Characterization of Schwann Cells in Self-Assembled Sheets from Thermoresponsive Substrates

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Schwann cells are the vital glial cells in the development and regeneration of the peripheral nervous system (PNS). Recently, Schwann cell transplantation has emerged as one of the attractive candidates in treating demyelinating diseases resulting from the PNS and central nervous system injuries. Schwann cells are usually injected as cell suspensions or transplanted after being seeded on extracellular matrix proteins or biodegradable polymeric scaffolds. In these approaches, the adherens junctions between Schwann cells present *in vivo* are not readily replicated as Schwann cells dispersed as individual cells. Here we describe a procedure to grow a large amount of Schwann cells in a sheet architecture that can be either transplanted or injected and provide some insights into the influence of a sheet-like cell organization on the function of Schwann cells, including their viability, proliferation, alignment, and migration. The Schwann cell sheet was successfully generated through coating the culture plate surfaces by layer-by-layer self-assembly of the thermoresponsive polymer poly-(*N*-isopropylacrylamide) (PNIPAAm). Further characterization of the Schwann cell sheet showed that Schwann cells in sheet were highly viable, but maintained a lower proliferation rate than individual Schwann cells. The levels of nerve growth factor and glial cell-derived neurotrophic factor were also maintained in Schwann cell sheets. The protein level of a cyclin-dependent kinase inhibitor, p27, was upregulated in the Schwann cell sheet. Both alignment with axon-like nanofibers and migration of Schwann cells are not significantly different between Schwann cells in a sheet-like organization and as individual cells. We conclude that Schwann cell sheet engineering presents a promising method for cell-based nerve injury therapy, as well as a model to study the control of Schwann cell proliferation in response to intercellular organization.

Introduction

SCHWANN CELLS PLAY an important role in the development, maintenance, and repair of the peripheral nervous system (PNS) and in nerve regeneration after injury by differentiation, migration, and proliferation.^{1,2} Schwann cell transplantation is also a potential therapy for spinal cord injury, in guiding both regrowth and remyelination of the damaged axons.^{1,3} Schwann cells also release growth factors, cell adhesion molecules, and extracellular matrix (ECM) proteins, which could stimulate and support axonal growth,^{2,4} including neurotrophins, such as nerve growth factor (NGF) and glial cell-derived neurotrophic factor (GDNF).^{5,6} Therefore, several nerve tissue-engineering procedures have been developed to tackle the issue, including enhancing the efficiency of the nerve grafting. Scaffold engineering is an example technique developed to bridge the peripheral nerve gaps after injury.^{7–9} Nonetheless, the technique inevitably requires scaffold materials that are surgically placed at the host tissue as a transient mechanical

support, resulting in delay in the integration of the tissue implant. Moreover, the scaffold must be compatible with the host tissue, and its supramolecular organization must match the ECM released by the host tissue.^{10,11}

As an alternative to the scaffold tissue engineering approaches, the cell sheet technique has been developed to form a tissue-like construct without using any scaffold materials to assist manipulation of the cultured Schwann cells. Poly-*N*-(isopropylacrylamide) (PNIPAAm)-based thermoresponsive substrates have been proven to be an effective substance to create cell sheets after detachment.^{12–14} The cell sheets harvested have been used for many tissue engineering applications, such as corneal epithelium,¹⁵ skin,¹⁶ esophageal lining,¹⁷ liver,¹⁸ periodontal ligaments,^{19,20} and myocardium.^{21,22} These applications use the deposited ECM on the basal sheet surface, which holds every cultured cell before being transferred onto another surface for grafting without trypsinization.^{10,23} Therefore, this method allows the cells that were cultured to maintain a variety of cellular proteins in the cell-to-cell and cell-to-ECM interactions that are

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indispensable to both proliferation and differentiation.²⁴ In contrast, conventional techniques such as proteolytic treatment cause degradation of cell surface proteins that are vital for the interactions.¹¹ In addition, many studies that compared isolated cell injection and cell sheet transplantation showed cell-void necrotic areas after direct cell injection, whereas the cell sheet construct exhibited homogenous tissue without observable necrosis.^{25,26} The Schwann cell sheet is therefore another promising candidate in nerve tissue regeneration.

It has been known that cell cycle exit is important for differentiation of Schwann cells.²⁷ With regard to the process, it needs the precise integration of both proliferation and regulated cell cycle exit in an appropriate manner. Schwann cell proliferation gradually decreases when the cells begin to differentiate.^{28,29} Also, at the time Schwann cells initiate myelination, they have exited the cell cycle and entered a quiescent state through regulation of many cyclin-dependent kinases (CDKs), which coordinate the progression of cell cycle via cyclins, the regulatory subunits.^{2,30-32} Meanwhile, negative regulation of the cell cycle takes place by the Cip/Kip proteins (p21, p27, and p57) and Ink family of protein (p16, p18, and p19), which acts as specific inhibitors of the cyclin-CDK enzyme complex.³³⁻³⁵ Previous studies have indicated that both CDK2 and inhibitor protein p27 are involved in the G1-S progression in glial cells.^{36,37} In addition, the CDK2 protein levels declined in contact-inhibited confluent Schwann cells, whereas p27 levels increased.³⁸ These discoveries proved the importance of p27 in regulating the cell cycle of Schwann cells, including differentiation activities.

Further, Schwann cells play another role in regulating the growth of axons during regeneration and presumably during development.⁴ N-cadherin was reported to increase during those activities and also mediate adhesion of Schwann cells to neurites and other Schwann cells.³⁹ N-cadherin and associated catenins (proteins that mediate adhesion at adherens junctions) were also proved to be associated with peripheral nerve migration and regeneration,^{40,41} especially for N-cadherin that is directly involved in the axon-to-axon and axon-to-Schwann cell attachment.⁴¹ E-cadherin, its cytoplasmic partners called catenins, and F-actin are the key components of adherens junctions in Schwann cells during myelination to help sustain the growth and function of compact myelin layers.⁴² Also, it was evident that N-cadherin was related to the formation of the Schwann cell-Schwann cell junctions in alignment with axons in mammals.⁴³ The alignment of Schwann cells has been known to induce oriented axonal growth as a result of classical contact guidance through the oriented nanofiber.⁴⁴ Also, another report found that a large number of Schwann cells are needed to fill injury-induced cystic cavities, especially if nerve damages are severe.³ Therefore, if we transplant many Schwann cells that maintain the cell-cell adhesion proteins and have already released their ECMs, those cells might be more advantageous in nerve regeneration than individual cells those obtained from trypsinization.

To characterize Schwann cells assembled in the form of a cell sheet, we used cell sheet engineering on Schwann cells cultured on the surface of polystyrene tissue plates coated by PNIPAAm and other thermoresponsive substrates by a layer-by-layer self-assembly technique. Then, the cell sheet was characterized to study proliferation, attachment, expression

of cellular proteins, profile of neurotrophins, as well as migration. Those fundamental behaviors of cells in the assembled form are considered as critical elements in proliferation and differentiation, as well as tissue regeneration, especially for cell-based therapy in nerve injuries.

Materials and Methods

Preparation of temperature-responsive surfaces

A layer-by-layer technique was used to create temperature-responsive thin films.²⁴ First, a primary layer of polyethylene imine (PEI, 0.5 mg/mL; Sigma) at pH 4.0 was deposited on the surface by transferring the solution directly into the plate and left overnight. After that, excess PEI was removed and washed with deionized water and waited until it was completely dry. The PEI as the primary layer acted as the positive surface charge necessary for buildup of the next layer. Tannic acid (2 mg/mL; Sigma) was dissolved in deionized water, and pH was adjusted to 9.0. Tannic acid was then deposited on the PEI layer for 10 min. The tannic acid being negatively charged interacts with the positively charged PEI and further introduces the surface negative charges for attachment of PNIPAAm via electrostatic interactions as well as hydrogen bonding interaction between them. Then, the tannic acid was removed, and the surface was washed thoroughly with deionized water, followed by deposition of PNIPAAm (2 mg/mL; Sigma), pH 2.0, for 10 min. Afterward, the PNIPAAm was removed and washed by deionized water again. The temperature-responsive well plate was dried at the room temperature and should not be stored for more than 2 days before use.²⁴

Cell culture

Rat Schwann cells (ATCC; CRL 2941) were cultured in a medium containing a 1:1 mixture of Ham's F12 medium (Gibco) and Dulbecco's modified Eagle's medium low-glucose (Cellgro) supplemented with an antibiotic solution (1% penicillin-streptomycin; Sigma) and 10% fetal bovine serum (Atlanta Biologicals). The cells were maintained in a 37°C incubator with 5% CO₂ and a relative humidity of 95%. The medium was changed every 3 days.

Cell seeding on temperature-responsive surfaces

Rat Schwann cells were trypsinized and seeded onto the surface of well plates coated with a temperature-responsive polymer, and then added with the medium. Each well plate was seeded with 100,000 cells in 2 mL medium. The cells were allowed to attach, and the medium was changed every alternate day.

Harvesting cell sheet

The plate was incubated at 4°C for 2 h. Lowering temperature tended to increase the hydrophilicity of the multi-layer construct that causes the presence of a water interface between the cells and well plate surface coated by the polymer, leading to detachment of the cell sheet from the surface. Schwann cell sheets could be manipulated by using cleaned forceps, pipetting by a large-orifice pipette, or placing a hard and flat surface material, such as a scaffold or cover glass, under the cell sheet and then lift the entire cell sheet and place onto another well plate.

FIG. 1. Optical microscopic images of a Schwann cell sheet: **(A)** Actual size after detachment (1-cm scale); **(B)** 1× microscopic image; **(C)** 63× images of stained Schwann cell sheet; **(C1)** Phalloidin, **(C2)** DAPI, and **(C3)** merged. Schwann cells in the sheet structure were obviously compacted and overlapped together; **(D)** Z-stack images of Schwann cells and Schwann cell sheet stained with S-100 (shown in green). **(D1)** The thickness of confluent Schwann cells on a regular culture plate was around 8–12 μm. **(D2)** The Schwann cell sheet after detachment was around 30-μm thick. The data suggested that Schwann cell sheets were multilayered. Color images available online at www.liebertpub.com/tea

Live–dead cell assay

Schwann cell sheets were studied by fluorescent staining according to the protocol described in the Live–Dead Reduced Biohazard Viability/Cytotoxicity kit (Invitrogen). The test was based on different permeability of live and dead cells to two fluorescent stains in the kit. After that, the cell sheet already stained was imaged by a fluorescence microscope.

Quantification of cell numbers on a cell sheet

After the cell sheet was detached, one cell sheet was counted by resuspending in phosphate-buffered saline (PBS) several

times to break the sheet into lots of small fragments followed by centrifugation at 1000 rpm to separate the cell fragment. Then, the supernatant was discarded and followed by trypsinization and counting the cell number by a hemocytometer.

Western blot analysis

Reagents. The bicinchoninic acid (BCA) protein assay kit was from Thermo Scientific. Antibodies against Beta-Catenin, E-Cadherin, N-Cadherin, and Pan- Cadherin, CDK2, Cyclin A, Cyclin E, and p27 were from Cell Signaling. Anti-actin antibody was from Sigma-Aldrich. Stabilized peroxidase-

FIG. 2. Microscopic images (×20) of primary antibody S-100 stained on **(A)** Schwann cells seeded on a regular well plate; **(B)** Schwann cells on a thermoresponsive substrate surface. The samples were then incubated with Alexa Fluor 488-conjugated secondary antibodies (shown in green). S-100 was highly expressed in the Schwann cell sheet, suggesting that thermoresponsive substrates in the study did not affect phenotypic expression of Schwann cells. Color images available online at www.liebertpub.com/tea

FIG. 3. Live-Dead Reduced Biohazard Viability/Cytotoxicity kit images: **(A)** Live-Dead image of Schwann cells seeded on a tissue well plate; **(B)** Live-Dead image of a Schwann cell sheet after detachment. Both images showed a significantly higher number of live cells (fluorescent green) than dead cells (fluorescent red). This result indicated that the thermoresponsive substrates and incubation in 4°C for 2 h to detach the cell sheet did not harm the cells. Color images available online at www.liebertpub.com/tea

conjugated monoclonal and polyclonal antibodies against the primary antibodies were from Thermo scientific, including Super Signal West Femto as the chemiluminescence reagent. Films were from Kodak Biomax XAR Films.

Procedures. One cell sheet was transferred into a microcentrifuge tube and then added by a lysis solution composed of 10 µg/mL of aprotinin, 10 µg/mL of leupeptin, 1 mM of phenylmethyl sulfonyl fluoride, and 1 mM of sodium orthovanadate. The cell sheet was lysed by a sonicator (Misonix) and centrifuged at 13,000 rpm for 10 min. As the protein concentration of each sample might be different from each other, the sample was normalized by using BCA protein assay. Each sample was measured for an absorbance value at 562 nm by the Spectracount Chemireader (Packard). The protein concentration was then determined from the standard curve established by standard bovine serum albumin (BSA) solutions with gradient concentrations. The protein concentration of each sample was adjusted by diluting those samples with a lysis solution to obtain an equal amount of protein in every sample. After normalization, the samples were mixed with 4× loading buffer with a 3:1 ratio, and beta-mercaptoethanol to obtain a final ratio of 5%. Further, anti-actin was used as a loading control.

The samples (15–50 µg/mL of protein amount) were loaded into 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel in a running buffer at 120 V for 2.5 h in ice, followed by transferring the protein onto a polyvinylidene fluoride membrane by a transfer kit (Biorad) at 100 V for an hour in 4°C. The membrane was blocked by 2% BSA solution for 2.5 h and incubated with a primary antibody at least 16 h in 4°C followed by a horseradish peroxidase-conjugated secondary antibody matching to the primary antibody. Finally, the protein was reacted with the chemiluminescence reagent for 5 min before developing the film.

RNA extraction and reverse transcription–polymerase chain reaction assay

To examine expression of the neurotrophin-related genes in Schwann cell sheets, reverse transcription–polymerase chain reaction (RT-PCR) was performed. Briefly, total RNA from Schwann cell sheets cultured for 24 h and the control group was extracted with commercial Trizol reagent (Invitrogen). One microgram of total RNA was reverse transcribed with reverse transcriptase (Promega) as per the manufacturer's instruction. All PCR experiments were then

performed with *Taq* polymerase (Promega). The following primer pairs were used: *NGF* (sense, 5'-GCCCCACTGGAC TAAACTTCAGC-3'; antisense, 5'-CCGTGGCTGTGGTCTT ATCTC-3'; 349 bp), *GDNF* (sense, 5'-ATGAAGTTATGGGAT GTCGT-3'; antisense, 5'-CAGGGTCAGATACATCCACA-3'; 631 bp), and rat *β-actin* (sense, 5'-ATTGTAACCAACTGG GACG-3'; antisense, 5'-TTGCCGATAGTGATGACCT-3'; 533 bp) as the housekeeping gene control. As for the PCR condition, initial denaturation was at 94°C for 5 min, then followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 1 min, and polymerization at 72°C for 1 min, and then a final 10-min extension at 72°C. The PCR products were separated and visualized on a 1.5% agarose gel containing 5 g/L ethidium bromide.

Coomassie Blue staining

The gel was stained with 0.1% Coomassie Blue R-250 in methanol:conc. acetic acid:water=4:1:5 for 1 h, followed by destaining the gel with a destaining solution (methanol:conc. acetic acid:water=4:1:5) for 2 h.

Preparation of electrospun polycaprolactone–chitosan nanofibers

Eight percent polycaprolactone (PCL; Sigma) in hexafluoroisopropanol (1,1,1,3,3,3-hexafluoro-2-propanol) and 0.8% chitosan in hexafluoroisopropanol (1,1,1,3,3,3-hexafluoro-2-propanol) were prepared as a stock solution. Then, both solutions were mixed together in a ratio of 10:1 (v/v). The condition of electrospinning was fixed at a 10-cm

FIG. 4. Polymerase chain reaction (PCR) analysis of Schwann cell sheets and Schwann cells on a regular culture plate as the control group. Both samples were cultured for 1 day before extracting RNA. Primers of nerve growth factor (*NGF*) and glial cell-derived neurotrophic factor (*GDNF*) were tested with the RNA by the reverse transcription–PCR assay. *Rat beta-actin* was used as a loading control. The result indicated that the Schwann cell sheets produced both neurotrophins as Schwann cells in the control group.

FIG. 5. Proliferation rate of individual Schwann cells on tissue culture plates on days 1–4 (blue) and those on the plates coated with the thermoresponsive substrates (green): 100,000 cells were cultured on day 0, and the cell number was counted on days 1–4 by a hemocytometer. The cells were confluent enough to harvest as a cell sheet on day 4 after detachment. The result indicated that the proliferation rate of Schwann cells (green) was significantly lower than the one in the control group (blue) due to the impact of thermoresponsive substrates. Color images available online at www.liebertpub.com/tea

working distance, 15 KV, and a flow rate of 10 μ L/min. Finally, nanofibers on a 1.5 $\times$ 1.5-cm cover glass slip were obtained and stored in the desiccator at room temperature.

Cell sheet transfer to the PCL–chitosan fibers

After detachment, the cell sheet was manipulated by gently placing a coverslip electrospun by PCL–chitosan fibers under the cell sheet and then transferring the coverslip with the whole cell sheet onto another well plate. After that, a sterile ring was put on top to hold the fiber in place before

gently filling the well plate with the medium. The cell sheet was allowed to reattach to the surface of cover glass placed under the cell sheet in a 37°C incubator.

Fluorescence staining of cell sheet

The cell sheet was fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 at room temperature for 3 min, and then blocked by 2% BSA in PBS with 0.1% azide solution for 2 h. The cell sheet was then stained with 10 μ M Hoechst dye (DAPI). In addition, 10 μ L of

FIG. 6. (A) A sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel after being stained with 0.1% Coomassie Blue solution showed similarity of two samples prepared from a Schwann cell lysate; (B) western blotting data showing the intensity of specific bands related to cell cycle markers, namely, p27, cyclin-dependent kinase 2 (CDK2), cyclin A, cyclin E, and actin as loading control respectively. The western blotting analysis reveals that p27 was extremely upregulated during the process of forming a Schwann cell sheet. Color images available online at www.liebertpub.com/tea

FIG. 7. Western blotting analysis that compared the cell-cell interaction proteins, Pan-cadherin, N-cadherin, Beta-catenin, E-cadherin, and Actin of individual Schwann cells (left) and Schwann cell sheet (right). Both samples were prepared from the cells that were cultured in the same period of time. The western blotting analysis revealed that the cadherins of the Schwann cell sheet after detachment were similar to those of individual Schwann cells. In other words, the process of generating cell sheets still maintained a high level of cadherins upregulated during the cell culture and important to many tissue engineering applications. Color images available online at www.liebertpub.com/tea

phalloidin (200 U/mL) in 1 mL of PBS/azide was used for actin staining. As for antibody S-100 staining, the sample was incubated with the primary antibody at room temperature for 2 h, rinsed with PBS, and followed by incubation with Alexa Flour 488-conjugated secondary antibody (Invitrogen) for an hour. The sample was analyzed using a Zeiss LSM 5 Pascal confocal microscope.

Statistical analysis

Statistical differences between two groups were determined by performing a Student's *t*-test. All the experiments were run in triplicate per sample type.

Results and Discussions

Schwann cells that detach and form a cell sheet are highly viable

In the study, the process was initially optimized to determine the most appropriate parameters for forming a Schwann cell sheet by a layer-by-layer technique. The key parameters

were composed of concentration of PEI, tannic acid, and poly(N-isopropylacrylamide) (PIAAM) (mg/mL), pH, the initial number of Schwann cells seeding on well plates, incubation, and harvesting period (shown in the Materials and Methods section). Those parameters are needed for Schwann cells to attach and detach properly by the effect of substrates, thus forming a Schwann cell sheet that is confluent enough to be manipulated. The PIAAM-coating solution was freshly prepared to prevent oxidation before spreading on the plate. Schwann cells were subsequently seeded and incubated until day 4. When the culture plate was put in a refrigerator to lower its temperature to 4°C (LCST) for 2 h, those cells were detached and formed as a sheet (Fig. 1A, B). The cell sheet harvested would tightly pack together (Fig. 1C). Further, we found that the cell sheet was multilayered, and its thickness was around 30 µm (Fig. D2), compared to the Schwann cells seeded on a regular culture plate that were 8–12-µm thick (Fig. D1). In addition, we stained the cell sheet with antibody S-100 (in green) to identify the phenotype of Schwann cells (Fig. 2A, B). We found that there was no significant difference of S-100 expression of the cells on the plates coated with thermoresponsive substrates and those on regular culture plates. The data suggested that thermoresponsive substrates used to create cell sheets did not affect Schwann cell phenotypic expression. The self-assembling property possessed by Schwann cells therefore allowed us to transfer the cell sheet without trypsinization by a large-orifice pipette, coverslip, or manipulator.

Next, to determine whether Schwann cells in a tightly packed sheet-like structure are viable, we stained the Schwann cell sheet with the Live-Dead Reduced Biohazard Viability/Cytotoxicity kit. As shown in Figure 3B, the number of live cells in green is much higher than that of dead cells in red (Fig. 3B). Also, there was no significant difference in Schwann cells seeded on a culture plate as the control group (Fig. 3A). We therefore concluded that the cell sheet organization and the procedure of cell sheet preparation did not have adverse effects on the health of Schwann cells.

Schwann cell sheets maintained secretion of some neurotrophins

One of the key factors of cell-based therapy in nerve regeneration is the ability of Schwann cells to release a variety of neurotrophic factors. Therefore, it is not unusual to find that the role of those molecules in the maintenance and survival of peripheral nerves has become the subject of numerous studies.^{45–48} To find out whether Schwann cells tightly packed in the sheet structure was able to release neurotrophic molecules, RT-PCR analysis was performed to test the presence of two main neurotrophins: NGF and GDNF. The result indicated that both neurotrophins were produced from the Schwann cell sheet and individual cultures (Fig. 4). Also, there was no significant difference in the expression of neurotrophins in both samples (Fig. 4). We therefore concluded that the Schwann cells in the study still possess the ability to release the neurotrophins, and the thermoresponsive substrates that induced self-assembling of Schwann cells and caused tightly-packed cells did not alter the level of neurotrophins. In other words, Schwann cell sheets still maintain the high amount of neurotrophins that are one of the crucial factors in supporting nerve regeneration.

FIG. 8. (A) Schwann cells on nanofibers stained with DAPI/Phalloidin (0.10-mm scale); (B) a phase-contrast image of the Schwann cell sheet on nanofibers after 48 h ($\times 10$) and (C) $\times 40$. The result showed that individual Schwann cells and those in the sheet form performed cell migration through nanofibers that would assist the cells to migrate to the target location if the cell sheet is placed for nerve tissue regeneration. Color images available online at www.liebertpub.com/tea

Schwann cells in the sheet structure demonstrate decreased proliferation and elevated level of p27 CDK inhibitor

Next, we studied the influence of the cell sheet structure on Schwann cell proliferation. As shown in Figure 5, the number of Schwann cells in the sheet-like structure was consistently less than that as individual cells during the course of culture (Fig. 5).

To investigate whether proteins that control cell cycle might play a role in the decreased proliferation of Schwann cells in the sheet-like structure, we examined the expression of selective proteins, including CDK inhibitor (CKI) p27, cyclin A, cyclin E, CDK2, and actin as loading control. The cell lysates were obtained from a Schwann cell sheet and individual Schwann cells as a control. The samples were run on two SDS-PAGE gels simultaneously. One was stained with 0.1% Coomassie Blue solution and washed by a destaining solution several times (Fig. 6A) to test the accuracy of protein loading. The other gel was used for a western blotting assay. The result indicated that the level of p27 was extremely elevated compared to that of the control group. However, the levels of CDK2, cyclin A, and cyclin E were not as significantly altered (Fig. 6B). These data suggest that the cell sheet organization decreased Schwann cell proliferation and increased the cellular amount of p27, an inhibitor of cell cycle progression.

If the Schwann cells in the cell sheet lose contact inhibition, they may pose the risk of tumorigenesis for *in vivo* applications. The contact inhibition is regarded as a key mechanism that arrests cell division when cells reach a high density.⁴⁹ In case of Schwann cells, there was a study of the relationship of CDK and CKI in the cell cycles of Schwann cells, indicating that once p27 was upregulated, it would suppress cell proliferation and induce the cells into a quiescent stage.³⁸ More interestingly, the elevated p27 was proved to be a positive marker of Schwann cell differentiation.⁵⁰ For our study, p27

in the Schwann cell sheets was elevated, causing lower proliferation than the control group. The data suggested that Schwann cells in the sheet tend to exit the cell cycle and were subject to a contact inhibition stage. We therefore conclude that the Schwann cell sheets might have a lower tendency of tumor formation. Moreover, there was no documented evidence that transplantation of cell sheets causes tumor formation, even though a lot of cells were grafted in the damaged tissues. For example, a myocardium tissue was constructed from 6 layers of cell sheets, and it could be successfully transplanted *in vivo* into the tissue without tumor formation.⁵¹ However, even though there have been several examples of clinical success of using cell sheets, the cell sheet from Schwann cells has not been used clinically for nerve regeneration. Further investigations, especially *in vivo* studies, are still needed to ensure that the Schwann cell sheets are safe and can be used as an alternative nerve regeneration candidate in the future.

Schwann cells in the sheet structure and as individual cells maintained comparable level of selective cadherins

To investigate whether Schwann cells in cell sheet have altered expression of cell adhesion proteins, we examine the amount of Pan-cadherin, N-cadherin, β -catenin, and E-cadherin in Schwann cells in sheet or as individual cells. There was a comparable level of these cadherins in Schwann cells in the cell sheet or as individual cells (Fig. 7), suggesting that expression of cadherins is not influenced by the cell sheet structure or the procedure.

Schwann cells in the sheet structure migrate along nanofibers

One of the most important functions of Schwann cells in the PNS is traveling along the axons. We questioned whether tightly packed Schwann cells in the sheet structure are able

to align and migrate along axon-like structures. Predictably, individual Schwann cells after trypsinization were able to align along the PCL–chitosan nanofibers (Fig. 8A). Interestingly, a complete Schwann cell sheet placed on the same nanofibers resulted in the outmigration of Schwann cells along the nanofibers (Fig. 8B, C). The data indicated that Schwann cells in the sheet can be guided by axon-like structures such as nanofibers to the target location. This finding could be beneficial to future studies of differentiation of Schwann cells into myelin sheets through migration along axons, including regeneration of both the axon and myelin sheet when damaged through cell-based therapies.

Conclusions

We successfully developed an approach to create a Schwann cell sheet using temperature-responsive substrates. The Schwann cell sheet shows the same character as individual Schwann cells, except for significantly elevated p27 levels, resulting in a lower proliferation rate than individual Schwann cells. Nonetheless, the Schwann cell sheet presents high viability and still maintains the cell–cell interaction indicated by high expression of Cadherins as well as the level of neurotrophins. We therefore conclude that cell sheet engineering is a promising method for treating nerve injuries through grafting Schwann cells in the sheet architecture. More interestingly, the Schwann cell sheet can be used as a model to study the role of the cell–cell interaction in the behavior of Schwann cells in the absence of the extrinsic ECM.

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Disclosure Statement

No competing financial interests exist.

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