixture of dichloromethane and *N,N*-dimethylformamide (both from Fisher Chemical, Waltham, MA) at a ratio of 6:4 (v/v) to prepare two solutions at concentrations of 10 and 12 wt %. The polymer solution was ejected at a flow rate of 1.0 mL/h through a syringe pump. A high voltage (DC) of 12 kV was applied between the nozzle (a 22-gauge needle) and a grounded collector. Different types of collectors were employed to obtain fibers with various orientations. Specifically, random fibers were directly collected on glass coverslips (22 mm $\times$ 22 mm). Uniaxially aligned fibers were electrospun onto a stainless steel frame (with an opening of 2 cm $\times$ 5 cm) and then transferred onto glass coverslips. The aligned PCL fibers were coated with

laminin (Millipore, Temecular, CA) as follows. The sample fixed on a glass coverslip was treated with oxygen for 2 min in a plasma cleaner (PlasmaEtch, Carson City, NV), sterilized under UV for 1 h, immersed in a 0.1% poly-L-lysine (Sigma-Aldrich) solution for 1 h at room temperature, followed by washing with phosphate-buffered saline (PBS) buffer (Invitrogen, Carlsbad, CA) three times. Subsequently, the sample was immersed in a laminin solution (500 μL , diluted from a 50 $\mu\text{g/mL}$ stock solution with 5 mL of PBS buffer) at 4 °C overnight and then rinsed with PBS buffer three times to wash off the loosely bound laminin. The amount of laminin deposited on the electrospun fibers was determined using a Micro BCA protein assay according to the manufacturer's protocol.

The morphologies of the fibers were characterized by scanning electron microscopy (SEM). The diameter distribution and average diameter of the fibers were measured using ImageJ software from 100 fibers in the SEM images. Fast Fourier transform (FFT) analysis of the SEM images was performed by utilizing the FFT function of the ImageJ software. A quantitative analysis of the alignment of the electrospun fibers was performed by summation of pixel intensities at different degrees. To determine the wettability of a specific type of scaffold, the water contact angle was measured with deionized water using a SL200A-type contact angle analyzer. All experiments were conducted under ambient conditions.

2.2. Isolation and Characterization of BMSCs

All animal procedures were done with approval from the Emory University institutional animal care and use committee (IACUC). We isolated BMSCs from the bone marrow of adult Sprague Dawley rats weighing 250–300 g by differential attachment and used them at passages 2–4. After euthanizing, the left and right femurs of a rat were dissected out, and the proximal and distal ends were removed to expose the marrow cavity. The marrow cavity was then flushed with 20 mL of α -modified Eagle's medium (α -MEM, Invitrogen) to remove the cells. The cells were spun down, re-suspended in 10 mL of complete α -MEM (α -MEM containing 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic (ABAM)), and plated in a T25 flask in an incubator at 37 °C under 5% CO_2 . After 24 h, the medium was removed to get rid of nonadherent hematopoietic cells and replaced with 5 mL of complete α -MEM. The adherent cells, assumed to be BMSCs, were passaged whenever the primary culture reached confluence. The cells were confirmed as BMSCs by the mesenchymal morphology, in addition to examining the expression of CD44, CD34, and CD45 on the cell surface by immunofluorescence staining.

2.3. Seeding, F-actin Staining, and Viabilities of BMSCs

The fibers fixed on glass coverslips were placed into six-well plates and afixed to the bottoms of the wells with a small dab of medical adhesive. The samples were then sterilized with UV for 1 h. Passage 2 rat BMSCs were seeded onto the different scaffolds at a density of 250 cells/ mm^2 . The cells cultured on tissue culture plate (TCP) were set as the control. As the cell density also influences the interaction between stem cells and fibers, we also seeded the BMSCs on the fibers at a low density of 50 cells/ mm^2 . After seeding the cells for 24 h on the different types of electrospun scaffolds, their viabilities were evaluated using the Cell Counting Kit-8 (CCK-8; Sigma-Aldrich) assay. The cells were washed with PBS, and then CCK-8 reagent (10%) in culture medium was added to each well. After 4 h, the well plate

was shaken for 15 min, and then the absorbance value of the supernatant at 450 nm was measured using a microplate reader. After incubation for 3 days, the F-actin of the BMSCs on different scaffolds was stained with Alexa Fluor 555 phalloidin (Invitrogen). The cells were fixed in 3.7% formaldehyde at room temperature for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. Staining solution was prepared by diluting 5 μ L methanolic stock solution into 200 μ L of PBS for each sample to be stained. To reduce nonspecific background staining, 1% bovine serum albumin (BSA) was added to the staining solution. Each sample was incubated in the staining solution for 20 min at room temperature. The nuclei of the cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. After staining, fluorescence micrographs were taken using a Leica DMI 6000 B inverted microscope (Buffalo Grove, IL). The number of cells on each group was counted from the fluorescence micrographs using MATLAB software. The viabilities of BMSCs on different scaffolds were evaluated by CCK-8 assay.

2.4. Differentiation of BMSCs into Schwann Cells

We performed in vitro chemical induction of the BMSCs into Schwann cell-like phenotypes in three steps.^{21,45} BMSCs of passage 2–4 were seeded onto the sterilized scaffolds in a six-well plate at a density of 250 cells/mm². At 24 h post seeding, the medium was replaced with complete α -MEM supplemented with 1 μ M β -mercaptoethanol (Invitrogen). After incubation for another 24 h, the cells were then cultured in the complete α -MEM supplemented with 35 ng/mL all-*trans*-retinoic acid for 72 h. Afterwards, the medium was replaced with differentiation medium (complete α -MEM supplemented with 5 ng/mL platelet-derived growth factor-AA, 10 ng/mL basic fibroblast growth factor, 126 ng/mL glial cell-derived growth factor, and 5.7 μ g/mL forskolin). The cells were then incubated for 2 weeks in the differentiation medium, with fresh medium replaced every 3 days.

2.5. Immunostaining and F-actin Staining of the Derived Cells

We performed immunofluorescence staining to monitor phenotypic changes to the BMSCs using S100 as a general indicator for the derived Schwann cells. The cells were fixed in 3.7% formaldehyde at room temperature for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, blocked with 3% BSA for 1 h, and then incubated with S100 antibody diluted (1:200) in PBS overnight at 4 °C. The anti-S100 marker was detected using Alexa Fluor 594 goat anti-rat IgG (1:200; Invitrogen) secondary antibody, followed by DAPI staining for cell nuclei. Immunofluorescence imaging was performed using the fluorescence microscope. The process performed for F-actin staining of the derived cells was the same as above described for that of the BMSCs. The average length of the filamentous actin of the cells derived on different types of electrospun scaffolds was quantitatively analyzed from the respective fluorescence micrographs using ImageJ software.

2.6. Gene Expression Analysis and Viabilities of the Derived Cells

We used real-time PCR analysis to quantify the mRNA expression levels of representative genes expressed by the derived cells on different scaffolds. Total RNA was extracted using TRIzol reagent (Invitrogen), and then 1 μ g RNA was used to synthesize cDNA using a reverse transcription reagents kit (Thermo Fisher). PCR was performed under the respective conditions with 100 ng cDNA, a SYBR Green PCR Master Mix (Thermo Fisher), and the

primers are listed in Table S1. Finally, the real-time PCR was conducted with an Applied Biosystems 7500 Fast Real-time PCR system with the following temperature profile: 95 °C for 2 min, 40 cycles at 95 °C for 3 s, and then 60 °C for 30 s. Five genes were evaluated in our study: BMPRIa (bone morphogenetic protein receptor, type IA), CD44, CD105, S100, and p75 along with the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH). The gene expression was normalized to GAPDH in the same sample.

The viability of the derived Schwann cells on the different types of scaffolds was tested by a CCK-8 assay with the same procedure as that described above. The survival of the derived Schwann cells on AF1000L was investigated as follows. The differentiation medium was switched to the Schwann cell medium, which was composed of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 1% ABAM, 2 μ M forskolin (Sigma-Aldrich), and 20 μ g/mL bovine pituitary extract (Biomedical Tech Inc., Stoughton, MA). The cells were then incubated for 7 days, with fresh medium replaced every 2 days. The cell viability was separately detected at the time of incubating the cells for 1, 3, 5, and 7 days by the CCK-8 assay.

2.7. Co-culture of the Derived Schwann Cells with PC12 Cells

We investigated the maturation of the derived Schwann cells from both the genetic level and secreted protein level. The gene expression levels of NGF and brain-derived neurotrophic factor (BDNF) by the BMSCs and the derived Schwann cells were quantitatively evaluated by real-time PCR analysis. The NGF contents secreted from the BMSCs on laminin-coated TCP and the derived Schwann cells on AF1000L were detected using an NGF Elisa kit (Invitrogen) according to the manufacturer's instructions. The BMSCs were incubated on laminin-coated TCP for 7 days to form a full cellular layer, and then the culture medium was collected to detect the secreted NGF content. For the derived Schwann cells, the secreted NGF content was detected after being incubated in the Schwann cell medium for 7 days.

We then co-cultured the derived Schwann cells with PC12 cells, which is commonly used as a model system for neuronal differentiation,⁴⁶ to investigate the function of the derived Schwann cells on the neurites extension from the PC12 cells. The PC12 cells were dissociated in DMEM supplemented with 10% horse serum (Gibco) and 5% new born calf serum (Gibco) and then seeded at a density of 500 cells/cm² on the substrate. After incubation for 24 h, the culture medium was replaced with DMEM supplemented with 1% horse serum and 50 ng/mL NGF (Thermo Fisher), and the cells were further incubated for 6 days. Thereafter, the neurites extruded from the PC12 cells were immunostained with anti- β III tubulin antibody (Tuj1) (Covance, San Diego, CA). Briefly, the samples were fixed, blocked, and then incubated with the primary antibody overnight at 4 °C, and then the Tuj1 marker was detected using Alexa Fluor 594 goat anti-mouse IgG (1:200; Invitrogen) secondary antibody. The cells were imaged under a fluorescent microscope. The average neurite lengths and the longest neurite lengths were quantitatively analyzed from the fluorescence micrographs by using ImageJ software. The expression levels of the neurite growth-relating genes in the PC12 cells were detected using real-time PCR analysis. Three genes were detected in our study: NEFH (neurofilament heavy), NEFL (neurofilament light),

and TUBB3 (tubulin β -3), along with the housekeeping gene GAPDH. The gene expression level was normalized to GAPDH in the same sample.

2.8. Co-culture of the Derived Schwann Cells with DRG

All DRG cells were isolated from the thoracic region of the spinal column in embryonic chick via sterile microdissection.⁴⁷ Embryonic day 8 (E8, stage HH35–36) chick was removed from the white leghorn egg and decapitated. DRG cells were dissected from the thoracic region, and seeded onto the center of the samples (1 DRG per sample) followed by culturing for 6 days in a modified neurobasal medium supplemented with 10% FBS, 1% N-2 supplement (Invitrogen), and 1% ABAM. Thereafter, fixed DRG neurons were immunostained with Tuj1 primary antibody and Alexa Fluor 488 secondary antibody, and the cell nuclei were stained with DAPI. Fluorescence micrographs were captured using a laser confocal scanning microscope (Zeiss LSM 700).

The behavior of neurite populations extending from the DRG was examined by quantitative analysis of the neurite field.²⁵ The average neurite lengths and the longest neurite lengths of the projected neurites from the DRG bodies were derived from the fluorescence micrographs using ImageJ software. The neurite's path was traced, and the pixel densities from the DRG body toward the end of the neurite were measured using the software to obtain the length of the neurite. Six DRG cells on each kind of sample were used to calculate the neurite length. Calculations of neurite field eccentricity (a measure of directed neurite growth along a given axis) were accomplished by separately fitting the leading edge of the neurite field and the perimeter of the DRG body to standard elliptical equation (eq 1) at point (h, k) of the form²⁵

$$\frac{(x-h)^2}{a^2} + \frac{(y-k)^2}{b^2} = 1 \quad (1)$$

where a and b are the ellipse's semimajor and semiminor axes, respectively.

Eccentricity of the neurite field was then calculated using eq 2

$$\text{Ecc.} = \frac{\sqrt{(a^2 - b^2)}}{a} \quad (2)$$

where values of a and b were obtained from the elliptical equation fit to the leading edge of the neurite field. Statistical analysis was performed using the t test.

3. RESULTS AND DISCUSSION

3.1. Characterization of the Electrospun Fibers

First of all, the differentiation of BMSCs into Schwann cells should be dependent on their viability and the configuration of their cytoskeletons, which are largely determined by the underlying fibers. To this end, we varied the alignment and diameter of the electrospun

fibers to evaluate their impact on the differentiation process. Specifically, random fibers with an average diameter of 488 ± 23 nm (labeled as RF500) were electrospun from a 10 wt % PCL solution and directly collected on glass coverslips. Uniaxially aligned fibers with average diameters controlled at 521 ± 15 and 986 ± 31 nm, respectively, were electrospun from 10 and 12 wt % PCL solutions and labeled as AF500 and AF1000. The aligned fibers were collected using metal frames and then transferred onto glass coverslips.^{48,49} Figure 1A shows an SEM image of a typical sample of the random fibers. The FFT pattern of the image confirms that the fibers were randomly oriented as the pixel intensities showed essentially no dependence on the direction.⁵⁰ Figure 1B,C shows SEM images of the fibers with a uniaxial alignment, which was further confirmed by the corresponding FFT pattern. The surface of the AF1000 scaffold was also coated with laminin to yield the scaffold referred to as AF1000L. As shown in Figure 1D, the morphology and alignment of the fibers in the laminin-coated sample were essentially identical to those in pristine AF1000, except for the slight increase in fiber diameter (Figure S1C,D). Quantitative analysis of the alignment of the electrospun fibers is shown in Figure S2. The amount of laminin coated on the electrospun fibers was $0.92 \mu\text{g}/\text{cm}^2$, as determined using the Micro BCA protein assay. The surface wettability of a scaffold can also affect the behavior of cells seeded on it. The contact angles were 128.2, 121.3, 119.0, and 78.1° , respectively, for RF500, AF500, AF1000, and AF1000L. The slight increase in wettability for the aligned fibers relative to that of the random fibers can be attributed to the acicular shape of the pores among the aligned fibers.⁵¹ After coating with laminin, the wettability of the aligned fibers was further improved.

3.2. Comparison of the BMSCs Cultured on Different Types of Scaffolds

The differentiation of BMSCs into Schwann cells is also dependent on the adhesion and density of BMSCs seeded on a scaffold.⁵² To improve nerve repair, the NGC should contain Schwann cells at the highest possible density. In this regard, we examined the morphology and viability of BMSCs cultured on the different types of scaffolds. Firstly, to confirm the phenotype of the isolated cells, immunofluorescence staining was performed to examine the expression of CD44, a biomarker of BMSCs. As shown in Figure S3, the cells expressed CD44. In addition, no expression of CD34 and CD45 was observed, further confirming that the cells were BMSCs. Then, BMSCs at passage 2 were seeded on the scaffolds, including TCP as a control, at a density of 250 cells/ mm^2 . The BMSCs were also seeded on the aligned, laminin-coated scaffolds at a lower density of 50 cells/ mm^2 (i.e., the group AF1000L-1). At 24 h post seeding, the cell viabilities on the different types of electrospun scaffolds were examined using the CCK-8 assay. As shown in Figure S4, the cells showed the highest viability on the AF1000L scaffold, indicating that the laminin coated on the fibers could improve the adhesion of cells to the fibers. To determine the biocompatibility of the electrospun fibers and their impact on the cell morphology, F-actin staining was applied to the BMSCs cultured for 3 days. Figure S5 compares the cytoskeletal arrangements in the BMSCs. In general, the BMSCs adhered to and grew healthily on all the scaffolds. The BMSCs cultured on TCP and random fibers showed a disorganized actin meshwork. In contrast, the BMSCs cultured on aligned fibers showed an actin network consisting of a large number of filaments aligned parallel to the long axis of the underlying fibers. The aspect ratios of cells cultured on the aligned fibers were obviously higher than those cultured

on the random fibers. The effect of fibers on the actin arrangement and thus cell morphology was similar to what was observed for tendon fibroblasts and Schwann cells in previous studies.^{24,53} The number of cells on the scaffolds appeared to be different. Figure S6A shows a comparison of the average numbers of cells on different types of scaffolds. When the fibers were roughly the same in diameter, the number of cells on aligned fibers (e.g., AF500) was higher relative to the case of random fibers (RF500) because of the enhancement in surface wettability. Compared with that for AF500, more alive cells were found on AF1000. When the surface of the fibers was coated with laminin, an important component of the ECM, the BMSCs reached almost 80% confluence within 3 days, which was even higher than that on TCP. Coating the surface of the fibers with laminin greatly promoted the adhesion and growth of the BMSCs, primarily due to the bioactivity of laminin and the improvement in surface wettability.⁵⁴ The same conclusions could also be drawn from the evaluation of cell viability using the CCK-8 assay, as shown in Figure S6B.

3.3. Differentiation of BMSCs on Different Types of Scaffolds

All transdifferentiation experiments were carried out in vitro with rat BMSCs at passage 2. The BMSCs were seeded separately at a cell density of 250 cells/mm² on TCP, RF500, AF500, AF1000, and AF1000L, together with 50 cells/mm² on AF1000L-1. After the cells were incubated for 19 days according to the in vitro chemical induction, we evaluated the expression of protein S100, which is one of the most important surface markers of Schwann cells. Figure 2 shows the representative immunofluorescence micrographs of the cells derived from the BMSCs, in which the S100 protein and cell nucleus were stained in red and blue, respectively. After transdifferentiation, the cells demonstrated morphological changes, showing a bipolar and spindle shape that resembles the typical morphology of Schwann cells.²⁴ In the images, cells on the scaffolds all expressed S100. The expression content of S100 in the derived cells was reflected by the fluorescence intensity. For the cells differentiated on RF500 and AF500, no obvious difference of fluorescence intensity was observed, indicating that the alignment of fibers did not affect the differentiation efficiency. The differentiation of BMSCs into Schwann cells is sensitive to the configuration of their cytoskeletons, which is dependent on the diameter of the fibers.²⁶ The derived Schwann cells indeed expressed a higher level of S100 on AF1000 than that of those on AF500, suggesting that thicker fibers were instrumental to the differentiation of BMSCs into Schwann cells. After surface coating with laminin, the differentiation efficiency was further promoted. The Schwann cells differentiated on the laminin-coated fibers showed the highest level of S100 expression. Surface coating with laminin not only improved BMSCs adhesion and proliferation but also promoted their differentiation.

From Figure 2, it is clear that the cells differentiated on TCP and the random fibers had a random distribution in terms of orientation. In contrast, the cells differentiated on the aligned fibers showed a uniaxial alignment parallel to the fibers, indicating the presence of contact guidance provided by the underlying fibers. Further, the overall cell viabilities for the different types of scaffolds appeared to vary by the end of the differentiation process. The RF500 gave a density of cells similar to that of AF500, which was lower than that of AF1000. AF1000L gave the largest number of cells among the different scaffolds. From these results, we can conclude that BMSCs could be successfully transdifferentiated into

Schwann cells on both TCP and electrospun fibers, with the AF1000L scaffold giving the highest differentiation efficiency and cell viability for the derived cells.

We further applied F-actin staining to the derived Schwann cells to determine whether the cellular changes in morphology and orientation induced by the electrospun fiber scaffolds were effectively translated into cytoskeletal remodeling. Figure 3 shows fluorescence micrographs of the as-derived Schwann cells after F-actin staining. The Schwann cells transdifferentiated on TCP and random fibers demonstrated a disorganized actin network, whereas those on the aligned fibers showed an aligned actin network. The average length of the filamentous actin filaments of the cells derived on different types of electrospun scaffolds was quantitatively analyzed from the fluorescence micrographs and is shown in Figure S7. On the aligned fibers, the derived cells showed longer filamentous actin than that of those on random fibers, whereas there was no significant difference in the actin fiber length among the different types of scaffolds made of aligned fibers. These results demonstrate that the derived Schwann cells indeed responded to the topographical cue presented by the underlying fibers. The cytoskeletal arrangement of the derived Schwann cells on different scaffolds was similar to what was observed for the BMSCs seeded on the corresponding scaffolds. The trend in cell number after differentiation among the different scaffolds also largely agreed with what was observed for the number of BMSCs after proliferation on the fibers.

3.4. Gene Expression and Cell Viability of the Derived Schwann Cells

The differentiation of BMSCs on different types of scaffolds was further evaluated at the genetic level by performing real-time PCR analysis. We detected the expression of the BMSC-related genes: BMPR1a, CD44, and CD105, and the Schwann cell-related genes: S100 and p75 in the derived cells. The relative transcript level expressed in the undifferentiated BMSCs was set as a control. From Figure 4A, it can be seen that the expression of BMPR1A, CD44, and CD105 was all greatly reduced in the derived Schwann cells when compared with that in the BMSCs. The expression levels of Schwann cell-related genes are shown in Figure 4B. Compared with that in BMSCs, regardless of the types of the substrates for the differentiation, upregulation of S100 and p75 was found in the derived Schwann cells. On the aligned fibers, the Schwann cells expressed higher levels of S100 and p75 than that of those on TCP, indicating that the biomimic structure of the aligned fibers promoted the differentiation. By increasing the diameter of the fibers from about 500 to 1000 nm, the gene expression levels of S100 and p75 were further upregulated. Upon surface coating with laminin, the expression of both S100 and p75 showed the highest levels among the different types of scaffolds, which was consistent with the results we observed from the immunofluorescence micrographs. Further, on the AF1000L-1 scaffold with a low cell seeding density, the derived cells expressed lower S100 and p75 levels than that of those on the AF1000L scaffold, which was attributed to the fact that the cellular interactions could affect the differentiation of BMSCs. These results clearly indicate that the morphology and differentiation of stem cells can be modulated by engineering the properties of the fiber scaffolds. From Figure 4C, the Schwann cells derived on AF1000L showed the highest viability. The biocompatibility of the electrospun fibers affected the viability of the BMSCs and thus the Schwann cells transdifferentiated from the BMSCs. Compared with the other

types of scaffolds, AF1000L resulted in enhanced adhesion of BMSCs to the scaffold, as well as enhancement of cell growth, because of the laminin coated on the fibers. Taken together, it can be concluded that the differentiation performed best when the BMSCs were seeded on AF1000L scaffolds.

It is of critical importance to ensure that the derived cells can survive for several days either for the purpose of transportation or for remaining viable after transplantation into the body. We further incubated the derived Schwann cells on AF1000L in the culture medium for 7 days. The cell viabilities were then analyzed using the CCK-8 assay and compared in Figure 4D. The cells remained alive, and even proliferated over time. The laminin coated on the surface of the fibers could interact with β 1-integrin on the surface of the derived Schwann cells, providing a Schwann cell survival signal and thus promoting cell proliferation and maturation.^{55–57} It has been reported that the differentiation of BMSCs into Schwann cells on TCP was a reversible process in that the BMSCs could be reversed to their original fibroblast-like morphology in vitro after 2 weeks.⁵⁸ From our observation, the derived Schwann cells on the aligned fibers showed no morphological changes, which could be attributed to the high degree of differentiation of BMSCs and the maturation of the derived Schwann cells. The analogy between the electrospun fibers and the collagen fibers in ECM also helped maintain the phenotype of the derived Schwann cells.⁵⁹ Besides, due to the presence of environmental factors in vivo, the derived Schwann cells should remain stable.⁵⁸ If the derived cells are released from the scaffold and recultured in a Petri dish, we can expect that the derived cells will remain as Schwann cells for a period of time of at least 2 weeks.

3.5. Co-culture of the Derived Schwann Cells with PC12 Cells

Evidence of morphological and phenotypic characteristics may not be enough to justify that the function of the derived cells is similar to that of the Schwann cells. Myelinating Schwann cells can secrete neurotrophic factors including NGF and BDNF, two of the most important neurotrophins to facilitate neuroregeneration.⁶⁰ As such, we evaluated the influence of electrospun fibers on modulating the expression of NGF and BDNF by the derived Schwann cells. Uniaxial alignment of the derived cells was obtained on the aligned fiber scaffolds; therefore, we selected the aligned fibers for this experiment. From Figure 5A, compared with that of the BMSCs, after transdifferentiation into Schwann cells on TCP, the expression of BDNF increased by almost 6-fold. Further, the expression of BDNF by the Schwann cells derived on electrospun fibers significantly increased in comparison with that of those derived on TCP. The uniaxially aligned fibers had positive effects on the maturation of the derived Schwann cells. Furthermore, the Schwann cells derived on AF1000 expressed a higher level of BDNF than that of those derived on AF500, suggesting that fibers with a micrometer size were better suited for the maturation of the derived Schwann cells. In addition, the expression of BDNF was enhanced by almost 2-fold when comparing the samples derived from AF1000L and AF1000, indicating that the surface-coated laminin was able to promote myelin gene expression for the derived Schwann cells. Figure 5B compares the expression of NGF from the Schwann cells derived on different types of scaffolds. Similar to BDNF, the Schwann cells derived on AF1000L showed the highest level of NGF gene expression, with an almost 4-fold enhancement relative to the sample based on TCP.

The NGF content secreted from the BMSCs and the derived Schwann cells was also analyzed by NGF Elisa. From Figure 5C, it can be seen that the derived Schwann cells secreted higher contents of NGF than the undifferentiated BMSCs. The amount of secreted NGF from the derived Schwann cells on AF1000L reached 213 pg. This result demonstrates that the Schwann cells derived on AF1000L had the ability to secrete NGF and that the aligned fibers promoted maturation of the derived Schwann cells. We can conclude that BMSCs were transdifferentiated into Schwann cells on electrospun fiber scaffolds and that the differentiation process was affected by the physical properties of the fibers, including the alignment, diameter, and surface properties. The enhanced Schwann cell differentiation can be attributed to both the topological effect and surface properties (surface coating of laminin). Using different types of materials, for example, incorporation of hydrophilic materials, such as gelatin, collagen, or laminin, into the PCL fibers will have an impact on the surface properties of the electrospun fibers, thus affecting the differentiation process. Therefore, the material types will also affect the differentiation of BMSCs into Schwann cells. In our present study, the AF1000L scaffold exhibited the best performance for both the differentiation of BMSCs and maturation of the derived Schwann cells. Therefore, we used the AF1000L scaffold to investigate the function of the derived Schwann cells in inducing the neurite extension from neurons.

It has been demonstrated that Schwann cells can induce the differentiation of PC12 cells and form a myelin sheath around the neurites.³⁰ To prove the function of the derived Schwann cells in inducing the differentiation of PC12 cells, we co-cultured them with the Schwann cells derived on AF1000L. In a typical process, PC12 cells were immediately seeded on the AF1000L scaffold covered by the as-derived Schwann cells, with TCP, AF1000L, and TCP covered by the as-derived Schwann cells serving as controls. Figure S8 compares the morphology and extension of neurites from PC12 cells cultured on the different substrates. After differentiation for 6 days, the neurites were observed to outgrow from the PC12 cells. The neurites were aligned along the direction of underlying fibers when cultured on AF1000L regardless of the absence/presence of the derived Schwann cells, whereas those on TCP showed no preference of orientation. The average neurite lengths and the longest neurite lengths from the different groups are compared in Figure S9. The average lengths of neurites extruded from the PC12 cells cultured on TCP and AF1000L were $64.5 \pm 25.2 \mu\text{m}$ and $119.4 \pm 36.7 \mu\text{m}$, respectively. When cultured on TCP covered by the derived Schwann cells, the neurites had an average length of $161.1 \pm 61.7 \mu\text{m}$, which was greater than that of those cultured on TCP. NGF is considered to play an important role in the differentiation of PC12 cells. When treated with NGF, PC12 cells could sprout neurites to form synapse structures and a neurite network, differentiating into neuron-like cells.⁶¹ In the current study, the derived Schwann cells improved the differentiation of PC12 cells by upregulating the expression of NGF. When comparing the neurites extruding from the PC12 cells cultured on pristine AF1000L and TCP covered by Schwann cells, we found that the average neurite length on the TCP covered by Schwann cells was greater. This result indicates that the effect of neurotrophic factors from the derived Schwann cells was more significant than the topographic guidance imposed by the underlying fibers. A combination of the topographic guidance of aligned fibers and the neurotrophic effect of the derived Schwann cells led to a significant increase in average neurite length to $256.4 \pm 68.3 \mu\text{m}$. In addition, the neurites

extruded from the PC12 cells cultured on AF1000L covered by the derived Schwann cells were thicker than that of those on other substrates.

We then compared the expression of genes related to neurite growth by the PC12 cells that were co-cultured with Schwann cells on both TCP and AF1000L (Figure 5D). All three genes, NEFH, NEFL, and TUBB3, were upregulated in the sample involving AF1000L when compared with that of the sample based on TCP; 1.8-fold for NEFH, 2.7-fold for NEFL, and 2.9-fold for TUBB3, respectively. This result agreed with the observation that the Schwann cells derived on AF1000L had an ability to improve neurite extension from PC12 cells.

3.6. Neurite Outgrowth from DRG Co-cultured with Derived Schwann Cells

We used DRG as another model neuron to evaluate the function of the derived Schwann cells. DRG conveys sensory information from the periphery to the central nerve system.⁶² Figure 6 shows fluorescence micrographs of the typical neurite fields extending from DRG seeded on laminin-coated TCP, TCP covered by the derived Schwann cells, AF1000L, and AF1000L covered by the derived Schwann cells, as well as a magnified view of the respective neurite field for each sample. The average and the longest neurite lengths, as well as the eccentricities of the neurite fields, were calculated from the micrographs and are summarized in Figure 7. When cultured on laminin-coated TCP, DRG extruded neurites with no preferred direction or orientation (Figure 6A,B). The distribution of neurite length was isotropic around the DRG body. For the DRG cultured on TCP covered by the derived Schwann cells, as shown in Figure 6C,D, the neurites also extended with a random pattern but with a locally ordered structure that was induced by the Schwann cells underneath. In the absence and presence of the derived Schwann cells, the average lengths of the neurites extended from DRG were 966 ± 130 and 1223 ± 150 μm , respectively. A longer neurite length was obtained by co-culturing DRG with the derived Schwann cells. From Figure 6E,F, it can be seen that the extension of neurites from DRG cultured on the scaffold comprising uniaxially aligned fibers displayed a different pattern compared to that from DRG cultured on TCP. The neurites projecting from the DRG body were guided to extend parallel to the alignment direction of the fibers (as indicated by a white line), which was similar to our previous report.⁴⁸ The average length and the longest length of the neurites were 1218 ± 280 and 1572 ± 221 μm , respectively. There was no significant difference in the average neurite length for the groups of TCP covered by the derived Schwann cells and AF1000L, indicating that the effect of surface morphology of the fibers on the improvement of neurite extension was more or less equivalent to the neurotrophic effect of the derived Schwann cells.

For DRG co-cultured with the derived Schwann cells on AF1000L (Figure 6G,H), the neurites extended along both the derived Schwann cells and the underlying aligned fibers. As a result, the neurite field displayed a high degree of eccentricity and directional specificity that was not observed for DRG cultured on TCP. In the presence of the derived Schwann cells, the average length and the longest length of the neurites were further enhanced to 3395 ± 391 and 3892 ± 408 μm , respectively. The average neurite growth rate reached an impressive level of 566 ± 65 μm per day. High-magnification images of the

neurites showed that thicker neurites were projected from DRG when co-cultured with the derived Schwann cells on the AF1000L scaffolds. The presence of Schwann cells notably increased both the length and thickness of the neurites projected from the DRG body, indicating the additional neurotrophic effect of the Schwann cells derived from BMSCs in inducing neurite extension. The aligned fibers not only promoted the differentiation of BMSCs into Schwann cells but also determined the alignment of the resulting cells to further direct and enhance the axonal extension. Taken together, we can conclude that the neurite extension from DRG is significantly improved by combining the morphological effect of the aligned fibers with the neurotrophic effect of the derived Schwann cells.

4. CONCLUSIONS

We have demonstrated that rat BMSCs could be induced to differentiate into Schwann cells at a high efficiency when seeded on electrospun fibers and cultured in a differentiation medium containing the growth factor cocktail. The differentiation process was optimized by altering the physical properties of the fibers, including their alignment, diameter, and surface properties. Uniaxially aligned fibers not only promoted the differentiation of BMSCs into Schwann cells but also determined the alignment of the resulting cells. Coating the surface of the fibers with laminin further improved the maturation and neurotrophin secretion of the derived Schwann cells. The as-derived Schwann cells showed the neurotrophic effect, which guided and greatly improved the neurite extension from both PC12 cells and chick DRG that were co-cultured with the derived Schwann cells on uniaxially aligned fibers. The results of this study point toward a platform that combines electrospun fibers and stem cell therapy to enhance the regenerative outcome of peripheral nerve injury repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

SEM micrographs of four different types of scaffolds comprising (A) random fibers with an average diameter of 488 ± 23 nm (RF500), (B) aligned fibers with an average diameter of 521 ± 15 nm (AF500), (C) aligned fibers with an average diameter of 986 ± 31 nm (AF1000), and (D) aligned fibers with an average diameter of 1001 ± 24 nm, whose surface was coated with laminin (AF1000L). Insets: two-dimensional FFT images generated from the SEM images.

Figure 2.

Representative immunofluorescence micrographs of the cells transdifferentiated from BMSCs on different types of scaffolds after culture for 19 days according to the in vitro chemical induction procedures. The BMSCs were seeded separately at a cell density of 250 cells/mm² on TCP, RF500, AF500, AF1000, and AF1000L, and at a density of 50 cells/mm² on AF1000L-1. S100 expressed from the derived cells was stained with anti-S100 (red) and the cell nuclei were stained with DAPI (blue). The double-headed arrows indicate the alignment directions of the underlying electrospun fibers.

Figure 3. Representative F-actin fluorescence micrographs of the cells transdifferentiated from BMSCs on different types of scaffolds after culture for 19 days according to the in vitro chemical induction procedures. The actin cytoskeleton was stained with Alexa Fluor 555 phalloidin (red) and the cell nuclei were stained with DAPI (blue). The double-headed arrows indicate the alignment directions of the underlying electrospun fibers.

Figure 4.

Real-time PCR analysis on the gene expression of (A) the BMSC-related genes: BMPRI1a, CD44, and CD105, and (B) the Schwann cell-related genes: S100 and p75 in the cells transdifferentiated from BMSCs on different types of scaffolds according to the in vitro chemical induction procedures. * $P < 0.05$ and ** $P < 0.01$ compared with that in BMSCs. (C) The viabilities of the derived cells on the scaffolds tested by CCK-8 assay. * $P < 0.05$ as compared with that on TCP. (D) The derived cells on AF1000L were further kept incubating for 7 days in the Schwann cell culture medium, and the cell viabilities at different incubation times were separately tested by the CCK-8 assay. * $P < 0.05$ compared with that at 1 day.

Figure 5.

Gene expression levels of (A) BDNF and (B) NGF in the cells transdifferentiated from BMSCs on different types of scaffolds. * $P < 0.05$ and ** $P < 0.01$ compared with that for BMSCs cultured on TCP. (C) Secretion of NGF from the BMSCs on TCP (TCP-BMSC), the derived Schwann cells on TCP (TCP-SC), and the derived Schwann cells on AF1000L (AF1000L-SC) as revealed by NGF Elisa. ** $P < 0.01$ as compared with that from the BMSCs cultured on TCP. (D) The expression levels of neurite extension-related genes in PC12 cells after incubating on TCP-SC and AF1000L-SC for 6 days. ** $P < 0.01$ for comparing group of PC12 cells incubating on AF1000L-SC with group of that on TCP-SC.

Figure 6.

Fluorescence micrographs of the typical neurite fields extending from DRG when cultured on (A) laminin-coated TCP, (C) TCP covered by the derived Schwann cells, (E) AF1000L, and (G) AF1000L covered by the derived Schwann cells. Magnified views of the neurite fields marked by red boxes in (A), (C), (E), and (G) are shown in (B), (D), (F), and (H), respectively. The neurites were stained with Tuj1 marker (green) and the nuclei were stained with DAPI (blue). The boundary of the DRG body in each field was labeled with black ellipses.

Figure 7.

Analyses of (A) the average neurite lengths, (B) the longest neurite lengths, and (C) the eccentricities of the neurites outgrowing from the chick DRG bodies cultured on different types of scaffolds: laminin-coated TCP, TCP covered by the derived Schwann cells (TCP-SC), AF1000L, and AF1000L covered by the derived Schwann cells (AF1000L-SC). * $P < 0.05$ and ** $P < 0.01$ as compared with that for chick DRG bodies cultured on laminin-coated TCP.