KLF7 overexpression in bone marrow stromal stem cells graft transplantation promotes sciatic nerve regeneration

Wen-Yuan Li1,4, Guan-Yu Zhu2,4, Wen-Jiang Yue3, Guang-Da Sun1, Xiao-Feng Zhu1,5,6 and Ying Wang1,5,6

1 Institute of Neural Tissue Engineering, Mudanjiang College of Medicine, Mudanjiang 157011, People’s Republic of China
2 Department of Neurosurgery, Beijing Tiantan Hospital, Capital Medical University, Beijing 100070, People’s Republic of China
3 Department of Otorhinolaryngology, The Second Affiliated Hospital, Mudanjiang College of Medicine, Mudanjiang 157011, People’s Republic of China

E-mail: yingwang2016@sina.com and zhuxiaofeng1227@163.com

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Abstract

Objective. Our previous study demonstrated that the transcription factor, Krüppel-like Factor 7 (KLF7), stimulates axon regeneration following peripheral nerve injury. In the present study, we used a gene therapy approach to overexpress KLF7 in bone marrow-derived stem/stromal cells (BMSCs) as support cells, combined with acellular nerve allografts (ANAs) and determined the potential therapeutic efficacy of a KLF7-transfected BMSC nerve graft transplantation in a rodent model for sciatic nerve injury and repair. Approach. We efficiently transfected BMSCs with adeno-associated virus (AAV)-KLF7, which were then seeded in ANAs for bridging sciatic nerve defects. Main results. KLF7 overexpression promotes proliferation, survival, and Schwann-like cell differentiation of BMSCs in vitro. In vivo, KLF7 overexpression promotes transplanted BMSCs survival and myelinated fiber regeneration in regenerating ANAs; however, KLF7 did not improve Schwann-like cell differentiation of BMSCs within the nerve grafts. KLF7-BMSCs significantly upregulated expression and secretion of neurotrophic factors by BMSCs, including nerve growth factor, ciliary neurotrophic factor, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor in regenerating ANA. KLF7-BMSCs also improved motor axon regeneration, and subsequent neuromuscular innervation and prevention of muscle atrophy. These benefits were associated with increased motor functional recovery of regenerating ANAs. Significance. Our findings suggest that KLF7-BMSCs promoted peripheral nerve axon regeneration and myelination, and ultimately, motor functional recovery. The mechanism of KLF7 action may be related to its ability to enhance transplanted BMSCs survival and secrete neurotrophic factors.

4 Equal contributors.
5 Author to whom any correspondence should be addressed.
6 Institute of Neural Tissue Engineering, Mudanjiang College of Medicine, Tongxiang St, Aimin District, Mudanjiang, Heilongjiang 157011, People’s Republic of China

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factors rather than Schwann-like cell differentiation. This study provides novel foundational data connecting the benefits of KLF7 in neural injury and repair to BMSC biology and function, and demonstrates a potential combination approach for the treatment of injured peripheral nerve via nerve graft transplant.

Keywords: KLF7, BMSCs, axonal regeneration, peripheral nerve injury

Supplementary material for this article is available online

(Some figures may appear in colour only in the online journal)

Abbreviations

| Abbreviation | Description                                |
|--------------|--------------------------------------------|
| ANA          | Acellular nerve allograft                  |
| AAV          | Adeno-associated virus                     |
| TA           | Tibialis anterior                          |
| BMSCs        | Bone marrow stromal stem cells             |
| BDNF         | Brain-derived neurotrophic factor          |
| CAMP         | Compound action potential amplitude        |
| CNTF         | Ciliary neurotrophic factor                |
| ChAT         | Choline acetyltransferase                  |
| d            | Day                                        |
| EVs          | Extracellular vesicles                     |
| EdU          | 5-ethyl-2-deoxuridine                      |
| GDNF         | Glial cell-derived neurotrophic factor      |
| HRP          | Horseradish peroxidase                     |
| KLF7         | Krüppel-like Factor 7                      |
| NF200        | Neurofilament 200                          |
| NGF          | Nerve growth factor                        |
| P0           | Peripheral myelin protein zero             |
| SFI          | Sciatic function index                     |
| TEM          | Transmission electron microscope           |
| TUNEL        | TdT-mediated dUTP nick-end labeling        |

1. Introduction

Peripheral nerves have the intrinsic mechanisms and extrinsic cellular and non-cellular support to promote regeneration, whereas damaged axons or tracts of the central nervous system (CNS) do not. A key difference in the regenerative capacity between the two systems is the plasticity and reparative abilities of the Schwann cell in the peripheral nerve [1, 2]. Schwann cells exhibit many properties that enhance axon regeneration following peripheral nerve injury and production of trophic factors, including insulin-like growth factor-1 (IGF-1), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) by the Schwann cell are understood to facilitate these functions [3–6]. Most peripheral nerve injuries are laceration or transection injuries, which complicates successful regeneration and targeting of damaged peripheral axons. As such, functional outcome is often poor, with evidence linking these reduced trophic factor production among other inhibitory characteristics in the microenvironment of the regenerating nerve [7–9]. These issues are amplified when a nerve graft is required to bridge a gap in a transected nerve, despite the value of this approach to providing a conduit for regenerating peripheral axons. Schwann cells are critical for this regenerative process, and our previous research has shown that enhancing beneficial aspects of Schwann cell responses to injury improves regeneration and subsequent functional outcomes in the transected and grafted sciatic nerve [10].

In particular, modifying SCs to over express the transcription activating factor, Krüppel-like factor 7 (KLF7) enhanced proliferation of transplanted and native Schwann cells in an acellular nerve allograft (ANA) following sciatic nerve transection injury [10]. This response led to enhanced regeneration and myelination of regenerating axons in the ANA, as well as increased neuromuscular innervation and functional recovery [10]. Much evidence in the literature supports various positive influences of KLF7 on axon regeneration and neuron survival, among other benefits following nervous system injury [11–15]. As a transcriptional activating factor, we found that mechanisms of KLF7-mediated benefits on regeneration and recovery in peripheral nerve injury and repair are likely mediated through enhanced expression of target genes including NGF, tyrosine kinase receptors A and B, growth associated protein-43 (GAP-43) [10], which supports known effects of KLF7 [15–19]. However, though Schwann cells proved useful as a starting point for investigating the function of KLF7 in peripheral nerve regeneration and the role it plays when overexpressed by Schwann cells, difficulties in using Schwann cells related to isolation, proliferation, purification, and donor site complications are known [20]. Our recent study of KLF7 overexpression by Schwann cells only spanned approximately one month post-repair, so it remains unclear what the proliferation and survival status of SCs overexpressing KLF7 and affected native Schwann cells are beyond this period of time. In light of this lack of information, and the limitations of SC transplantation in ANA, utilizing a stem cell to study the reparative and cellular effects of KLF7 is a logical and valuable next step in improving the therapeutic efficacy of ANA and enhancing our understanding of the role and benefits of KLF7 in peripheral nerve injury.

Mesenchymal stem cells (MSCs) have been widely studied as a therapy in neural injury models, including spinal cord injury (SCI), traumatic brain injury (TBI) and peripheral nerve injury [21–25]. Concerning the relationship between KLF7 and MSCs, very little is known. Recent research indicated extracellular vesicles (EVs) secreted from MSCs contain mRNA for KLF7 expression, among various other factors [26]. It is believed MSCs primarily secrete transcription factor genes and mRNAs to influence processes and cellular behavior, such as for angiogenesis and other tissue formation via the cells in the surrounding microenvironment [26]. However, MSCs, especially bone marrow stromal stem cells
trolled by the CMV promoter (Vector BioLabs, Pennsylvania, mouse KLF7 was sub-cloned into an AA V vector cassette designated as a negative control. For AA V-KLF7 viral production, Biolabs, Philadelphia, USA) was created, and AA V-NC was des-

2. Materials and methods

2.1. Preparation of viral vectors

In preparing adeno-associated virus (AAV) virus for use in the present study, a virus containing both empty AAV serotype 2 capsid and the cytomegalovirus immediate-early promoter (CMV) (AAV2-NC, 1.0 × 10^13 viral particles ml⁻¹; Vector Biolabs, Philadelphia, USA) was created, and AAV-NC was designated as a negative control. For AAV-KLF7 viral production, mouse KLF7 was sub-cloned into an AAV vector cassette controlled by the CMV promoter (Vector BioLabs, Pennsylvania, USA; AAV-m-KLF7) as previously described [10].

2.2. BMSC isolation and culture

BMSCs were isolated from adult female mice and cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS, Gibco, US) (culture medium) as previously described [31]. Briefly, bone marrow was flushed out of the marrow cavity with culture medium, followed by centrifugation and supernatant removal. Then, the pelleted cells were re-suspended in culture medium, incubated for 48 h, and the non-adherent cells were removed. Then, the adherent cells were cultured by replacing the medium with fresh culture medium every 3 d, the adherent cells were labeled passage 0 (P0), and cells that had reached 85% confluence were passed into 6-well plates and grown to approximately 90% confluence. Then, the cells were pre-treated with 4–6 µg ml⁻¹ polybrene (Sigma, USA) for 30–60 min followed by infection with AAV-NC and AAV-KLF7 (6.5 × 10^13 viral particle ml⁻¹) (MOI) (150 µl/well) of 4 for 12 h [32]. After 3 d, the infection medium was replaced with fresh growth medium. Then, cells seeded in 6-well plates were lysed and collected for protein analysis. The expression of KLF7 was assessed via Western blot and RT-PCR. The stable infected BMSCs were divided into three groups: (1) normal BMSCs, (2) AAV-NC-BMSCs, and (3) AAV-KLF7-BMSCs.

2.4. Western blot analysis

Western blot for protein analysis was performed as described in prior studies [33]. In brief, for in vivo studies, each BMSCs sample was homogenized 3 d following transfection with AAV-KLF7. Protein samples (20 µg) were loaded and separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Next, the membranes were incubated overnight at 4 °C with primary antibodies against KLF7 (1:500; Novus Biologicals, Colorado, USA) followed by incubation with a horse-radish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature (1:5000). Labeled proteins were visualized on the membranes using an enhanced chemiluminescence (ECL) plus kit (GE Healthcare, Little Chalfont, UK).

2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA analysis to quantify protein was performed as described in prior studies [20]. In brief, to examine whether NGF was secreted from BMSCs infected with KLF7, conditioned medium all groups of cells were collected and quickly frozen in a dry ice/ethanol bath and kept at −80 °C. The amount of NGF protein was determined by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocol. The experiments were performed in triplicate.

2.6. BMSCs proliferation assay

To assess the effects of KLF7 transfection on BMSC proliferation, all groups of cells were seeded at a density of 1.25 × 10^4 cell per well in 96-well plates and incubated with complete culture medium for different time periods (1, 2, 4, 6, and 8 d). Fresh medium was replaced every 24 h. At the designated time point, cell viability was assessed using a 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay (Promega, USA).

Following eight days of transfection, the cells from each group were quantified and then seeded onto glass coverslips (2.0 × 10^2 cell ml⁻¹). The glass coverslips were pre-coated with poly-D-lysine (200 µg ml⁻¹) after sterilization in alcohol overnight. Following seeding, the cells were incubated with 5-ethyl-2-deoxyuridine (EdU) for 4 h to label proliferating cells. After EdU treatment, the cells were fixed with 4% paraformaldehyde solution for 10 min at room temperature, followed by EdU immunostaining using the
Cell-Light™ EdU DNA Cell Proliferation Kit according to the manufacturer’s instructions (Ribobio, USA). To evaluate BMSC proliferation, the EdU labeling index was calculated by dividing the number of EdU+ nuclei by the total number of DAPI+nuclei from five random fields. All experiments were performed in triplicate.

2.7 Assessing BMSCs viability in starvation conditions

To determine the viability of BMSCs under conditions of nutrient deprivation, cells from each group were seeded into 96-well plates (1.25 × 10^6 cells/well) and incubated with DMEM lacking FBS for 1, 2, 4, 6, or 8 d [34]. Medium was refreshed daily, and cell viability was assessed by MTT assay at the designated time points as described above.

A TdT-mediated dUTP nick end labeling (TUNEL) apoptosis kit (Roche) was utilized to analyze the extent of apoptosis in the different BMSC groups. Briefly, the cells were incubated in the dark with the provided TUNEL reaction mixture for 1 h at 37 °C in a humidified incubator. Following labeling, the cell samples were coverslip mounted using ProLong Gold antifade reagent (Invitrogen) and analyzed using an epifluorescent microscope, at least 100 BMSCs were counted in eight random fields of each group under a fluorescence microscope to calculate the percentage of Tunel-positive cells. All analyses were performed by an investigator blinded to the treatment conditions.

2.8. Differentiation of BMSCs into Schwann cell-like cells

For the evaluation of the effects of KLF7 transfection on BMSC differentiation status, BMSCs were incubated in serum-free DMEM containing 1 mM β-mercaptoethanol (β-ME) (Sigma-Aldrich, USA) for 24 h. The following day, the culture medium was replaced with DMEM+10% FBS and 35 ng ml⁻¹ all-trans-retinoic acid (RA) (Sigma-Aldrich) to stimulate differentiation. Three days later, the cell groups were treated with induction medium that consisted of DMEM, 10% FBS, 5 μM forskolin (Sigma), 200 ng ml⁻¹ recombinant human heregulin-β1 (HRG) (PeproTech), 10 ng ml⁻¹ basic fibroblast growth factor (bFGF) (R&D Systems), and 5 ng ml⁻¹ recombinant rat platelet-derived growth factor (PDGF)-AB (R&D Systems) for 10 d [20].

Differenated cells were harvested by trypsinization (0.25% trypsin) before immunofluorescence labeling. The cells were plated onto glass cover slips at a density of 10^5 cells ml⁻¹. BMSCs were incubated continuously for 12 h, fixed in 4% paraformaldehyde for 20 min at room temperature, and washed with 0.01 M PBS three times. All sections were first blocked with 2% normal bovine serum for 1 h, followed by overnight incubation at 4 °C with primary antibody against S-100 (1:200; Sigma Aldrich) and DAPI (1:200; Sigma Aldrich). After several washes, incubation with a fluorescent goat anti-rabbit IgG (FITC) (1:200; Sigma Aldrich) was performed followed analysis of the ratio of S100+cells using an Olympus BX41 epifluorescent microscope. All analyses were performed by an investigator blinded to the treatment conditions.

2.9. Animals

For in vivo experiments, 48 C57BL/6 (24 males and 24 females; weight 18–22 g; eight weeks old) and 24 adult female CD1 (ICR) mice were obtained from the Experimental Animal Center of China Medical University (Certification No. SCXK Liao 2003-0009). The mice were housed with up to four animals per cage per cage, all with ad libitum access to food and water. ICR mice served as nerve and BMSC donors, and C57BL/6 mice were utilized as graft recipients. All animal experiments were reviewed and approved by the Animal Care and Use Committee of China Medical University.

2.10. Seeding of KLF7-BMSCs into ANA

ANA were prepared as previously described [10]. Briefly, 24 CD1 (ICR) mice were anesthetized and both sciatic nerves were surgically exposed and 10 mm nerve segments were removed from lumbosacral region of the sciatic nerve to its terminal divisions [35]. Both ends of the 18 isolated nerve segments were fixed and incubated for 4 d at 4 °C with 0.05 M Tris-HCl buffer with added protease inhibitors (aprotinin at 0.1 μg ml⁻¹, leupeptin at 0.5 μg ml⁻¹, pepstatin A at 0.6 g ml⁻¹). After incubation, DNase I (5 ng ml⁻¹) and RNase A (1 U ml⁻¹) were added to Tris-HCl buffer with 3% Triton X-100 (pH 7.4), and this solution was used to digest the nerves for 10 h.

To label cultured BMSCs, fluorescent tracking dye PKH26 was added to the cultures prior to engraftment, according to the manufacturer’s instructions (Sigma-Aldrich). For BMSC engraftment into the ANA, a total of 2 × 10⁶ BMSCs in 10 μl complete medium was injected at four evenly spaced locations along the nerve segment using a Hamilton syringe with a 30 gauge needle and a micro-injector. Leakage of liquid from the nerve was avoided, and the nerve appeared distended immediately after injection. The ANA treatment groups were as follows: (1) injection of DMEM only, (2) BMSCs only (BMSCs), and (3) BMSCs infected with AAV-KLF7 (KLF7-BMSCs).
Following injections, the ANAs were then incubated in complete medium in 5% CO₂ humidified environment at 37 °C for 48 h. After this incubation period, the ANAs were then used for in vivo experiments.

2.11. Experimental groups and surgical procedures

All mice were randomly divided into three groups (n = 16/group): Group I: ANA group (ANA treated with DMEM); Group II: ANA+BMSCs group (ANA treated with BMSCs); Group III: ANA+KLF7-BMSCs group (ANA treated with KLF7-BMSCs). Establishment of a sciatic nerve gap defect and ANA implantation were performed as described previously [10, 36]. In brief, mice were shaved over the legs using electric clippers and the skin with scrubbed with betadine solution and 70% alcohol wipes before surgery. Mice were anesthetized via intraperitoneal (i.p.) injection of 10 g l⁻¹ chloral hydrate (100 mg kg⁻¹) and the right sciatic nerve was exposed. The nerve segment was transected, gently retracted, and transected again to establish a 1 cm sciatic nerve gap, and the nerve gap was bridged in the three groups using one of the three designated ANA engraftment paradigms. To bridge the nerve gap with an ANA, the nerve graft was sutured to the proximal and distal nerve stumps using a 2/0 nylon suture (figure 1). Muscles and skin were closed in layers, and mice were placed on a heated blanket and maintained at 37 °C until complete recovery from anesthesia.

2.12. HRP retrograde neural tracing

We performed retrograde tracing using HRP-conjugated cholera toxin B subunit (BHRP) based on previously published methods [37]. In brief, 26 d post-surgery, all mice were re-anesthetized. Tibialis anterior (TA) muscles were exposed and BHRP (2 µl, 0.2%; List Biological, Inc) was distributed by injection over three regions within each muscle using a Hamilton microsyringe and a 25-gauge needle. The injection needle was coupled to the microsyringe by a polyethylene tube. The BHRP were delivered slowly by means of a mechanical microdrive and the needle left in place for at least 2 min after injection. Using this approach, leakage of dye from the muscle belly was avoided. L3–L5 ipsilateral ventral horns were retrogradely labeled, which showed detailed morphology of the associated motoneurons and dendritic arbors. Forty-eight hours post-BHRP injection, the mice were weighed and euthanized via Nembutal (60 mg kg⁻¹, i.p.) followed by transcranial perfusion with saline and ice-cold fixative solution (1% paraformaldehyde/1.25% glutaraldehyde). In each tissue section, all labeled cells were quantified and a total calculated per animal, a section incubated without BHRP was stained as a negative control (supplemental figure 1). All analyses were performed by an investigated blinded to the treatment conditions.

2.13. Real-time PCR analysis

To analyze KLF7 and neurotrophic factor gene expression by BMSCs in vitro, BMSC samples were homogenized 3d following viral transfection. For assessment of in vivo expression, ANAs from the different groups were isolated and frozen on dry ice four weeks post-engraftment.

Primer sequences used for real-time quantitative PCR (RT-PCR) (Sangon Biotech Co., Ltd (China) are detailed in table 1. To assess gene expression in isolated ANAs, total RNA was extracted with Trizol reagent (Life Technologies, US), and purified RNA was diluted to 500 ng µl⁻¹. For the synthesis of cDNA, 3 µl purified diluted RNA was processed with a PrimeScript® RT reagent kit (TaKaRa, China) according to the manufacturer’s instructions. Once the amplification curve crossed the threshold line, this was designated as the critical threshold (CT), and the cycle number at this point was noted. Gene expression level was determined using the comparative CT (2⁻ΔΔCT) method. β-actin was used as the housekeeping control gene.

2.14. Immunofluorescence tissue labeling

For determining innervation and Schwann cell migration into the ANA following engraftment, immunofluorescence labeling was performed based on method utilized in our previous work [10]. Briefly, 25 µm-thick longitudinal or transverse sections of the grafted nerve were serially cut using a cryostat and mounted onto Superfrost Plus microscope slides (Menzel-Glaser, Braunschweig, Germany). Tissues were then

Table 1. Oligonucleotide sequences and product sizes of primers.

| Gene  | Sequence                       | Product size |
|-------|--------------------------------|--------------|
| NGF   | ACC TCT CGA ACT CTG GA          | 168 bp       |
| BDNF  | GTC CGT GCC TGT GGG CTG AT      | 214 bp       |
| CNTF  | GGT CAC AGT CAG GGA AAA AG      | 519 bp       |
| KLF7  | CCCATAATGGCTCCTCATGTC           | 219 bp       |
| GDNF  | TTTCTCTGGCAGTCATCCTGC           | 147 bp       |
| β-actin | CCGATCTATGAGGTTACG             | 150 bp       |

Oligonucleotide sequences and product sizes of primers.
immunolabeled with antibodies against neurofilament (NF; Rabbit polyclonal IgG; N4142; 1:200; Sigma Aldrich) to label axons, S-100 (Rabbit polyclonal IgG:1:200; HPA015768; Sigma Aldrich) to label Schwann cells, and ChAT (Mouse monoclonal IgG:1:200; AMAB91130; Sigma Aldrich) to label motor axons. Positive immunolabeling was visualized by incubation with FITC-conjugated anti-rabbit secondary antibody (1:200; Goat Polyclonal IgG; F0382; Sigma Aldrich) or FITC conjugated anti-mouse secondary antibody (1:200; Goat Polyclonal IgG; F5262, Sigma Aldrich) and imaged using an Olympus BX41 epifluorescent microscope. MetaMorph software (Molecular Devices, Inc.) was used acquire images and analyze the fluorescent signal by semi-quantification of the integrated optical density of positive immunolabeling. All analyses were performed by an investigated blinded to the treatment conditions.

2.15. Histologic analysis

Nerve regeneration was histologically assessed utilizing previously published methods [9, 10]. In brief, tissue samples excised from the middle of regenerating ANAs were fixed overnight in a solution containing 2% glutaraldehyde and 5% sucrose in 0.1 M sodium cacodylate buffer (pH 7.4). The following day, a 1h incubation was performed in 1% osmium tetroxide in the same buffer. The tissue was embedded in Spurr’s epoxy resin and cured at 70 °C, and then cut into 1 μm thick semi-thin sections and 70 nm thick ultrathin sections. The semi-thin sections were stained with Toluidine Blue O solution (1% in sodium borate; 198161; Sigma Aldrich). Ten randomly selected fields were captured for each section from each group to assess axon myelination, thickness of the myelin sheath, and axon diameter by Stereo Investigator software (MicroBrightField). Subsequently, the ultrathin sections were collected on copper mesh grids (600 bars per inch), and counterstained with 4% uranyl acetate in 50% ethanol and Reynolds’ lead citrate. Finally, sections were examined using Hitachi H600 electron microscope with an accelerating voltage of 75 kV.

2.16. Wet weight ratio and motor endplate analysis in skeletal muscle

Twenty-eight days post engraftment, TA muscles on the injured and contralateral sides were collected from the anesthetized mice, and immediately weighed. A muscle wet weight ratio was determined as the wet weight of TA muscle on the injured side divided by the wet weight of the contralateral TA muscle.

A muscle sample was harvested from mid-TA muscle belly, and motor endplate densities were assessed after staining for acetylcholinesterase using the Roots–Karnovsky method, as previously described [10, 38]. In Brief, The TA muscles were fixed overnight and then transferred to sucrose phosphate buffer (10% w/v, pH 7.4). Muscles were then rinsed in distilled water, blocked into proximal and distal segments, and flash-frozen in 2-methylbutane. Muscle segments were sectioned longitudinally and thaw-mounted onto glass slides. Motor endplate density was assessed after staining for acetylcholinesterase using the Roots–Karnovsky method. An average of 35 muscle fibers and 30 endplates were measured per animal at a final magnification of 500×. The number of motor endplates per muscle fiber was estimated by counting the number of muscle fibers and endplates in a grid (1 mm × 1 mm) from a randomly selected region of the muscle section (one sample field per section, five muscle sections per animal). An average of 150 muscle fibers per animal was examined. Fiber and endplate areas within each animal were then averaged for statistical analysis.

2.17. Sciatic function index (SFI) assessment

For the assessment of hindlimb function following sciatic nerve engraftment, the SFI was utilized [39]. Scoring for the SFI was performed weekly post-surgery by a research assistant blinded to animal treatment groups. The hindpaws of the mice were coated in ink, and the mice were allowed to freely walk across a plastic tunnel covered in paper. The pawprints left by the mice on the paper during crossing were evaluated, and the following parameters were measured: distances between the third toe and heel (PL), first and fifth toe (TS), and second and fourth toe (ITS) of the hindpaw on the side that received surgery (EPL, ETS, and EITS, respectively) and on the unaffected contralateral side (NPL, NTS, and NITS, respectively). The SFI was calculated according to the following formula: SFI = −38.3 × (EPL − NPL)/NPL + 109.5 × (ETS − NTS)/NITS + 13.3 × (EITS − NITS)/NITS − 8.8.

2.18. Electrophysiological analysis of neuromuscular function

Electrophysiological analysis was performed on all animals on day 28 prior to sacrifice to determine the level of neuromuscular function following sciatic nerve graft. All animals were placed under anesthesia (pentobarbital 40 mg kg−1 i.p.), and the sciatic nerve was exposed and stimulated via a pair of needle electrodes positioned at the sciatic notch with single electrical stimulations of 0.05 ms until achieving supramaximal intensity. Amplitude measurements above 0.15 mV and latencies shorter than 25 ms were considered physiological responses. Small needle electrode was placed in the medial gastrocnemius and plantar muscles (at the third interosseous space) for recording compound action potentials following sciatic nerve stimulation. A storage oscilloscope was used to visualize evoked action potentials (Sapphire 4M, Medelec Vickers) at the appropriate settings to measure amplitude from baseline to peak and latency to onset. Next, compound action potential amplitude (CAMP) and latency period were recorded [40].

2.19. Statistical analysis

Statistical analyses were performed using SPSS software (v.13.0; SPSS, Illinois, USA). And all values were expressed as mean ± standard deviation. Determination of sample sizes
was achieved through calculation of the minimum number of animals or assessments required for adequate power of analysis using statistical software. All groups in this study included greater than the calculated minimum number of animals/experiments.

Statistical tests included a two-tailed Student’s t tests for comparison between two groups, and a one-way ANOVA with Tukey’s post hoc test was used to compare three or more groups in an individual experiment. A P value < 0.05 was considered statistically significant.

3. Results

3.1. BMSC morphology and phenotype

At passage 1, 2 and 4, cellular morphology was consistent with the morphology of BMSCs as observed under inverted microscope (figure 2(A)). For identification of BMSCs, cells were labeled using DAPI (for the nucleus) and either CD29, CD90, CD34, or CD45 immunolabeling. Positive labeling of BMSCs showed double-labeling by DAPI and cytoplasmic MSC markers CD29 and CD90 (figures 2(B) and (C)); however, BMSCs were negative for hematopoietic stem cell markers CD34 and CD45 (figures 2(D) and (E)).

3.2. AAV-KLF7 increased KLF7 and NGF expression in infected BMSCs in vitro

In comparison to the AAV-NC and normal groups, the KLF7 protein and mRNA expression levels were significantly elevated in the AAV-KLF7 group, as anticipated (protein, $F_{2,6} = 91.31, P < 0.001$; mRNA, $F_{2,15} = 46.88, P < 0.001$) (figures 3(A) and (B)).

Likewise, NGF mRNA expression was also higher in AAV-KLF7 cells compared to the other groups ($F_{2,15} = 16, P < 0.01$) (figure 3(C)). However, no significant difference
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in KLF7 and NGF mRNA expression was observed between cells in the normal and AA V-NC groups. Moreover, NGF expression in secretions from the three groups of BMSCs was similar ($F_{2,15} = 11.20, P < 0.01$) (figure 3(D)).

These results clearly indicate successful infection of BMSCs by AA V-KLF7 resulting in significantly increased expression of KLF7 and its target gene, NGF, compared to the control groups not overexpressing KLF7.

3.3. AAV-KLF7 promoted BMSCs proliferation in vitro

The proliferation capabilities of KLF7-BMSCs was investigated through EdU labeling at 8 d post-transfection (figure 4(A)). BMSCs in the AAV-KLF7 group exhibited a greater number of EdU+cells than the AAV-NC and normal groups ($F_{2,15} = 40.95, P < 0.001$) (figure 4(B)), suggesting overexpression of KLF7 enhances BMSC proliferation. Cell proliferation was also analyzed via MTT assay. Proliferation of BMSCs was significantly increased at day 8 in AAV-KLF7 cells compared with cells in the control groups ($F_{2,15} = 40.95, P < 0.001$) (figure 4(C)). These findings further indicate that KLF7 overexpression promotes the proliferation of BMSCs.

3.4. AAV-KLF7 enhanced survival of BMSCs in vitro

To determine the influence of KLF7 on BMSC survival, AAV-KLF7, AAV-NC and normal BMSCs were starved using DMEM alone without serum. Apoptotic BMSCs in all three groups of cultured BMSCs are shown at 3 days in vitro ($n = 3$). Graphs from the respective analyses represent the relative density of KLF7 protein expression, (B) and (C) RT-PCR revealed the relative expression of KLF7 and NGF mRNA in the three treatment groups of BMSCs at 3 d in vitro ($n = 3$), (D) ELISA analysis determined NGF levels secreted from the three groups of BMSCs at 3 d in vitro ($n = 3$). Error bars = SD. "P < 0.01, ""P < 0.001. NS, not significant. Data were analyzed with a one-way ANOVA followed by Tukey’s post hoc test.

Figure 3. Efficacy of in vitro AAV-KLF7 gene transfer to BMSCs (A) Representative Western blots of KLF7 expression in the three groups of cultured BMSCs are shown at 3 days in vitro ($n = 3$). Graphs from the respective analyses represent the relative density of KLF7 protein expression, (B) and (C) RT-PCR revealed the relative expression of KLF7 and NGF mRNA in the three treatment groups of BMSCs at 3 d in vitro ($n = 3$), (D) ELISA analysis determined NGF levels secreted from the three groups of BMSCs at 3 d in vitro ($n = 3$). Error bars = SD. **P < 0.01, ***P < 0.001. NS, not significant. Data were analyzed with a one-way ANOVA followed by Tukey’s post hoc test.
An MTT assay indicated that cell survival in the AA V-KLF7 group was significantly higher than the AA V-NC and normal BMSC groups at days 2, 4, 6 or 8 (2d: $F_{2,6} = 32.25, P < 0.001$; 4d: $F_{2,6} = 27.56, P < 0.001$; 6d: $F_{2,6} = 36.69, P < 0.001$; 8d: $F_{2,6} = 41.37, P < 0.001$) (figure 5(C)). These results suggest that KLF7 overexpression is beneficial for BMSC survival and protects against starvation-induced apoptosis in vitro.

**Figure 4.** KLF7-overexpressing BMSCs exhibit increased proliferation in vitro (A) The total number of the cells shown by DAPI (blue) staining, the number of proliferating BMSCs was determined through EdU (red) staining. (B) EdU labeling index of AAV-KLF7, AAV-NC and Normal group at day 8. (C) AAV-KLF7, AAV-NC and Normal group BMSCs were incubated for days 1, 2, 4, 6, or 8. Cell proliferation was assessed by MTT assay. Error bars = SD. ***$P < 0.001$, NS, not significant. Data were analyzed with a one-way ANOVA followed by Tukey’s post hoc test. Scale bars = 100 µm.

**Figure 5.** KLF7 enhances BMSCs survival in vitro (A) AAV-KLF7, AAV-NC and normal BMSCs were serum-starved and treated with only DMEM for 8 d. A TUNEL assay was used to detect apoptotic cells. (B) The percentage of apoptotic cells in each treatment group. (F) Each group BMSCs were incubated with DMEM only (without serum) for 1, 2, 4, 6, or 8 d. An MTT test was used to assess cell viability. Error bars = SD. ***$P < 0.001$, NS, not significant. Data were analyzed with a one-way ANOVA followed by Tukey’s post hoc test. Scale bars = 100 µm.

An MTT assay indicated that cell survival in the AAV-KLF7 group was significantly higher than the AAV-NC and normal BMSC groups at days 2, 4, 6 or 8 (2d: $F_{2,6} = 32.25, P < 0.001$; 4d: $F_{2,6} = 27.56, P < 0.001$; 6d: $F_{2,6} = 36.69, P < 0.001$; 8d: $F_{2,6} = 41.37, P < 0.001$) (figure 5(C)). These results suggest that KLF7 overexpression is beneficial for BMSC survival and protects against starvation-induced apoptosis in vitro.
3.5. AAV-KLF7 improved Schwann-like cell differentiation of BMSCs

Ten days following induction, BMSCs were morphologically similar to SCs by day 8, presenting a narrow fusiform-like shape. Immunofluorescence images show S100+ cells labeled in green, and cell nuclei labeled in blue. Graphs represent the quantification and analysis of the ratio of S100+ cells (B) and surviving cell number (C). n = 3, Error bars denote SD. **P < 0.01, NS, not significant. Data were analyzed with a one-way ANOVA followed by Tukey’s post hoc test. Scale bars = 100 µm.

Figure 6. KLF7 improves Schwann-like cell differentiation of BMSCs (A) in the photomicrographs, each group BMSCs showed similar morphology to SCs by day 8, presenting a narrow fusiform-like shape. Immunofluorescence images show S100+ cells labeled in green, and cell nuclei labeled in blue. Graphs represent that quantification and analysis of the ratio of S100+ cells (B) and surviving cell number (C). n = 3, Error bars denote SD. **P < 0.01, NS, not significant. Data were analyzed with a one-way ANOVA followed by Tukey’s post hoc test. Scale bars = 100 µm.

3.6. KLF7-overexpression promoted transplanted BMSCs survival in regenerating ANAs

ANAs were sectioned longitudinally to assess myelinated axon regeneration and survival of transplanted BMSCs in regenerating ANA, and visualized by immunohistochemistry (figure 7(A)). The BMSCs within the ANA from the ANA+KLF7-BMSCs group presented significantly increased PKH26-labeling compared to ANA+BMSCs groups (t = 8.828, df = 6, P < 0.001); however, no PKH26-labeling of BMSCs was detectable in ANA only groups (figure 7(B)). The merged images show yellow fluorescence indicating colocalization of PKH26 and S100. From this evidence, we inquired whether implanted BMSCs may differentiate into Schwann-like cells in vivo. Differentiated
Figure 7. KLF7-BMSCs significantly increased S100 (myelinated fiber) expression and transplanted BMSCs survival (PKH26) in regenerating ANAs. Representative immunofluorescence images of S100 (green) and a fluorescent tracking dye PKH26-labeled BMSCs (red) in ANA in longitudinal sections from all three ANA transplant groups 28 d post-surgery. The high magnification inserts of boxed area of interest show red labeled BMSC do not co-localize much with green S100 staining (A). n = 6 mice/group. The number of PKH26 positive cells (B), the number of double-labeled PKH26 and S100+ cells (C), PKH26 and S100 colocalization ratio of the total PKH26 positive cells (D), S100 protein expression (E) immunohistochemical labeling for S100. Error bars = SD. ***P < 0.001, NS, not significant. Data were analyzed with a one-way ANOVA followed by Tukey’s post hoc test. Scale bars = 20 μm.
cells exhibited double labeling between PKH26 and S100 in the ANA+KLF7-BMSCs group, and this co-localization was significantly elevated compared to ANA+BMSCs groups ($t = 6.349$, $df = 6$, $P < 0.001$) (figure 7(C)). We identified no significant difference in Schwann-like differentiated ratio between the ANA+KLF7-BMSCs and ANA+BMSCs groups ($t = 0.58$, $df = 6$, $P > 0.05$) (figure 7(D)).

Intensity quantification of S100 labeling demonstrated that sectioned nerve tissue from ANA+KLF7-BMSCs and ANA+BMSCs groups showed significantly increased levels of peripheral myelin compared to the ANA group. Furthermore, S-100 expression was significantly greater in the ANA+KLF7-BMSCs group compared to the ANA+BMSCs group ($F_{2,15} = 66.10$, $P < 0.001$) (figure 7(E)).

Taken together, the above results indicate that ANA+KLF7-BMSCs treatment increases survival of BMSCs in ANAs; however, KLF7 overexpression was not able to increase the differentiation of BMSCs into Schwann-like cells in the nerve grafts.

### 3.7 KLF7-BMSCs enhanced axon and myelinated fiber regeneration in regenerating ANAs

To evaluate the regeneration of peripheral axons into the ANAs, a marker of neurofilament labeling of axons (NF200) and PKH26-labeled BMSCs in transverse sections in the middle of grafts were analyzed. We found that NF200 was significantly elevated in ANA+KLF7-BMSCs and ANA+BMSCs groups compared to the group that received the ANA transplantation alone. In addition, NF200+labeling in the ANA+KLF7-BMSCs group was significantly greater than in the ANA+BMSCs group ($F_{2,15} = 114.8$, $P < 0.001$) (figures 8(A) and (B)).

To assess the number and of myelination quality of axons within the ANAs, toluidine blue staining and TEM was performed. The result showed that the number of myelinated axons in the ANA+BMSCs and ANA+KLF7-BMSCs groups were increased compared to the ANA group. Importantly, total myelinated axons observed in the ANA of the ANA+KLF7-BMSCs group was significantly greater in comparison to the ANA+BMSCs group ($F_{2,15} = 100.0$, $P < 0.001$) (figures 8(A) and (C)). Myelin thickness and axon diameter within the ANA corresponded with the number of myelinated axons across the three groups (myelin sheath thickness: $F_{2,15} = 40.41$, $P < 0.001$; axon diameter: $F_{2,15} = 19.56$, $P < 0.001$) (figures 8(A), (D) and (E)). Furthermore, TEM indicated that myelin sheath degeneration was more pronounced in the ANA group compared to the ANA+BMSCs and ANA+KLF7-BMSCs groups (figure 8(A)). These results demonstrate that the quantity and quality of myelinated nerve fibers were increased in the ANA+KLF7-BMSCs group compared to other groups.

### 3.8 KLF7-BMSCs significantly increased KLF7, NGF, CNTF, BDNF, and GDNF expression in regenerating ANA

To verify successful transplantation of KLF7-BMSCs, KLF7 expression was assessed using RT-PCR in ANA tissues harvested at 28 d following surgery (figure 9(A)). In the ANA and ANA+BMSCs groups, KLF7 was expressed at a low level; however, animals receiving the KLF7-BMSCs nerve allograft showed increased KLF7 protein expression ($F_{2,6} = 46.88$, $P < 0.001$) (figure 9(A)). Meanwhile, to identify a possible mechanism of treatment effects of KLF7-BMSCs in nerve regeneration, we assessed expression of the trophic factors NGF, CNTF, BDNF, and GDNF in ANA tissues (figure 9(B)). We found that the ANA+KLF7-BMSCs and ANA+BMSCs groups exhibited a dramatic increase in NGF, CNTF, BDNF, and GDNF expression in the graft compared to engrafted ANA lacking both BMSCs and KLF7. Notably, The expression of NGF, CNTF, BDNF, and GDNF in the ANA+KLF7-BMSCs group significantly elevated over levels observed in the ANA+BMSCs group (NGF: $F_{2,6} = 88.93$, $P < 0.05$; CNTF: $F_{2,6} = 51.23$, $P < 0.001$; BDNF: $F_{2,6} = 27.31$, $P < 0.001$; GDNF: $F_{2,6} = 23.15$, $P < 0.001$) (figures 9(B)–(F)).

These results show that KLF7-BMSCs upregulate KLF7 expression and induced increased NGF, CNTF, BDNF, and GDNF expression in regenerating ANAs. We believe KLF7-BMSCs can promote axonal myelination though strengthening the survival of BMSCs by promoting them to produce neurotrophic factors rather than Schwann-like cell differentiation.

### 3.9 KLF7-BMSCs enhance motor axon regeneration and protects target musculature

When we assessed the number of HRP-labeled motor neurons in the ipsilateral ventral horns (figure 9(A)), HRP-labeled motor neurons were significantly increased in the ANA+KLF7-BMSCs and ANA+BMSCs groups compared to the ANA group. Furthermore, HRP-labeled motor neurons in the ANA+KLF7-BMSCs group was elevated compared to the ANA+BMSCs group ($F_{2,6} = 79.25$, $P < 0.001$) (figure 10(D)).

To determine whether KLF7-BMSCs could beneficially affect TA muscle motor function recovery, TA motor endplate density was assessed post-injury (figure 10(B)). The number of motor end plates was elevated in the ANA+BMSCs and ANA+KLF7-BMSCs groups in comparison to the ANA group. In particular, the number of observable motor end plates was significantly less in the ANA+BMSCs group compared to the ANA+KLF7-BMSCs group ($F_{2,15} = 18.12$, $P < 0.001$) (figure 10(E)). ChAT immunostaining provide a reliable method to evaluate the outgrowth rate of motor axons. The expression of ChAT in regenerating ANAs showed similar patterns of change in the three groups ($F_{2,15} = 28.33$, $P < 0.001$) (figure 10(G)). These results demonstrated that KLF7-BMSCs positively influenced motor neuron axonal regeneration and reinnervation of denervated TA muscle.
Figure 8. KLF7-BMSCs promote axon and myelinated fiber regeneration in regenerating ANAs (A) representative immunofluorescent images for neurofilament (NF200, green) and a fluorescent tracking dye PKH26-labeled BMSCs (red) in transverse sections, histologic photomicrographs of toluidine blue staining, and transmission electron microscope (TEM) analysis of axon and myelinated fibers in regenerating ANAs of the three treatment groups (n = 6). Quantification of NF200 protein expression (B), myelinated fiber number (C), myelin sheath thickness (D) and axon diameter (E) in the center of ANAs are shown. Error bars = SD. *P < 0.05, **P < 0.01, ***P < 0.001. Data were analyzed with a one-way ANOVA followed by Tukey’s post hoc test. Scale bars = 50 µm or 5 µm.
The TA muscles began to undergo atrophy following sciatic nerve injury. The weight ratios of target TA muscles were higher in the ANA+BMSCs and ANA+KLF7-BMSCs groups compared to the ANA group, and the mean ratios of TA muscle wet weight were significantly increased in the ANA+KLF7-BMSCs group in comparison to the ANA+BMSCs group ($F_{2,15} = 44.05$, $P < 0.001$) (figure 10(F)). Our findings indicate that KLF7-BMSCs prevent or reverse target muscle atrophy following sciatic nerve injury.

Figure 9. KLF7-BMSCs significantly increased KLF7, NGF, CNTF, BDNF, and GDNF expression in regenerating ANA. (A) Representative RT-PCR images of KLF7 (A), NGF (B), CNTF (C), BDNF (D), GDNF (E) mRNA expression in harvested ANA tissues from the three treatment groups 28 d post-injury. $n = 3$ mice/group. Error bars = SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS, not significant. Data were analyzed with a one-way ANOVA followed by Tukey’s post hoc test.
3.10. KLF7-BMSCs significantly improves motor functional recovery of regenerating ANAs

We evaluated sciatic nerve function utilizing the SFI. The documented SFI values were significantly increased on day 28 in the ANA+KLF7-BMSCs and ANA+BMSCs groups compared to the ANA group (figure 11(A)). Significantly increased SFI scores were observed in the ANA+KLF7-BMSCs group in comparison to the ANA+BMSCs group ($F_{2,21} = 21.11, P < 0.001$).

Next, we measure functional recovery in the groups using electrophysiology (figure 11(B)). The ANA+KLF7-BMSCs and ANA+BMSCs groups demonstrated significantly elevated CAMP values compared to the ANA group ($F_{2,21} = 216.1, P < 0.001$) (figure 11(C)). Meanwhile, a downregulated latency period in the ANA+KLF7-BMSCs and ANA+BMSCs groups was observed ($F_{2,21} = 71.81, P < 0.001$) (figure 11(D)). Furthermore, the group engrafted with ANA+KLF7-BMSCs exhibited an increased electrophysiological response compared to the ANA+BMSCs group. These data demonstrate that KLF7-BMSCs showed the most beneficial effects on motor function recovery.

4. Discussion

This is the first study to investigate the effects of transplantation of an ANA seeded with KLF7-overexpressing BMSCs following sciatic nerve transection injury. Through examination of the effects on both the BMSCs as well as nerve...
regeneration, neuromuscular targeting and muscular atrophy, and functional recovery, our results demonstrate a marked influence on BMSC proliferation and viability in vitro, as well as enhanced regeneration, reduced neuromuscular pathology and recovery of function in vivo following sciatic nerve injury and nerve graft repair. It is known that KLF7 serves major roles in the nervous system, with prior research demonstrating involvement of KLF7 in neuronal sensory-associated functions including olfactory bulb development and protein expression in sensory neurons [18, 41]. KLF7 is well documented to be expressed within the nervous system, and plays a role in development of the brain and spinal cord, as well as the peripheral nervous system [42]. In addition to our own work [10, 43], other research has tied KLF7 to the promotion of regeneration and growth of peripheral neurons [12, 44].

Research has shown that MSCs release EVs that mediate at least in part the paracrine effects of the parental cells. The characterization of the RNA cargo of EVs revealed that EVs are selectively enriched for distinct classes of RNAs such as preferentially expressing mRNA and miRNA targeting transcription factors, which suggests that EVs transport gene regulatory information to modulate cell pathways in recipient cells [26]. However, the influence of KLF7 on BMSCs is not well characterized. Therefore, we first needed to assess how overexpression of KLF7 by BMSCs could affect their viability, proliferation, metabolism, and differentiation. This was important for the full implementation of seeding an acellular nerve graft with KLF7-BMSCs. Our results showed that AAV-meditated expression of KLF7 positively influenced proliferation and viability BMSCs, as well as differentiation status toward Schwann cell lineage. This was encouraging for the potential therapeutic value and success of utilizing the KLF7-BMSCs seeded ANA in our sciatic nerve injury model.

Our prior research showed that Schwann cells engineered to overexpress KLF7 successfully promoted axon regeneration and myelination, and ultimately improved hindlimb function recovery, in our ANA repair model [10, 43]. Other properties of Schwann cells such as proliferation and viability were also influenced by KLF7, suggesting that KLF7 promotion of BMSCs in the current study could affect these properties at the stem cell stage which would increase the pool of BMSCs in the ANA. As KLF7 has been shown to stimulate cellular expression of various trophic factors, these factors also likely played a role in the cell-specific functions within the transplanted BMSC seeded nerve ANA.

We observed upregulation of NGF mRNA expression in KLF7-overexpressing BMSCs in vitro, though in the nerve graft 28 post-surgery, NGF, BDNF, GDNF and CTNF mRNA were all increased over KLF7-BMSCs (figure 9), as was the amount of S100+ cells in the ANA (figure 7), which provides an explanation for enhanced axon regeneration in the nerve grafts seeded with KLF7-BMSCs compared to the non-KLF7 expressing cell groups. All of these trophic factors play some role in the promotion of axonal regeneration.

In the current study, BMSCs were chemically induced to differentiate into Schwann-like cells in vitro (figure 6), using differentiation factors β-ME and RA, followed by a mixture of forskolin, HRG, bFGF, and PDGF[45]. BMSCs have recently been shown to revert Schwann cells into an activated state similar to that caused by nerve injury, which may have contributed to the increased trophic factor gene expression [46]. However, since the activation of myelinating Schwann cells involves de-differentiation to a non-myelinating repair Schwann cell [47], the fact that increased axonal myelination was observed in the KLF7-BMSCs seeded ANA suggest this BMSC-meditated induction this may not have been a major contributor to the observed gene expression pattern, which may be attributable to the production of neurotrophic factors by BMSCs. Still, increased S100+ Schwann cells were observed in the KLF7-BMSC seeded ANA, so KLF7 stimulated either enhanced migration of Schwann cells or their proliferation once in the graft, and the cells likely matured toward a myelinating phenotype upon interaction with regenerating axons [48]. However, other impacts of the ANA microenvironment on the differentiation of BMSCs into Schwann cells is yet to be investigated. As described in our recent study, one of the upregulated trophic factors, NGF is a known target gene for KLF7 [16] as well as its receptor, TrkA [17, 18]. When we injected a cellular label to track BMSCs following seeding into the ANA, we observed significantly more PKH26-labeled BMSCs within the graft at 28 d post-surgery in the KLF7-BMSCs group compared to the BMSCs group, which correlates with the increased proliferation and viability of KLF7-overexpressing BMSCs observed in vitro (figures 4 and 5). Taken together, more viable and replicating BMSCs mediated by KLF7 in vivo may increase overall NGF and TrkA expression by the cells and in the graft microenvironment. So, both KLF7 and the potential effects on BMSCs to induce increased presence in the graft played a considerable role in axonal regeneration, myelination, and ultimately, functional recovery. The precise mechanisms of how this complex interaction of factors achieved these beneficial effects remains unclear.

In our assessments of neuromuscular targeting and functional recovery, KLF7-BMSCs increased numbers of ChAT-positive labeled motor axons sprouted and regenerated into the ANA (figure 10). This type of axonal sprouting and regeneration plays an important role in the rebuilding of new motor end plates in denervated muscles [49]. Combined, KLF7-BMSCs promoted a significant reduction in muscle atrophy and increased motor end plates (figure 10), which reflects the increased axon regeneration and myelination observed in this group compared to other groups. These findings strongly indicate that KLF7-BMSCs could indirectly promote motor axonal regeneration and reinnervation of denervated TA muscle, as non-innervated end-plates were also observed.

Likewise, animals that received ANA seeded with BMSCs overexpressing KLF7 showed improved SFI scores, as well as CMAP amplitude and decreased latency in muscle response to electrical stimulation of the sciatic nerve compared to the control groups (figure 11). Since CMAP amplitude is directly proportional to the number of nerve fibers innervating the target muscle and allows the conduction velocity of motor nerves to be calculated, CMAP examinations offer an important index for the conduction function of peripheral nerve [40].
In the spinal cord, retrogradely labeled motor neurons in the KLF7-BMSCs group was significantly greater than observed in the control groups, increased labeling of motor neurons in the L3–L5 ipsilateral ventral horns was likely due to the influence of KLF7-BMSCs in promoting and supporting a regenerated nerve pathway and axonal transport function of regenerated nerve fibers in ANA [40]. Pervious study confirmed the regenerating motor axons preferentially reinnervated the targeted muscles, increased the size of their motor units and, thus, of the CMAP. Still, the presence of BMSCs in the graft increased endplate and motor neuron numbers compared to ANA only, indicating the BMSCs provide some benefit in these parameters without the need for overexpressed KLF7. Nevertheless, our study indicates that the presence of KLF7-BMSCs in an ANA enhanced axonal regeneration and myelination via increased promotion of native Schwann cell migration into the graft. As a result, neuromuscular targeting and subsequent functional recovery of the hindlimb on the injured side was increased. As neurotrophic factors such as NGF, GDNF, BDNF, and CNTF were affected by KLF7-overexpression in BMSCs in vivo, our data strongly suggest KLF7-BMSCs can promote axonal myelination through strengthening the survival of BMSCs by inducing them to produce neurotrophic factors rather than undergo Schwann cell-like differentiation.

4.1. Conclusion

Our study represents a novel investigation of the effects of KLF7 overexpression on BMSC replication, viability and differentiation capacity. Importantly, seeding an acellular nerve graft with KLF7-overexpressing BMSCs promoted enhanced axonal regeneration, myelination, muscular innervation and functional recovery compared to transplantations of BMSCs alone. Together with our previous studies showing similar benefits by transplanting nerve grafts containing KLF7-overexpressing Schwann cells, our research has established a valuable foundation of knowledge concerning the cellular effects of supraphysiologic levels of KLF7, as well its physiologic benefits on different cell types.
and the therapeutic value of treating sciatic nerve injury with nerve grafts transplanted with cells engineered to over-express KLF7. Future studies will further investigate individual cellular mechanisms of the observed benefits, as well as other ways cell-seeded nerve grafts may be manipulated to increase regeneration and functional recovery in peripheral nerve injury.

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ORCID iDs

Guan-Yu Zhu  https://orcid.org/0000-0002-5632-486X
Ying Wang  https://orcid.org/0000-0002-8679-7473
Wen-yuan Li  https://orcid.org/0000-0002-5632-486X

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