ORIGINAL

Effective *in vitro* differentiation of adipose-derived stem cells into Schwann-like cells with folic acid supplementation

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Abstract : Peripheral nerve injury (PNI) after pelvic surgery is a common issue with a significant impact on patients. Autologous nerve grafting is the gold standard treatment for PNI, but this technique cannot be applied to fine nerve fibers in the pelvis. Schwann-like cell (SLC) differentiation is a novel therapeutic strategy for this clinical condition. However, the efficiency of SLC differentiation remains unsatisfactory. We modified an SLC differentiation protocol using adipose-derived stem cells (ADSCs) and folic acid. Morphology, gene expression and secretion of neurotrophic factors were examined to assess the differentiation quality and phenotypic characteristics. Our new modified protocol effectively induced a Schwann cell (SC) phenotype in ADSCs as assessed by morphology and expression of SC markers [S100 calcium-binding protein B (*S100B*), $P<0.01$; p75 neurotrophic receptor (*p75NTR*), $P<0.05$]. SLCs produced by the new protocol displayed a repair phenotype with decreased expression of *ERBB2* and early growth response protein 2 (*EGR2*)/KROX20 ($P<0.01$). Furthermore, our new protocol enhanced both mRNA expression and secretion of nerve growth factors by SLCs ($P<0.01$). This protocol enhanced the SC characteristics and functions of ADSC-derived SLCs. This promising protocol requires further research and may contribute to SC-based nerve regeneration. J. Med. Invest. 68 : 347-353, August, 2021

Keywords **:** *Adipose-derived stem cell, Schwann-like cells, Folic acid, Nerve growth factor, Regenerative medicine*

INTRODUCTION

Peripheral nerve injury (PNI) is a common issue that affects more than 1 million people every year worldwide and causes a significant degree of pain in the affected individuals (1). Autologous nerve transplantation is currently the best method for treating PNI, but its application is limited owing to the scarcity of donors and the risk of neuroma formation and other complications due to a second operation (2). Moreover, PNI after pelvic surgery needs to be addressed to improve patients' quality of life (3). Nerve transplantation is one option ; however, injured nerve fibers are generally fine and complex. Therefore, another strategy is required.

Cell transplantation is another option that has progressively gained popularity. Schwann cells (SCs) contribute to the removal of damaged myelin, secretion of nerve growth factors (NGFs) and the formation of new myelin. These properties are beneficial for the regeneration of peripheral nerves (4, 5). However, owing to limitations in SC separation, purification and proliferation technology, there is an urgent requirement for abundant alternative cells with lower immunogenicity. Regenerative medicine has recently been applied to solve these issues (6). Adipose-derived stem cells (ADSCs) have been focused on because of their multipotency and ease of harvest. We have investigated ADSC multipotency and reported scientific evidence of insulin producing cells (IPCs) for clinical applications (7-11). Thus, we hypothesized that ADSCs can also self-renew and differentiate into ectodermal lineage cells, including Schwann-like cells (SLCs). The most commonly used differentiation protocol for producing SLCs from ADSCs has been described by Kingham *et al*. (12). However, this method is still not ideal, considering the scope for further improvement with regard to the secretion of neurotrophic factors by differentiated SLCs. Thus, it is imperative to find methods that will ensure the optimal performance of SLCs.

Folic acid (FA) is an inexpensive and safe substance with regulatory properties in various cells in the nervous system. It can promote chemotactic responses in glial cells, induce neurotrophin release and stimulate neuronal differentiation (13). FA can effectively promote growth, repair and recovery of damaged adult central nervous system (CNS) (14), provide neuroprotection and promote nerve regeneration after ischemic injury (15). The FA/Dihydrofolate reductase (DHFR)/AMP-activated protein kinase (AMPK)α axis improves survival and induces myelination of oligodendrocytes, and thus, enhances the maturity of oligodendrocytes *in vivo* and *in vitro* (16). FA supplementation has been shown to be effective in PNI treatment (17). A recent study on a rat PNI model has demonstrated that FA promoted proliferation and migration of SCs and secretion of NGF to improve the repair of peripheral nerve damage (18). SCs transform into a repair phenotype at the site of the injured nerve to promote nerve regeneration (6). Then, NGFs promote autophagy of SCs through the p75 neurotrophin receptor (p75NTR)/AMPK/mechanistic target of rapamycin (mTOR) signaling pathway to accelerate removal and phagocytosis of myelin debris and promote the regeneration of axons and myelin in the early stages of PNI (19). The close relationship between FA and NGFs indicates they play a crucial role in SC-mediated nerve regeneration. However, there is no research on the application of FA in the differentiation process of SLC from stem cells. And the appropriate concentration of FA also needs to be investigated.

Our new modified protocol effectively induced an SC phenotype in ADSCs as assessed by morphology and expression of SC markers. Furthermore, new generated SLCs display a repair phenotype as decreases of Erb-B2 receptor tyrosine kinase 2 (*ERBB2*) and early growth response 2 (*EGR2*) gene expression,

Received for publication March 1, 2021 ; accepted August 19, 2021.

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and show enhanced secretion capacity of NGFs. This promising new protocol requires further research for SC-mediated nerve regeneration.

MATERIALS AND METHODS

Cell Culturing and Differentiation Protocols

Human ADSCs were purchased from Invitrogen (Grand Island, NY, USA) and cultured in ADSC basal medium with added growth supplement (Gibco, Carlsbad, CA, USA) and GlutaMAX-I (Gibco). Primary human SCs (ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured in 6-well flat-bottom plates precoated with poly-L-lysine (2 μg/cm², P4707, Sigma-Aldrich) and maintained in complete SC medium supplemented with 5% fetal bovine serum (FBS), 1% SC growth supplement cocktail and 1% penicillin/streptomycin (ScienCell Research Laboratories) following the manufacturer's instructions. To produce SCs (50 μg/ml) group, 50 μg/ml of FA was added into the growth medium to stimulate SCs for 3 days. For inducing conventional SLCs differentiation, a three-step protocol reported by Kingham *et al*. (12) was used. Briefly, ADSCs were seeded in collagen I-coated 6-well plates (4810-010, IWAKI, Tokyo, Japan), cultured in α-modified Eagle's medium (α-MEM, Gibco) without FBS (Gibco) and treated with 1 mM β-mercaptoethanol (β-ME, 133-14571, Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) for 24 h. Next, α-MEM with 10% FBS, 35 ng/ml all-trans-retinoic acid (ATRA, 3027949, PeproTech Ltd., Cranbury, NJ, USA) were followed to treat cells for 72 h. Then, α-MEM with 10% FBS and a mixture of growth factors including 252 ng/ml glial growth factor-2 (GGF-2, 90255-B, BPS Bioscience, Inc., San Diego, CA, USA), 10 ng/ml basic fibroblast growth factor (bFGF, 100-18B, PeproTech Ltd.), 5 ng/ml platelet-derived growth factor (PDGF, 100-14B, PeproTech Ltd.) and 14 μM Forskolin (F3917, Sigma-Aldrich) were used to culture cells for 14 days with a medium change for every 3 days. For our new protocol, 50 μg/ml FA (F8758-5G, Sigma-Aldrich, St. Louis, MO, USA) was added as supplementation in the step 3 as the schematic representation shown in Fig. 1. We called these SLCs as SLCs (FA). In addition, for checking the effects of different concertation of FA on the differentiation of SLCs, 10 μg/ml and 200 μg/ml FA were also used in the step 3 of differentiation protocol and these SLCs were named as SLCs (FA, 10 μg/ml) and SLCs (FA, 200 μg/ml), respectively.

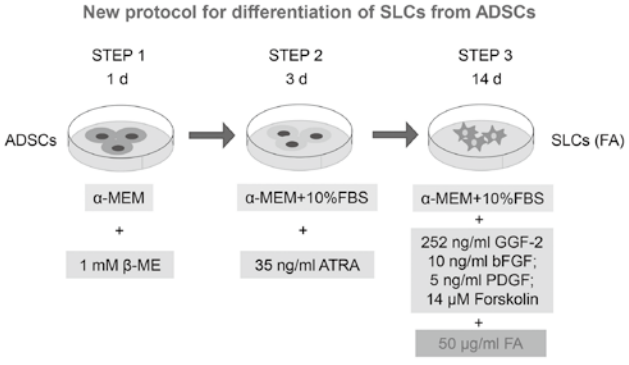

Fig 1. Schematic representation of the modified protocol for differentiating ADSCs into SLCs. adipose-derived stem cells, ADSCs ; Schwann-like cells, SLCs ; β-mercaptoethanol, β-ME ; all-trans-retinoic acid, ATRA ; basic fibroblast growth factor, bFGF ; FA, folic acid ; glial growth factor-2, GGF-2 ; platelet-derived growth factor, PDGF.

Morphological Analysis

Cells were seeded in a Nunc Lab-Tek II Chamber Slide System (154526PK, Thermo Fisher Scientific, Waltham, MA, USA) and stained with hematoxylin and eosin (H&E). Groups of cells were compared under an optical microscope.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

An RNeasy Mini Kit (Qiagen, Hilden, Germany) and a reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific) were used to extract total RNA, which was reverse transcribed to obtain cDNA. qRT-PCR analyses were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). The primers (TaqMan gene expression assays, Thermo Fisher Scientific) used were : *S100B* (Hs00902901_m1), *p75NTR* (Hs00609976_m1), *ERBB2* (Hs01001580_m1), *EGR2*/*KROX20* (Hs00166165_m1), *NGF* (Hs00171458_m1) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Hs99999905_m1). Results are presented as relative mRNA expression normalized to *GAPDH* as an internal control.

Immunofluorescence Staining

Indicated cells were digested by trypsin and seeded for 2×10^4/well in a Nunc Lab-Tek II Chamber Slide System until adhesion. After being washed twice with PBS, 4% paraformaldehyde (163-20145, FUJIFILM) was used to fix the cells at 4°C for 30 min. Next, incubated the slides with 0.1% Triton X-100 (HFH10, Thermo Fisher Scientific, Inc.) for 2 min at room temperature for permeabilization and followed with 3% bovine serum albumin (BSA) diluted from MACS BSA Stock Solution (130-091-376, Miltenyibiotec, Bergisch Gladbach, Germany) in PBS for 1 h. all slides were incubated. Then, the slides were incubated with an Anti-S100 beta antibody (ab52642, 1 : 100, Abcam, Cambridge, UK) or Anti-p75NTR (sc-271708, 1 : 50, Santa Cruz Biotechnology, Inc. Paso Robles, Ca, USA) at 4°C overnight. The next day, after being washed three times with PBS, Alexa Fluor 488 conjugated secondary antibodies (A-11008, 1 : 500, Thermo Fisher Scientific, Inc.) was used to incubate slides for S100B and Alexa Fluor 594 conjugated secondary antibodies (A-11005, 1 : 800, Thermo Fisher Scientific, Inc.) was for p75NTR, for 1 h at 37°C in the dark. Then, slides were washed with PBS for three times protected from light and stained the nuclei by ProLong™ Gold Antifade Mountant with DAPI (P36931, Thermo Fisher Scientific, Inc.) for 10 min in the dark. A fluorescence microscope (BZ-X700 KEYENCE, Tokyo, Japan) was used to observe and photograph the slides. As for negative controls, the primary antibody was replaced with BSA.

Enzyme-Linked Immunosorbent Assay (ELISA)

Human NGF ELISA kits (BEK-2212-1P/2P, Biosensis, Thebarton, Australia) were used to assess NGF secretion in the supernatant of different groups of cells following the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader and the concentration of NGF was calculated using a standard curve. Relative secretion capacity was normalized to the cell number.

Statistical Analysis

For statistical analysis and plotting, we used Prism 7.0 software (GraphPad, San Diego, CA, USA). Data are expressed as the mean ± standard deviation. Comparisons between two groups were evaluated using Mann-Whitney U test. Differences among multiple groups were tested using one way analysis of variance (ANOVA) followed by Tukey's post hoc test. All experiments were performed in triplicate. All P-values are two-sided

and P<0.05 was considered significant (*P<0.05 ; **P<0.01).

RESULTS

Improved protocol for generating SLCs

We searched the literature for methods to enhance differentiation and function of SCs or SLCs using small molecules (Table 1) (13, 18, 20-26). FA was identified as a small molecule that may promote NGF secretion by primary SCs. Secretion of neurotrophic factors is a crucial function of SCs for promoting nerve regeneration, hence we hypothesized that FA would be an inexpensive, convenient and promising candidate for enhancing the function of SLCs. Thus, we modified our protocol by adding 50 μg/ml FA supplementation and generated SLCs (FA) (Fig. 1).

New protocol effectively induces the SC phenotype in ADSCs

Undifferentiated ADSCs grew in clusters and displayed a rounded fibroblast-like shape. In contrast, SCs exhibited a classic spindle-shaped morphology with a higher aspect ratio. SLCs (FA) became morphologically similar to SCs after 18 days of differentiation, exhibiting small and elongated spindle shapes with a bipolar or tripolar structure (Fig. 2A).

New protocol enhances the SC characteristics of generated SLCs

The generated SLCs (FA) cultured with the new protocol displayed significant upregulation of the SC markers, *S100B* and *p75NTR* (P<0.05 and P<0.01, respectively, Mann-Whitney U test, Fig. 2B) compared with ADSCs, suggesting that the improved protocol effectively induced the SC phenotype in SLCs derived from ADSCs. Moreover, the immunofluorescence staining results showed that ADSCs had an extremely low expression of S100B and a moderate expression of p75NTR, while both high level of S100B and p75NTR were observed in SCs (Fig. 2C). SLCs (FA) generated by new protocol showed significantly up-regulated levels of S100B and p75NTR, indicating that SLCs (FA) were similar in phenotype to SCs (Fig. 2C). Taken together, a large proportion of ADSCs were differentiated into SLCs (FA) with SC characteristics through our modified protocol.

SLCs produced by the new protocol display a repair phenotype

ERBB2 is a subtype of Neuregulin (NRG) receptor that can activate downstream signal transduction and facilitate the myelinating phenotype of SCs (27). EGR2 is also a marker of myelinating SCs that is downregulated in the repair phenotype and upregulated in the re-myelinating phenotype of SCs (28). Next, we checked the effect of different concentration of FA supplementation on the phenotypic characteristics of SLCs. Compared with conventional SLCs, SLCs (FA) generated by our new protocol with adding 50 μg/ml FA in the step 3 displayed lower mRNA expression of both *ERBB2* and *EGR2* (P<0.01, Mann-Whitney U test, Fig. 3).

New protocol enhances the mRNA expression and the secretion of NGF from SLCs

PCR analysis showed that SLCs (FA) generated using the new protocol had the highest level of *NGF* mRNA than that in ADSCs, primary SCs and other groups of SLCs (P<0.01, one way-ANOVA, Fig. 4A). Furthermore, we collected supernatants from the above groups to compare NGF expression at the protein level. The new protocol significantly increased the NGF secretion capacity of SLCs (FA) compared with conventional SLCs (40.380 ± 0.803 pg/ml vs. 19.056 ± 0.580 pg/ml, P<0.01, one way-ANOVA). In primary SCs, after stimulation with FA for 3 days, the NGF secretion also increased (12.461 ± 0.103 pg/ml vs. 49.115 ± 1.854 pg/ml, P<0.01, one way-ANOVA).

Table 1. Summary of activators for promoting SC or SLC functions

Activator	Functions of SCs or SLCs	Feasible	Ref.
Folic acid (100 μg/ml)	▪ promotes proliferation ▪ promotes migration ▪ promotes NGF secretion ▪ promotes NT3/NT4/5 secretion	High	Kim GB, *et al*. (13) Kang WB, *et al*. (18)
Carboxymethylated chitosan (200 μg/ml)	▪ promotes proliferation via the activation of Wnt/β-catenin signaling ▪ induces biosynthesis of NGF	Moderate	Tao HY, *et al*. (20)
Lithium (30 nM)	▪ promotes proliferation ▪ suppresses migration	Moderate	Gu XK, *et al*. (21)
Matrix metalloproteinase-7 (10 nM)	▪ promotes migration ▪ no effect on proliferation ▪ promotes SCs move from the proximal nerve segment and myelin sheath formation	Moderate	Wang HK, *et al*. (22)
Melatonin (1 nM)	▪ promotes proliferation via ERK1/2 pathway	Moderate	Chang HM, *et al*. (23)
M2 receptor agonist, Arecaidine Propargyl Ester (100μM)	▪ increases NGF production and maturation ▪ decreases proliferation and migration ▪ increases SC marker and myelinating marker expression ▪ enhances changes in morphology	Side effect	Piovesana R, *et al*. (24, 25)
Immunoglobulin (20 mg/ml)	▪ reduces SC proliferation rates and accelerates growth of cellular protrusions ▪ stimulates SCs maturation. ▪ boosts myelin gene expression and myelination-related signaling pathways ▪ secretes IL-18 to promote axonal growth	Immunogenicity problemz	Tzekova N, *et al*. (26)

Abbreviation : nerve growth factor, NGF ; neurotrophin, NT ; extracellular signal-regulated kinase, ERK ; interleukin 18, IL-18

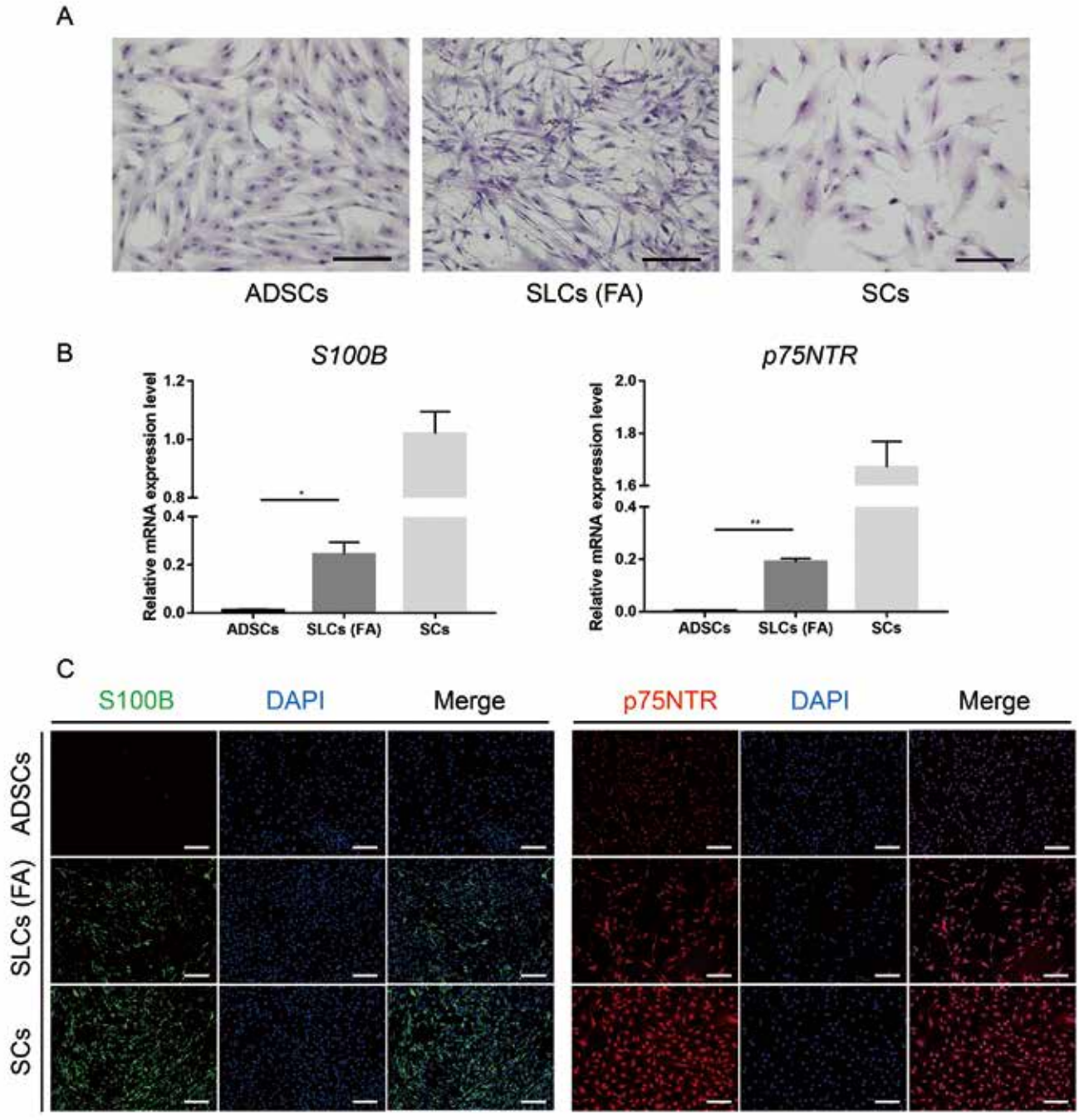

Fig 2. Morphological changes and expression of SC markers in SLCs (FA). (A) Microphotographs of H&E staining of ADSCs, SLCs (FA) and primary SCs (scale bar, 100 μm). (B) Relative mRNA expression of *S100B* (left) and *p75NTR* (right) in ADSCs, SLCs (FA) and primary SCs. (C) Confocal images showing the immunofluorescence staining for S100B and p75NTR in ADSCs, SLCs (FA) and primary SCs (scale bar, 200 μm). *P<0.05 ; **P<0.01, Mann-Whitney U test. adipose-derived stem cells, ADSCs ; Schwann cells, SCs ; Schwann-like cells, SLCs ; folic acid, FA ; S100 calcium-binding protein B, *S100B* ; p75 neurotrophin receptor, *p75NTR*.

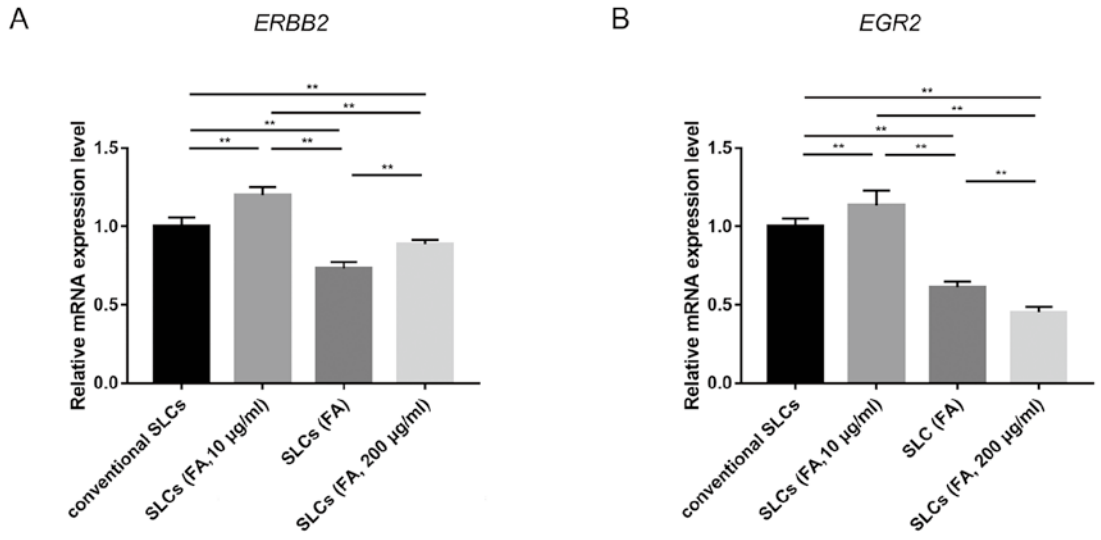

Fig 3. Relative expression of *ErbB2* and *EGR2* in different groups of SLCs. Relative mRNA expression of *ERBB2* (A) and *EGR2* (B) in conventional SLCs, SLCs (FA, 10 μg/ml), SLCs (FA) and SLCs (FA, 200 μg/ml) groups. **P<0.01, Mann-Whitney U test. adipose-derived stem cells, ADSCs ; Schwann-like cells, SLCs ; Erb-B2 receptor tyrosine kinase 2, *ERBB2* ; early growth response 2, *EGR2* ; folic acid, FA.

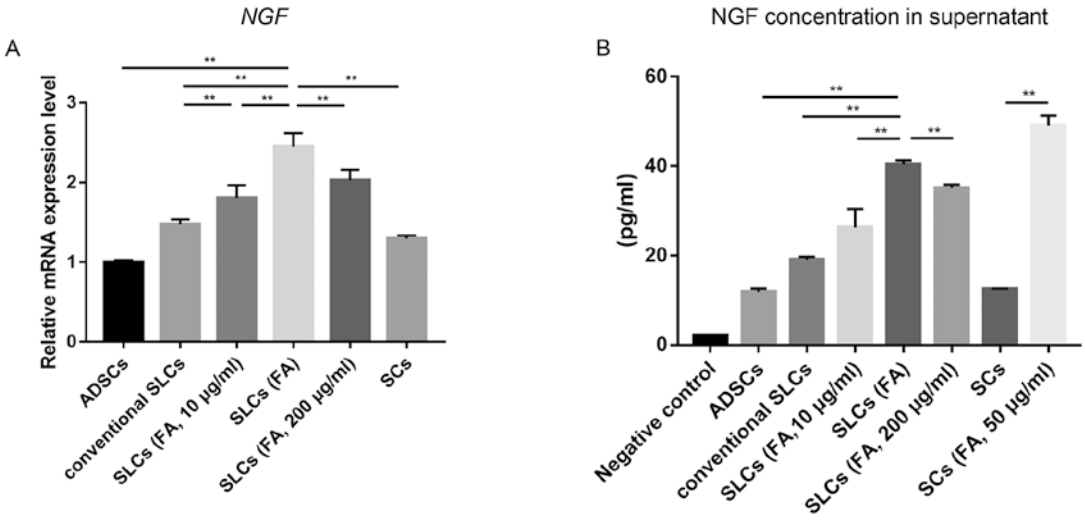

Fig 4. Gene expression and secretion of NGF by ADSCs, SLCs and SCs. (A) Gene expression of *NGF* in ADSCs, different groups of SLCs and SCs. (B) NGF concentration in the supernatants of ADSCs, different groups of SLCs, SCs and SCs (50 μg/ml) groups. **P<0.01, one way-ANOVA. adipose-derived stem cells, ADSCs ; Schwann-like cells, SLCs ; SCs ; Schwann-like cells ; folic acid, FA ; nerve growth factor, NGF.

DISCUSSION

Many studies have reported using differentiated SLCs from various sources of stem cells to promote nerve regeneration (29-31). We focused on ADSCs, which exhibit several advantages : 1) a sufficient number of ADSCs can be easily obtained from the patient's own adipose tissue through a minimally invasive procedure ; 2) autologous ADSC transplantation poses fewer ethical issues and lower risk of tumorigenicity compared with the use of induced pluripotent or embryonic stem cells (32) ; and 3) previous studies have shown excellent differentiation capabilities of ADSCs, including differentiation into powerful IPCs and hepatocyte-like cells (8, 33). However, the protocols used in these studies are similar, and an improved protocol to obtain more useful SLCs requires further exploration.

This study used FA to induce stem cell differentiation. FA is a common water-soluble vitamin derivative. FA plays a key role in the growth, differentiation and regeneration of the CNS (34) and aids peripheral nerve repair via its neurotrophic effects. Yilmaz *et al*. have shown that FA protects diabetic rats from diabetic peripheral neuropathy by reducing malondialdehyde levels and upregulating NGF expression (35). Harma *et al*. have found that FA improved peripheral nerve healing and increased axon myelination in a rat sciatic nerve injury model (17). Bridging the

sciatic nerve defect using an FA-coated nerve conduit improved the sciatic nerve function index and the nerve conduction velocity. Moreover, FA promoted proliferation, migration and NGF secretion by SCs (18). Our results showed that the new differentiation cocktail induced a change in ASCD morphology into bipolar and tripolar spindle shapes along with the expression of typical SC markers, such as *S100B* and *p75NTR*, indicating effective differentiation into an SC phenotype.

After nerve injury, SCs transdifferentiate into so-called repair SCs, and promote lysis of myelin, attract macrophages to participate in phagocytosis and secrete neurotrophic factors, such as NGF, to support and control neuronal function and guide axonal regeneration and axonal re-myelination (6, 28, 36, 37). Repair SCs are considerably different from immature SCs. Immature SCs fail to express mature SC markers, such as S100B and p75NTR. Studies have shown that ERBB2 may act as a maturation signal to negatively regulate the growth of axons, allowing SCs to start re-myelination (38, 39). EGR2 is downregulated in repair SCs and upregulated during re-myelination (28, 40). Interestingly, different conditions used to induce SLC differentiation determined the cell characteristics ; our new protocol seems to generate repair SLCs. Our results show that the dose of FA is related to the phenotypic characteristics of SLCs. Compared with 10 μg/ml and 200 μg/ml, the concentration of 50 μg/ml could decrease the gene expression of both *ERBB2* and *EGR2*. There is little discussion in the literature on the state of the SLCs ; it is reasonable to infer that the repair phenotype of SLCs is the most beneficial for nerve regeneration when used to treat PNI. NGF may activate autophagy in SCs, accelerate removal and phagocytosis of myelin fragments, and promote regeneration of axons and myelin during the early stages of PNI (19). Some studies have added neurotrophic factors, such as NGF, into nerve conduits to improve the quality of nerve regeneration (41). Other studies have also changed the microenvironment of transplanted cells, e.g., using self-assembling hydrogels, to stimulate higher secretion of brain-derived neurotrophic factors and NGF (42). However, these approaches were limited by the short half-life and unstable properties of neurotrophic factors and by difficulties in preparing the graft. Therefore, there is an urgent need to find economical neurotrophic factors with long half-lives and gradual release. According to our results, adding FA into the differentiation protocol could promote the release of NGF by SLCs. However, the secretion of NGF by SLCs (FA, 200 μg/ml) is not higher than SLCs (FA). We believe that due to the vulnerability of stem cells, excessive amount of FA might cause potential damage to SLCs. So, we set the concentration of FA as 50 μg/ml in our new protocol.

However, this study still has some shortcomings as theses SLCs (FA) generated by our modified protocol are still in conventional two-dimensional pattern. Many researchers including our team (8, 9, 11, 43) have illustrated that the three-dimensional (3D) cell culture pattern could promote the differentiation efficiency of stem cells and improve the final therapeutic effect of cell transplantation and cell therapy, which is considered to be the trend of tissue engineering and regeneration medicine. Therefore, we decide and have already begun to apply this new protocol combined with 3D culture technology for the generation of more powerful SLCs and explore its potential on the nerve regeneration *in vivo* and *in vitro*.

CONCLUSION

Our modified protocol effectively induced an SC phenotype in ADSCs both in morphology and expression of SC markers. Furthermore, the addition of FA induced a repair phenotype typical of SLCs with decreased *ERBB2* and *EGR2* gene expression and enhanced NGF secretion. This new protocol improved the functions of SLCs by using a stable and inexpensive vitamin and is promising for SC-mediated nerve regeneration.

CONFLICTING INTERESTS

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

This study was partly supported by a Grant-in-Aid for Scientific Research (grant no. 19K09045 to T. I., S. I., Y. M. and Y. S., and grant no. 19K09072 to T.T. and Y.S.). S. C., O. S., K. M., K. T., S. Y., and M. S. declare no potential conflict of interest. We thank Edanz Group (https://en-author-services.edanz.com/ac) for editing a draft of this manuscript.

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