KLF7-transfected Schwann cell graft transplantation promotes sciatic nerve regeneration

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Abbreviations:

acellular nerve allograft (ANA)
adeno-associated virus 2 (AAV2)
anterior tibial (TA)
compound action potential amplitude (CMAP)
calcitonin gene-related peptide (CGRP)
central nervous system (CNS)
corticospinal tract (CST)
cholera toxin B (CTB)
choline acetyltransferase (ChAT)
day (d)
dorsal root ganglia (DRG)
5-ethynyl-2-deoxyuridine (EdU)
growth-associated protein (GAP43)
Horseradish peroxidase (HRP)
Krüppel-like Factor 7 (KLF7)
neurofilaments (NF)
nerve growth factor (NGF)
peripheral myelin protein zero (P0)
proliferating cell nuclear antigen (PCNA)
retinal ganglion cells (RGC)
Schwann cell (SCs)
sciatic function index (SFI)
Transmission electron microscope (TEM)
tyrosine kinase receptor A (TrkA)
tyrosine kinase receptor B (TrkB)
Abstract:

Our former study demonstrated that Krüppel-like Factor 7 (KLF7) is a transcription factor that stimulates axonal regeneration after peripheral nerve injury. Currently, we used a gene therapy approach to overexpress KLF7 in Schwann Cells (SCs) and assessed whether KLF7-transfected SCs graft could promote sciatic nerve regeneration. SCs were transfected by AAV2-KLF7 *in vitro*. Mice was allografted by an acellular nerve (ANA) with either an injection of DMEM (ANA group), SCs (ANA+SCs group) or AAV2-KLF7-transfected SCs (ANA+KLF7-SCs group) to assess repair of a sciatic nerve gap. The results indicate that KLF7 overexpression promoted the proliferation of both transfected SCs and native SCs. The neurite length of the DRG explants was enhanced. Several beneficial effects were detected in the ANA+KLF7-SCs group including an increase in the compound action potential amplitude, sciatic function index score, enhanced expression of PKH26-labeling transplant SCs, peripheral myelin protein 0, neurofilaments, S-100, and myelinated regeneration nerve. Additionally, HRP-labeled motoneurons in the spinal cord, CTB-labeled sensory neurons in the DRG, motor endplate density and the weight ratios of target muscles were increased by the treatment while thermal hyperalgesia was diminished. Finally, expression of KLF7, NGF, GAP43, TrkA and TrkB were enhanced in the grafted SCs, which may indicate that several signal pathways may be involved in conferring the beneficial effects from KLF7 overexpression. We concluded that KLF7-overexpressing SCs promoted axonal regeneration of the peripheral nerve and enhanced myelination, which collectively proved KLF-SCs as a novel therapeutic strategy for injured nerves.

Keywords: KLF7; SCs; axonal regeneration; myelination; peripheral nerve injury
INTRODUCTION

Although peripheral nerves can endogenously regenerate after injury, a complete nerve transection or proximal nerve injury are generally associated with poor outcomes with limited motor recovery (Lundborg, 2000; Ma et al., 2011). The reason has been ascribed to multiple factors including the shortage of growth-promoting molecules and an inhibitory microenvironment (Grinspan et al., 1996; J et al., 2004; Fang et al., 2010; Wang et al., 2012). Accordingly, a combinatorial strategy targeting multiple deficits is often necessary (Zhao et al., 2011; Gao et al., 2014; Ghayemi et al., 2014; Zhao et al., 2014).

As a member of the zinc-finger DNA-binding protein family, Krüppel-like factors (KLFs) transcriptionally control numerous signaling pathways responsible for cell growth, proliferation, differentiation, migration, apoptosis, and inflammation (Moore et al., 2009; Moore et al., 2011; Yin et al., 2015). As a transcriptional activator, KLF7 is predominantly involved in many process of the nervous systems (Laub et al., 2006; Zhang et al., 2013). Particularly, KLF7 are key transcription factors or robust axon regeneration, neuronal morphogenesis, neuroblast differentiation and survival of sensory neurons (Lei et al., 2006; Kajimura et al., 2007; Veldman et al., 2007; Yin et al., 2015; Zhang et al., 2013).

KLF7 has been found to be strongly activated in response to nerve injury, mediating axonal response to the injury (Veldman et al., 2007; Zou et al., 2009). KLF7 can promote both sprouting and regenerative axon growth in the corticospinal tract (CST) of adult mice (Blackmore et al., 2012). Our previous studies demonstrated that KLF7 promotes the axonal regeneration of the peripheral motor and sensory nerve (Wang et al., 2016), which suggests that KLF7 may play a role in enhancing the neuronal intrinsic growth capacity (Moore et al., 2009). Additionally, it has been noted that NGF (Caiazzo et al., 2010), TrkA (Lei et al., 2001;
Lei et al., 2006), TrkB (Kingsbury and Krueger, 2007) and GAP43 (Blackmore et al., 2012; Kajimura et al., 2007) are direct KLF7 target genes.

Schwann cells (SCs) are widely considered to be one of the best candidates for cell therapy of peripheral nerve injury due to their crucial role in axon regeneration, remyelination, producing neurotrophic factors, and organizing Büngner bands after injury (Shakhbazau et al., 2012; Lehmann and Hoke, 2010). The success of ANA in combination with SCs has revealed a promising strategy for peripheral nerve injury repair (Gravvanis et al., 2007; Biazar and Heidari Keshel, 2013). SCs are a strong candidate for transplantation in ANA in nerve gap repair due to their effectiveness in axonal regeneration in short distance (Moore et al., 2011). However, for nerve gaps with lengths of 10 mm or more in mice, there is an increasing SCs replication burden due to the low survival rate of transplanted SCs and a longer delay in the repopulation of the graft, likely causing reduced regeneration (Hayashi et al., 2007). Transcription factors have been found to benefit SCs survival in the peripheral nervous (Wang et al., 2015; Huang et al., 2015; Hoben et al., 2015). KLF7-SCs delivery strategies have been previously employed to improve SCs quantity toward the goal of enhancing the efficiency of cell transplantation (Hoben et al., 2015). However, there has been no investigation regarding the use of KLF7-transfected SC in anchoring or enhancing the therapeutic capacity of ANAs. In this study, SCs were infected by AAV2-KLF7 in vitro. We examined the effect of KLF7-transfected SCs on SCs proliferation and neurite outgrowth in DRG explants. Next, we transplanted KLF7-transduced SCs engineered into ANA to repair sciatic nerve gap in vivo and investigated the SCs’ survival, myelination, motor and sensory axon regeneration. Additionally, the expression of KLF7 and its target genes were evaluated in the regenerated nerve, along with locomotor outcome and electrophysiological activity. We hypothesized that overexpression KLF7 could up-regulate the expression of target genes such as NGF in transplanted SCs, enhancing SCs survival and proliferation and promoting axonal
regeneration, myelination and locomotor functional recovery. We report that the therapeutic strategy was efficacious, exhibiting superior outcomes than ANA+SCs treatment alone.

EXPERIMENTAL PROCEDURES

Viral vector preparation

AAV-GFP virus containing both AAV serotype 2 Capsid and GFP was used. GFP expression was under the control of Cytomegalovirus immediate-early promoter (CMV) (AAV2-GFP, 1.0 × 10^{13} viral particles/ml; Vector Biolabs, Philadelphia, USA) which was used as a control. Mouse KLF7 sub-cloned into an AAV2 vector cassette was under the control of the CMV promoter (Vector BioLabs, Pennsylvania, USA; AAV-m-KLF7).

KLF7 transduction SCs

SCs were seeded into a 6-well plate (5 × 10^5 cells/well). When cells growth has reached over 90% confluence, 4-6 μg/ml polybrene were added (Sigma, USA) for 30-60 min. Then cells were infected by AAV2-GFP (final concentration 6.5 × 10^9 viral particle/ml) and AAV2-KLF7 (6.5 × 10^9 viral particle/ml) for 12 h at a multiplicity of infection (MOI) (150 μl/well) of 4. More than 50% cells were infected (Abdellatif et al., 2006). After three days, SCs were fixed and immunostained with an anti-S-100 (1:200; Sigma Aldrich, USA) and anti-KLF7 antibody (1:200; Novus Biologicals, Colorado, USA).

SCs proliferation assay

SCs were plated with a density of 3 × 10^5 cells/mL on coverslips. 4 h after 5-ethyl-2-deoxyuridine (EdU) was applied, we fixed the cells for the EdU immunostaining. EdU Cell Proliferation Kit was used by following the manufacturer’s protocol (Ribobio, USA). To determine the EdU labeling index, We counted the number of EdU-labeled nuclei from five
random fields, which then was divided by the number of DAPI-labeled nuclei. The assays were conducted in triplicate.

We used coculture system to detect the effect of KLF7 transfection on native SC proliferation and the outgrowth of dorsal root ganglion (DRG) (Huang et al., 2015). To determine whether KLF7-SCs influenced the SCs proliferation, we seeded purified SCs or DRG in the bottom chamber and AAV2-GFP-SCs or AAV2-KLF7- in the top chamber. Three days later, the SCs proliferation in the bottom chambers was investigated.

**DRG explant cultures**

We used a coculture system to evaluate the effect of KLF7-SCs on neurite axonal outgrowth (Huang et al., 2015). Briefly, neonatal rats DRGs were digested, then placed on coverslips, the coverslips were carefully moved to the bottom chamber, subsequently KLF7-SCs and AAV-GFP-SCs were transferred to the top of each transwell chamber as described. The DRG was fixation and immunostained with an antibody of neuronal marker β-III-tubulin (1:200; Sigma Aldrich, USA) (to identify neurite outgrowth) and DAPI (1:200; Sigma, USA). Neurite axonal outgrowth was measured by the average length of the five longest axons as described previously (Zhang et al., 2009).

**Animals**

Forty-eight C57BL/6 female mice and 24 CD1 (ICR) mice (male = 24, female = 24, weight range 18-22 g, eight weeks-old,) were purchased from the Experimental Animal Center of China Medical University (Certification No. SCXK Liao 2003-0009). Nerve donors were ICR mice and graft recipients were C57BL/6 mice. The Animal Care and Use Committee of China Medical University approved our experimental protocols.

**Seeding KLF7-SCs into ANA**
Acellular nerve allografts (ANA) preparations followed previous method (Jia et al., 2012). Briefly, bilateral sciatic nerves of CD1 mice were exposed. 15-mm nerve segments were taken. Both ends of nerves were fixed and incubated with 0.05 M Tris–HCl buffer supplemented with protease inhibitors (aprotinin at 0.1 μg/ml; leupeptin at 0.5 μg/ml; pepstatin A at 0.6 g/ml) for 4 days at 4°C. Next, DNase I (5 ng/ml) and RNase A (1 U/ml) were added to digest the nerves for 10 h in Tris-HCl buffer containing 3% Triton X-100 (pH 7.4). We used distilled water to wash the nerves and immersed the ANAs in antibiotic-supplemented phosphate-buffered saline (pH 7.4; with 100 U/ml penicillin and 26 mg/L gentamycin sulfate).

Cultured SCs were labeled with a fluorescent tracking dye PKH26 tracking dye before grafting. The procedure for PKH26 labeling followed the supplier's instructions (Sigma Chemical Co., St. Louis, USA). A total of 2×10^7 SCs in 100 μl complete medium was injected into four evenly spaced points of the nerve section using a micro-injector and Hamilton syringe with a 30 gauge needle. The ANA contents included 1) DMEM alone, 2) SCs alone (SCs), or 3) SCs infected with AAV2-KLF7 (KLF7-SCs). The ANA were then incubated in complete medium in a humidified atmosphere with 5% CO₂ at 37°C for 48 h after which they were collected for in vivo experiments.

**Study groups and surgical procedures**

All mice were randomly divided into three groups. Group I: ANA group, ANA treated with DMEM alone (n = 16); Group II: ANA+SCs group, ANA treated with SCs alone control (n = 16); Group III: ANA+KLF7-SCs group, ANA treated with KLF7-SCs (n = 16). The procedures for sciatic nerve defect and ANA implantation, as well as for pre- and post-operative animal care, are described in detail in previous publications (Li et al., 2008; Gao et al., 2014). Briefly, the right sciatic nerve was exposed. The nerve was completely cut. The resect of stumps created a 10-mm sciatic nerve defect. The nerve defect was bridged by the
three different ANA graft paradigms previously described. The implant was sutured to both the proximal and distal nerve stumps. Post-operative cares were taken to all mice according to the research guideline.

**HRP and CTB retrograde neural tracing**

Retrograde tracing was performed by horseradish peroxidase conjugated to the cholera toxin B subunit (BHRP) according to previously published protocols (Byers et al., 2012). Briefly, on the 26th post-operative day, animals were re-anesthetized and the TA muscle was exposed and injected with BHRP (0.5 μl, 0.2%; List Biological, Inc). BHRP labeling permits population-level quantitative analysis of motor neurons in the L3-L5 ipsilateral ventral horns. To obtain an optimal labeling of motor neurons, animals were sacrificed by a lethal dose of Nembutal (60 mg/kg, i.p.) twenty eight hours after BHRP injection. Animals were perfused intracardially and fixed by cold 1% paraformaldehyde/1.25% glutaraldehyde (n = 3/each group).

On the 22nd post-operative day, animals were anesthetized, when CTB (a 2% solution of Alexa Fluor labeled (594) cholera toxin B (CTB) (Invitrogen, USA) in PBS) was injected into the TA muscle (Hirakawa et al., 1992). Mice were euthanized six days after injection of neural tracer. (n = 3/each group).

Cells labeled by HRP and CTB were identified and counted under microscope at a final magnification of × 400. All labeled cells were counted in each section and the numbers of labeled cells totaled. Measurements of more than 200 labeled motor neurons in L3-L5 Rexed lamina IX of the ipsilateral ventral horns and sensory neurons in the ipsilateral L3-L6 DRG had an average cell diameter of 27.2 and 25.6/μm, respectively (da Silva et al., 1985).

**Analysis of sciatic function index (SFI)**
The sciatic function index (SFI) was used to measure the function of regenerated sciatic nerve (Kanaya et al., 1996). A experimental performer blinded to treatment evaluated sciatic nerve function every week. The rats’ hind feet were inked and the rats walked across a tunnel to record the footprints on paper paved on the bottom of the tunnel. The length between the third toe and heel (PL), first and fifth toe (TS), and second and fourth toe (ITS) were measured on the experimental side (EPL, ETS, and EITS, respectively) and the contralateral normal side (NPL, NTS, and NITS, respectively). The SFI was calculated as follows: 

\[
SFI = -38.3 \times \frac{EPL - NPL}{NPL} + 109.5 \times \frac{ETS - NTS}{NTS} + 13.3 \times \frac{EITS - NITS}{NITS} - 8.8. 
\]

In general, an SFI value around 0 indicated normal nerve function and a value around -100 indicated total dysfunction.

**Hargreaves’ test**

We used a plantar test apparatus to assess heat hyperalgesia. We measured the forepaw withdrawal latency responding to heat stimulation (Woodland Hills, CA, USA) (Hargreaves et al., 1988). Testing was performed by blind performer. Assessments were made including baseline and different time points after treatment. We calculated baseline values of paw withdrawal by an average of 2 - 3 consecutive withdrawal latencies of both hind paws detected at 15-min intervals. The difference of latency between the bilateral paws was considered to be an index of heat sensitivity.

**Electrophysiological analysis**

All animals received electrophysiological test before sacrifice (Day 28) under anesthesia (pentobarbital 40 mg/kg i.p.). A pair of needle electrodes were placed at the sciatic notch to stimulate the nerve with single electrical pulses of 0.05 ms until supramaximal intensity was achieved. The compound muscle action potentials were recorded from the medial gastrocnemius muscle and from the plantar muscles (at the third interosseous space). The evoked action potentials were displayed at settings appropriate for the measurement of
the amplitude from baseline to peak and the latency to the onset. To normalize the data, values obtained from the operating hind limb were expressed as percentage of values of the contralateral limb for each animal. Latency period and compound action potential amplitude (CMAP) were then recorded (Yang et al. 2007).

Western blotting

Western blot analysis was performed in accordance with previous reports (Liu and Xu, 2006). In vivo, the SCs samples were was homogenized 3 d after AAV2-KLF7 transfected. To evaluation the expression changes of KLF7 after grafting, normal sciatic nerve or the distal segments of injured sciatic nerve samples at different time points after grafting was homogenized in tissue homogenization. To evaluation the expression of KLF7 and its target gene in ANA in different group. Four weeks after grafting, ANAs were dissected and washed in saline, the dura mater was removed and snap-frozen on dry ice, protein samples (20 μg) were electrophoresed on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blots were incubated with primary antibodies against KLF7 (1:500; Novus Biologicals, Colorado, USA), GAP43 (1:1000; Sigma Aldrich, Missouri, USA), NGF (1:500; sigma, USA), TrkA (1:1000; sigma, USA), and TrkB (1:1000; sigma, USA) overnight followed by incubation with an HRP-conjugated secondary antibody for 1 h at room temperature (1:5000). Blots were visualized using the enhanced chemiluminescence (ECL) plus detection system (GE Healthcare, Little Chalfont, UK).

Immunohistochemistry

cryostat sections were cut in 25 μm-thick from the middle of the grafted nerve (Menzel-Glaser, Braunschweig, Germany). Tissues were immune-stained for peripheral myelin or P0 (1:200; Sigma Aldrich, Missouri, USA), neurofilaments or NF (1:200; Sigma Aldrich), S-100 (1:200; Sigma Aldrich), choline acetyltransferase or ChAT (1:500; Sigma Aldrich), or
calcitonin gene-related peptide or CGPR (1:1000; Sigma Aldrich). The signals were visualized by secondary antibodies such as goat anti-rabbit IgG (FITC) or anti-mouse IgG (TRITC) (1:200; Jackson ImmunoResearch Laboratories, Pennsylvania, USA). The tissues were scanned by MetaMorph/DP10/BX41 analytical imaging system to generate figures. The optical density of positive immunological signal was also analyzed.

**Histopathological analysis**

Histological analysis of nerve regeneration was performed as previously described (Jia et al., 2012). A transmission electron microscope (TEM) and toluidine blue staining were used to evaluate the regeneration of myelinated nerve, myelin sheath thickness, and axon diameter of nerve graft.

**Muscle wet weight ratio and motor endplate analysis**

At 28 d, the muscles of gastrocnemius and anterior tibial on both sides were harvested, and immediately weighed, respectively, to measure the muscles wet weight ratio (the muscle wet weight of injured side/the muscle wet weight of uninjured side).

The muscle sample was harvested from the mid-belly of TA muscles, motor endplate density densities were assessed after staining for acetylcholinesterase using the cholinesterase staining method (Byers et al, 2012).

**Statistical analysis**

Statistical analysis was conducted using SPSS software (version 13.0; SPSS, Illinois, USA) where values were expressed as the mean with standard deviation. Sample sizes were initially determined using statistical software to calculate the minimum total required number of animals or assays. All reported groups were above the minimum calculated sample size. Kolmogorov-Smirnov test was used to distinguish between parametric or nonparametric data, all parametric values were analyzed with a two-tailed Student’s t tests, or one-way ANOVA,
and Tukey’s post hoc test with GraphPad Prism 6.0 software. A P value of <0.05 was considered statistically significant. A P value of <0.05 was considered statistically significant.

RESULTS

Efficacy of AAV2-KLF7 gene transfer to SCs in vitro

To identify SCs, cells were stained by S-100 and GFAP. The cytoplasm of the positively stained SC cells was apparent by double-labeling by S-100 and GFAP (Figure 1A). Additionally, the morphology of the positively labeled cells was consistent with that of SCs observed under inverted microscope.

We then examined AAV2-GFP infected SCs in vitro (Figure 1B). The AAV2-GFP transfection of SCs was successful, with a transfection efficiency of 93.7 ± 3.09% (Figure 1C). Next, the transfection efficiency of AAV2-KLF7 was tested in SCs using immunocytochemistry and Western blotting (Figure 2). We found that control SCs expressed a low level of KLF7, 3 d after AAV2-KLF7 infection, the KLF7 was widely colocalized with SCs labeled by S100, and KLF7 was distributed both in the nuclear and cytoplasm of SCs in vitro (Figure 2A). Compare to AAV-GFP-SCs, the expression of KLF7 had increased significantly in KLF7-SCs as detected by immunocytochemistry (Figure 2B).

The expression of KLF7 after AAV2-KLF7 transfected was further confirmed by Western blots (Figure 2C). Compare to AAV2-GFP group, the expression level of KLF7 and NGF protein were increased in the AAV2-KLF7 group (KLF7: t4 = 19.09, P<0.001; NGF: t4 = 4.01, P<0.05) (Figure 2D, E). This result verified that SCs have been successfully transfected by AAV2-KLF7 vector producing higher levels of KLF7 and its target genes NGF expression than the control groups.

KLF7 overexpression promotes infected SCs proliferation, and KLF7-SCs promote native SCs proliferation and neurite length of DRG explants in vitro
We first detected the proliferation of KLF7 overexpression SCs by EdU staining at 3 days transfection (Figure 3A, B). The EdU-positive SCs in the AAV2-KLF7 group was 2.1 fold higher than in the AAV2-GFP groups ($t_6 = 14.03$, $P<0.001$) (Figure 3E), suggesting that KLF7 overexpression promotes infected SCs proliferation.

We next verified whether the transplanted SCs can affect native SCs (Figure 3C, D). There is significantly increase in the percentage of EdU-positive SCs in the Co-AAV-KLF7 group as compared with Co-AAV-GFP group at 3 days post-transfection ($t_6 = 4.668$, $P<0.01$) (Figure 3F). The results clearly showed KLF7-SCs promote native SCs proliferation.

Finally, we detected whether the KLF7-SCs can promote neurite axonal outgrowth by applying a coculture system of KLF7-SCs and DRG explants (Figure 4A). The average length of the longest axons in Co-AAV-KLF7-SCs group (1329 ± 282.5 μm) is more than that in Co-AAV-GFP-SCs (620.4 ± 99.88 μm) ($t_8 = 5.285$, $P<0.001$) (Figure 4B).

These results supported the interpretation that KLF7 overexpression promotes infected SCs proliferation, and KLF7-SCs can promote proliferation of native SCs and neurite axonal outgrowth.

**The expression changes of KLF7 following ANA grafting**

The expression changes of KLF7 after ANA grafting was confirmed by Western blots (Figure 5A). Our results showed that KLF7 protein levels were very low in the normal sciatic nerve. The expression of KLF7 in the distal segments of injured sciatic nerve was induced by 1 day post-graft, peak around 1-2 week and return to normal level of the normal sciatic nerve at about 4 week ($F_{5,12} = 173.5$, $P<0.001$) (Figure 5B). Which indicated that the KLF7 is reactivated and involved the process of sciatic nerve regeneration after ANA grafting.

**KLF7-SCs significantly increased the expression of KLF7, NGF, GAP43, TrkA, and TrkB in regenerating ANA**
We examined KLF7 expression by Western blot in ANA tissues harvested 28 days after injury (Figure 6A). In the ANA and ANA+SCs groups, a very low expression of KLF7 was found, however, animals receiving the KLF7-SCs showed an increase in KLF7 protein expression \((F_{2,6} = 1114, P<0.001)\) (Figure 6B). Meanwhile, to test the effect mechanism of KLF7-SCs on promotion of nerve regeneration, we examined the expression of KLF7 target genes such as NGF, GAP43, TrkA, and TrkB in ANA tissues (Figure 6A). The results demonstrated that ANA+KLF7-SCs and ANA+SCs group dramatically promoted NGF, GAP43, TrkA, and TrkB expression in the nerve graft compared to ANA groups. Notably, the NGF, GAP43, TrkA, and TrkB expression in the ANA+KLF7-SCs group was more prominent than in the ANA+SCs group \((P<0.05)\) (Figure 6C-F).

The above results show that KLF7-SCs can promote expression of KLF7 and induce an increase in expression of target gene such as NGF, GAP43, TrkA, and TrkB in regenerating ANA tissues.

**KLF7-SCs promote myelinated fiber regeneration and transplant SCs survival in regenerating ANAs**

To further assess myelinated fiber regeneration and transplant SC survival in regenerating ANA, ANAs were sectioned longitudinally and visualized by immunohistochemistry (Figure 7A-C). The ANA from the ANA+KLF7-SCs group showed increased PKH26-labeling in SCs than the ANA+SCs groups, however, no expression of PKH26-labeling in SCs was detected in the ANA groups \((F_{2.15} = 70.22, P<0.001)\) (Figure 7A-D). In the ANA+KLF7-SC and ANA+SCs tissues, peripheral myelin (P0) occupied the total length of the ANA (Figure 7A, B), while, the ANA control tissues had shorter myelinated fibers across the ANA (Figure 7C). Quantification of P0 intensity verified that peripheral myelin in ANA+KLF7-SCs and ANA+SCs tissues were higher than that in ANA control group. Moreover, the peripheral
myelin in the ANA+KLF7-SCs group was higher than that in ANA+SCs group ($F_{2, 15} = 41.59$, $P<0.001$) (Figure 7E).

Toluidine blue staining further verified that the number of myelinated fibers in the ANA+SCs and ANA+KLF7-SCs group was greater than in ANA group. Notably, the number of myelinated fibers in the ANA+KLF7-SCs group was significantly higher than the ANA+SCs group ($F_{2, 15} = 24.77$, $P<0.001$) (Figure 8A, C). Similarly, the nerve myelin sheath thickness and axon diameter demonstrated similar patterns of changes in the three groups (myelin sheath thickness: $F_{2, 15} = 30.29$, $P<0.001$; axon diameter: $F_{2, 15} = 13.58$, $P<0.001$) (Figure 8D, E). Furthermore, TEM analysis indicated that the degeneration of myelin sheath in the ANA group was pronounced than in ANA+SCs and ANA+KLF7-SCs groups (Figure 8B). These results verified that the quantity, construction and myelination of regenerated nerve fibers in the ANA+KLF7-SCs group were best among all groups.

Collectively, the above results indicate that ANA+KLF7-SCs treatment promotes transplant SCs survival and myelinated fiber regeneration in regenerating ANAs.

**KLF7-SCs enhance motor and sensory axonal regeneration in regenerating ANAs**

We found that S100 (a marker of myelinated SC cells) surround NF-positive axons (a marker of pan-neuronal) (Figure 9A), which labeled myelinated axonal. the expression of S-100 and NF was significantly increased in the ANA+SCs and ANA+KLF7-SCs group than ANA group. Moreover, the expression of NF and S-100 in the ANA+KLF7-SCs group was higher than in the ANA+SCs group (NF: $F_{2, 15} = 26.29$, $P<0.001$; S 100: $F_{2, 15} = 55.72$, $P<0.001$) (Figure 9B, C). Which showed that KLF7-SCs promote the myelinated axonal regeneration in regenerating ANA.

ChAT (a marker of motoneurons) and CGRP (a marker of the peptidergic sensory neurons) immunostaining provide a reliable method to evaluate the outgrowth rate of motor
and sensory axon. The expression of ChAT and CGRP in regenerating ANAs showed similar patterns of change in the three groups (ChAT: $F_{2, 15} = 29.17, P<0.001$; CGRP: $F_{2, 15} = 27.40, P<0.001$) (Figure 10A-C). These results demonstrated that KLF7-SCs enhances the expression of motor and sensory axonal regeneration markers in regenerating ANAs.

Retrograde tracer can label neuronal cell bodies in the lumbar L3-L6 DRGs and the ventral horn of the L3-L5 spinal cord. When we examined the HRP-labeled motor neurons in Rexed lamina IX of the ipsilateral ventral horns (Figure 11A), we found the number of HRP-labeled motor neurons in the ANA+KLF7-SCs and ANA+SCs groups was more than that in the ANA control group. Moreover, the HRP-labeled motor neurons in the ANA+KLF7-SCs group was markedly higher than in ANA+SCs group ($F_{2, 6} = 28.27, P<0.001$) (Figure 11C).

We found the presentation of a number of CTB-labeled sensory neurons in the L3-L6 ipsilateral DRG showed similar patterns of change across each group ($F_{2, 6} = 27.62, P<0.001$) (Figure 11B, D). This finding confirmed that motor and sensory axonal regeneration was promoted in the ANA+KLF7-SCs group as evaluated by the retrograde method.

**KLF7-SCs promote target muscle motor endplate and wet weight ratio**

The reinnervation between the regenerated nerve fibers and the target muscle cells was verified by the cholinesterase staining of the motor endplate. To assess KLF7-SC’s effects on TA muscle motor function recovery, the motor endplate density in the TA was assessed following injury (Figure 12A). We found that the number of motor end plates was increased in the ANA+SCs and ANA+KLF7-SCs compared to the ANA group. Motor end plate numbers were particularly higher in the ANA+KLF7-SCs group compared to the ANA+SCs group ($F_{2, 15} = 31.61, P<0.001$) (Figure 12B). The above results indicate that KLF7-SC’s provide a positive effect on the reinnervation of denervated TA muscle.
After sciatic nerve injury, the gastrocnemius and anterior tibial muscles began to atrophy. We found the weight ratios of target gastrocnemius and anterior tibial muscles were increased in the ANA+SCs and ANA+KLF7-SCs compared to the ANA group, the mean ratios of gastrocnemius and anterior tibial muscle wet weight were particularly higher in the ANA+KLF7-SCs group compared to the ANA+SCs group (gastrocnemius muscle, $F_{2,15} = 58.34, P<0.001$; anterior tibial muscle, $F_{2,15} = 44.05, P<0.001$) (Figure 12C, D). The above results indicate that KLF7-SC’s provide a positive effect on the reverse of the target muscle atrophy.

**KLF7-SCs significantly enhances motor function**

Evaluation of sciatic function was carried out according to the sciatic function index (SFI). The results showed that SFI values were increased significantly in the ANA+KLF7-SCs and ANA+SCs group compared to the ANA group on day 28 (Figure 13A). Between these groups, a significant increase in SFI value was observed in the ANA+KLF7-SCs group compared to the ANA+SCs group ($F_{2,21} = 23.03, P<0.001$). We next measured the functional recovery of the ANAs (Figure 13C) by electrophysiology. The ANA+KLF7-SCs and ANA+SCs groups exhibited an increased CMAP amplitude compared to the ANA group ($F_{2, 21} = 24.71, P<0.001$) (Figure 13D). In contrast, a decreased latency period was found in the ANA+KLF7-SCs and ANA+SCs group ($F_{2,21} = 30.95, P<0.001$) (Figure 13E). Furthermore, ANA+KLF7-SCs showed a better electrophysiological response compared to the ANA+SCs group.

The thermal withdraw latency of the ipsilateral hind limb were assessed to determine the effect of KLF7-SCs on the pain sensation by Hargreaves’ test (Figure 13B). The thermal hyperalgesia evidenced by the shortening of the withdrawal latencies against thermal stimulation was found. The results showed significant hyperalgesia was observed of all
treatment groups from the 2 week to 3 week after ANA grafting (2 w, $F_{3, 28} = 36.35, P<0.001$; 3 w, $F_{3, 28} = 11.78, P<0.001$), the withdrawal latencies in the ANA+KLF7-SCs group showed significantly increase on 21 day compare to ANA+SCs and ANA group ($F_{3, 28} = 11.78, P<0.001$). However, the thermal hyperalgesia was not seen from days 21 to 28.

Altogether, these data indicated that the KLF7-SCs exhibited the most beneficial effects in the context of systematic motor function recovery and pain related behavior.

**DISCUSSION**

KLF7 is a transcriptional activator whose expression predominantly plays a role in the nervous system (Laub et al., 2006; Zhang et al., 2013). KLF7 can regulate several important neurobiological activities such as neurite outgrowth (Caiazzo et al. 2010, Laub et al., 2006), cell proliferation (Caiazzo et al., 2010; Zhang et al., 2013), neuronal differentiation (Caiazzo et al., 2010), and olfactory bulb development (Caiazzo et al., 2011). Previous studies have also confirmed that KLF7 can be activated by nerve injury and can regulate axonal injury responses. For example, up-regulation of KLF7 in response to axotomy was previously observed during which injured neurons such as RGCs and peripheral DRGs experienced regeneration (Veldman et al., 2007; Zou et al., 2009).

Our previous studies indicated that acellular nerve allografts (ANA) are highly biocompatible, capable of reducing the cellular and humoral immunologic response and adequately sparing nerve structure and extracellular matrix components which are necessary for nerve regeneration (Jia et al., 2012; Wang et al., 2012). We injected AAV2-KLF7 into ANA to repair sciatic nerve gap directly, since overexpression of KLF7 has been shown to promote motor and sensory axonal regeneration (Wang et al., 2016), indicating that KLF7 may enhance axonal growth (Veldman et al., 2007; Moore et al., 2009).
As a promising seed cell of nerve grafts, SCs can provide protection and nutrition to the axon and promote the formation of myelin sheaths, aiding in physiological and functional recovery. SCs also play a crucial role in neural protection, induction of axon growth, guidance and production of many neurotrophic factors. Neurotrophic and transcription factors have been transferred to SCs and used to enhance the endogenous SCs’ capability to promote nerve regeneration and positively affect functional recovery (Fang et al., 2010; Enomoto et al., 2013; Gravvanis et al., 2007; Deng et al., 2011). However, for the giant nerve gap, there is an increasing burden placed on SCs to replicate and survive (Hayashi et al., 2007). Interestingly, transcription factors have been previously shown to benefit SCs survival in the peripheral nervous system (Wang et al., 2015; Huang et al., 2015; Hoben et al., 2015). KLF7-SCs delivery strategies can thus be employed to improve SCs quantity toward the goal of enhancing the efficiency of cell transplantation (Hoben et al., 2015).

In our study, we used engineered SCs overexpression of KLF7 in ANAs to evaluate repair of sciatic nerve injury. We found that KLF7-SCs promote native SCs proliferation, consequently enhancing axonal growth and myelination. While control SCs expressed a low level of KLF7, 3 d after AAV2-KLF7 infection, KLF7 was widely co-localized with SCs (labeled by S100) and distributed both in the nucleus and cytoplasm of SCs in vitro (Fig. 2A) in the experimental group. Further, EdU staining results corroborated that KLF7 overexpression promoted SCs proliferation (Fig. 3). Enhanced SC proliferation in response to KLF7 overexpression may be rooted in the subsequent up-regulation of the trophic and growth KLF7 target genes such as NGF, which is known to enhance SCs proliferation (Lei et al., 2006; Caiazzo et al., 2010).

In vivo, our results demonstrated that KLF7 protein levels were very low in the intact sciatic nerve. The expression of KLF7 in the distal stump of the injured nerve, however, was induced by 1 day post-graft, peaking around 1-2 weeks, and returning to normal levels at
about 4 week (Fig. 5), indicating that the KLF7 is activated and involved in sciatic nerve injury responses after ANA grafting. These results are consistent with former results from similar studies performed previously (Veldman et al., 2007).

KLF7 can regulate several target genes, including the NGF (Caiazzo et al., 2010), TrkA (Lei et al., 2001; Lei et al., 2006), TrkB (Kingsbury and Krueger, 2007) and GAP43 (Blackmore et al., 2012; Kajimura et al., 2007). As stated earlier, KLF7 overexpression in SCs likely functions therapeutically through the onset of downstream signaling through its various growth and repair targets. Indeed, our data confirmed that implantation of KLF7-SCs increased the protein expression of KLF7 targets including NGF, TrkA, TrkB and GAP-43 in regenerated ANAs (Fig. 6). We therefore speculate that KLF7-SCs may increase regeneration-associated genes such as NGF, TrkA, TrkB and GAP-43 and their downstream mechanisms, subsequently protecting the transplant from further injury, supplementing neurotrophic factors, promoting SCs survival and proliferation—collectively increasing SCs’ capability to promote nerve regeneration and functional recovery. That the expression of TrkA, TrkB, KLF7, NGF and GAP43 were increased in the grafted SCs may indicate that several signaling pathways may converge to mediate a complex injury response, and that KLF7 overexpression can influence a range of growth and repair mechanisms. Moreover, the number of PKH26-labeled (a transplanted cell tracer) SCs in the ANA were increased in the KLF7-SCs group compared to control groups (Fig. 7), indicating that KLF7 improves cellular grafting of transplanted SC into the host tissue.

Various axonal regeneration evaluations were performed in DRG explant cultures. First, we note that there was a significant increase in the average length of the longest axons in the AAV-KLF7-SCs group compared to AAV-GFP-SCs (Fig.4), indicating that KLF7-SCs promote neurite outgrowth in an enhanced capacity compared to SC-transplants alone. In vivo, axonal regeneration tests revealed that compared to normal SCs, KLF7-SCs resulted in
higher expression of S-100, NF, myelin (P0) proteins, number of myelinated fibers, myelin sheath thickness and axon diameters, along with decreased myelin sheath degeneration (Figs.7-9). All of these findings indicate that KLF7-SCs treatment, in addition to enhancing axonal regeneration through enhancement of SC growth functions, promotes myelinated fiber regeneration and myelination in regenerating ANA, aiding in conduction of the fibers at the graft site and consequently in functional recovery.

Sciatic nerve injury is capable of severely impacting sensory and motor functions of the lower limbs, impeding functional movement and increasing pain sensation in some cases. As such, motor and sensory nerve axons were more closely examined in this study. The motor axons at the injured nerve were detected by the motoneuron marker ChAT (Tannemaat et al., 2008). Additionally, peptidergic sensory neurons expressing calcitonin gene related peptide (CGRP) were detected through the CGRP marker. ChAT and CGRP co-immunolabeling are reliable methods to evaluate the outgrowth rate of motor and sensory axons (Li et al., 2004; Di Giorgio et al., 2008). In this study, we found that KLF7-SCs treatment increased the density of ChAT and CGRP in regenerating ANAs compared to SCs alone (Fig. 10). Additionally, we used retrograde tracers to identify motor and sensory nerve regeneration across the lesion gap as previously described (da Silva et al., 1985; Koussoulakos et al., 2003). In our study, the retrograde tracer HRP and CTB identified an increased number of motor neurons on the L3-L5 ipsilateral ventral horns and an increased number of sensory neurons in the L3-L6 DRG in the KLF7-SCs treatment group compared to the ANA+SCs group (Fig. 11). These findings suggest that KLF7-SCs may have a significant effect on the preservation and/or promotion of motor and sensory axon regeneration, bearing important implications for the recovery of critical motor and sensory functions.

Former studies have reported that sciatic nerve injury induced pain-related behavior (Bester et al., 2000). We found here that ANA grafting of the sciatic nerve gap produces a transient
period of thermal hyperalgesia (indicator of nociceptive response), however, the KLF7-SCs treatment decreased thermal hyperalgesia 3 weeks after ANA grafting. Due to the expression of CGRP receptors on adult SCs, we propose that SC injury response may in part be mediated by peripheral nerve injury cues transduced through CGRP signaling sensory neurons (Toth et al., 2009). Consequently, considering that CGRP is also a critical player in pain perception (Hirose et al., 2010), the upregulation of CGRP expression in ANA may be correlated with the observed thermal hyperalgesia which was mitigated by KLF7-SCs (Fig. 13).

Finally, therapeutic strategies for peripheral nerve injury bear the burden of recuperation of critical functions. In addition to an improved pain response, our data demonstrate that KLF7-SCs treatment resulted in a significant and progressive recovery of physiological and behavioral responses. ANAs grafted with KLF7-SCs demonstrated an increased compound action potential amplitude recorded from the medial gastrocnemius and plantar muscles. Additionally, latency of the evoked potentials was reduced by the treatment compared to controls. Sciatic nerve function was monitored weekly after surgery according to the sciatic function index (SFI). As hypothesized, KLF7-SC overexpression improved SFI scores, indicating enhancement of sciatic functions. Another parameter of nerve regeneration with functional implications was the evaluation of motor endplates and weight of target muscles. The experimental treatment resulted in increased fiber density at the endplate in addition to increased wet weights of injured muscle at both the gastrocnemius and anterior tibial (Figure 12). These findings indicate that muscular atrophy was reduced by the novel treatment strategy compared to control treatments. Importantly, across various measures of functional outcomes, the KLF7-SC strategy demonstrated superior performance.

CONCLUSION
In summary, here we report a novel method of transplanting SCs engineered for the overexpression of KLF7 into ANAs for the repair of peripheral nerve injury. This promising therapeutic strategy relies mainly on the provision of prolonged transcriptional activation of targets that are responsible for the therapeutic mechanisms of SCs within the injured spinal cord, leading to the enhanced motor and sensory axon regeneration, myelination and functional recovery reported here.

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References


Biazar E, Heidari Keshel S (2013) A nanofibrous PHBV tube with Schwann cell as artificial nerve graft contributing to rat sciatic nerve regeneration across a 30-mm defect bridge. CELL COMMUN ADHES 20:41-49.


Figure Legends

**Figure 1. Immunohistochemistry of Schwann cells and AAV2-GFP infected** (A) SCs cell immunohistochemical staining by S-100 (green) and GFAP (red) with nuclear staining (DAPI; blue). Merged image showed that SCs were double labeled by S-100 and GFAP. Scale bars: 100 μm. (B) AAV2-GFP infected cultured SCs in vitro. Scale bars: 50 μm. (C) Quantitative analysis of AAV2-GFP infection rate.

**Figure 2. Efficacy of AAV2-KLF7 gene transfer to SCs in vitro**
(A) Immunohistochemical staining by KLF7 (pink) and S100 (red) with nuclear staining (DAPI; blue) of KLF7 transduced SCs. Merged image showed that SCs were double labeled by KLF7 and S100. Scale bars: 100 μm. (B) The graphs for the analyses represent the relative density of KLF7 in the cultured SCs of two groups are shown at 3 days in vitro. (C) Representative Western blot analysis of KLF7 and NGF expression in the cultured SCs of two groups are shown at 3 days in vitro (n = 3). The graphs for the analyses represent the relative density of KLF7 (D) and NGF (E). Error bars denote SD. *P < 0.05, *** P < 0.001, vs. the AAV2-GFP group. Student’s t tests.

Figure 3. The proliferation ability of KLF7-SCs and the influence of KLF7-SCs on native SCs proliferation

(A-D) The total number of the cells shown by DAPI (blue) staining, the number of proliferating SCs was determined through EdU (red) staining. (E) EdU labeling index of AAV2-KLF7 and AAV2-GFP group. (F) In the coculture system, the EdU labeling index of SCs in the bottom chambers represented the influence of AAV2-KLF7-SCs and AAV2-GFP-SCs on native SC proliferation. Error bars denote SD. **P < 0.01, Student’s t tests. Scale bars: 100 μm.

Figure 4.Effect of KLF7-SCs on neurite outgrowth. (A) Representative immunostaining images of DRG explants in each group. Axons were labeled with β-III-tubulin (green), cell nuclei were labeled with DAPI (blue). (B) A comparison of the average length of the five longest axons. Error bars denote SD. *** P < 0.001, vs. the Co-AAV2-GFP group. Student’s t tests. Scale bars: 100 μm.
Figure 5. The expression changes of KLF7 following ANA grafting (A) Western blot analysis revealed protein level of KLF7 in normal sciatic nerve (N) and the change of KLF7 expression in the distal segments of injured sciatic nerve after ANA grafting. (B) The graphs for the analyses represent the KLF7 relative density of the bands normalized to GAPDH in different time. \( n = 3 \), Error bars denote SD. \( ***P < 0.001 \) vs. the normal group. One-way ANOVA, Tukey’s post hoc test.

Figure 6. KLF7-SCs significantly increase the expression of KLF7, NGF, GAP43, TrkA, and TrkB in regenerating ANAs

(A) Representative Western blot analyses of KLF7, NGF, GAP43, TrkA, and TrkB expression in ANA tissues harvested 28 days post-injury across three treatment group. The graphs for the analyses represent the relative density of KLF7 (B), NGF (C), GAP43 (D), TrkA (E), TrkB (F). \( n = 3 \) mice/group. Error bars denote SD. \( *P < 0.05, **P < 0.01, ***P < 0.001 \), NS, not significant. One-way ANOVA, Tukey’s post hoc test.

Figure 7. In longitudinal sections, KLF-SCs significantly increase the expression of P0 protein (myelination) and transplant SCs survival (PKH26) in regenerating ANAs

At 28 days post-injury, representative immunohistochemical stainings for P0 (green) and a fluorescent tracking dye PKH26 labeled SCs (red) in ANA in longitudinal sections from the ANA+KLF7-SCs (A), ANA+SCs group (B) and ANA group (C). \( n = 6 \) mice/group. Quantitative analyses of PKH26 (D) or P0 (E) expression in immunohistochemical sections.
Error bars denote SD. *$P < 0.05$ ***$P < 0.001$, One-way ANOVA, Tukey’s post hoc test. Bar indicates 100 μm.

**Figure 8. KLF7-SCs promote myelinated fiber regeneration in regenerating ANAs**

Representative Toluidine blue staining (A) and transmission electron microscope (TEM) (B) analysis of myelinated fiber regeneration in the regenerating ANAs of three treatment groups are shown (n = 6). Quantitative analyses of the number of myelinated fibers (C), the nerve myelin sheath thickness (D) and axon diameter (E) in the middle of the ANAs is shown. Error bars denote SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, One-way ANOVA, Tukey’s post hoc test. Bar indicates 50 μm.

**Figure 9. KLF7-SCs significantly increase the expression of S-100 protein (myelination) and neurofilaments (NF) in ANAs**

(A) Representative immunohistochemical stainings of S-100 and NF protein in ANAs from the three treatment groups (n = 8). Merged images combine S-100 (green) and NF (red). Quantitative analyses of NF (B) and S-100 (C) expression in immunohistochemical sections. Error bars denote SD. ***$P < 0.001$, One-way ANOVA, Tukey’s post hoc test. Bar bars: 100 μm.

**Figure 10. KLF7-SCs significantly increase motor and sensory axonal regeneration**

ChAT and CGRP immunostaining evaluated the rate of motor and sensory axon outgrowth. (A) Representative immunohistochemical stainings of ChAT and CGRP protein in ANAs from the three treatment groups (n = 8). Merged images combine ChAT (red), CGRP (green)
and DAPI (bule). Quantitative analyses of ChAT (B) and CGRP (C) expression in immunohistochemical sections. Error bars denote SD. **$P < 0.01$, ***$P < 0.001$, One-way ANOVA, Tukey’s post hoc test. Bar bars: 50 μm.

**Figure 11. Retrograde tracing with HRP and CTB**

Retrograde HRP and CTB labeling from the TA muscle to motor neurons in L3-L5 ipsilateral ventral horns or sensory neurons in the L3-L6 DRG were used to evaluate motor and sensory axon regeneration. Representative images of the HRP-labeled motor neurons in the ventral horns (A) and CTB-labeled DRG sensory neurons (red) (B). Quantitative analyses of the number of HRP-labeled motor neurons detected in L3-L5 spinal cords (C) and CTB labeled sensory neurons in the L3-L6 DRG (D). Error bars denote SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, One-way ANOVA, Tukey’s post hoc test. Bar indicates 200 μm.

**Figure 12. Muscle wet weight and motor endplate analysis.** (A) Representative images of the motor end plate in the longitudinal section of injured side of TA muscle of each treatment group ($n = 3$). Scale bar, 20 mm. (B) Quantitative analyses of the number of motor end plate per TA muscle fiber. Histograms comparing the wet weight ratio of gastrocnemius (C) and anterior tibialis muscles (D) of each treatment group ($n = 3$). Error bars denote SD. **$P < 0.01$, ***$P < 0.001$, One-way ANOVA, Tukey’s post hoc test.

**Figure 13. KLF7-SCs significantly enhanced motor functional recovery of regenerating ANAs** Quantitative analyses of the sciatic function index (SFI) (A), withdrawal latencies according to Hargreaves’ test (B), and electrophysiological index
(C), including the compound action potential amplitude (mV) (D) and latency period (ms) (E) in ANAs from each group (n = 8) are shown. Error bars denote SD. *P < 0.05, **P < 0.01, ***P < 0.001, One-way ANOVA, Tukey’s post hoc test.