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MiR-494-3p regulates cell proliferation and apoptosis via KLF7 in Schwann cells

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Abstract: Peripheral nerve injury is a common neurodegenerative disease, which causes disability and a huge economic burden for patients. MicroRNAs (miRNAs) have been acknowledged as major regulators and therapeutic targets of neurological disease. Thus, the functional studies of miRNAs in neurological disease will contribute to discover new therapeutic targets for peripheral nerve injury. Sprague Dawley rats treated sciatic nerve surgical injury were regarded as peripheral nerve injury model *in vivo*. The expression of miR-494-3p and Kruppel like factor7 (KLF7) were measured by Real-time quantitative polymerase chain reaction (RT-qPCR) assay. In addition, western blot analysis was conducted to measure the protein levels of KLF7, Bax, Bcl-2, and C-caspase 3. Cell viability and apoptosis were detected in Schwann cells by EdU stain and flow cytometry, respectively. The interaction between miR-494-3p and KLF7 was investigated by dual-luciferase reporter assay. The expression of miR-494-3p was reduced at the beginning, but KLF7 was enhanced in Sprague Dawley rats with peripheral nerve injury. Knockdown of miR-494-3p promoted cell proliferation and suppressed apoptosis, while overexpression of miR-494-3p or silencing KLF7 led to opposite results. Moreover, the upregulation of KLF7 attenuated miR-494-3p overexpression-induced suppressive effects on viability and promotion of apoptosis in Schwann cells. MiR-494-3p negatively regulates KLF7 in Schwann cells to mediate proliferation and apoptosis.

Introduction

Previous reports indicated that mechanical compression, ischemia, penetrating injury, and stretch injury could result in nerve injury that is a worldwide trouble and largely affect patients' life quality (Gu *et al.*, 2011). The peripheral nervous system has discrimination from the central nervous system is its intrinsic regenerative power and the repair function of spontaneous peripheral nerve repair after injury, although the regeneration rate is slow and usually far from satisfactory (Chen *et al.*, 2007; Battiston *et al.*, 2009). With the advance of neurological disease pathogenesis in recent decades, multiple targets have been regarded as promising avenues, such as brain-derived neurotrophic factor (BDNF) (Yi *et al.*, 2016), methyl-CpG binding protein 2 (Chahrour *et al.*, 2008), and L-carnitine (Wang *et al.*, 2007). Additionally, microRNAs (miRNAs or miRs) may be key therapeutic targets.

MiRNAs, a novel class of non-coding RNAs, play crucial roles in cancer development (Liz and Esteller, 2016; Zhao *et al.*, 2010). Moreover, deep sequencing profile of miRNAs following sciatic nerve injury had revealed that differential expression of miRNAs was associated with proliferation and differentiation of neural stem cells (Yu *et al.*, 2011; Li *et al.*, 2011). For instance, miR-182 directly targeted fibroblast growth factor 9 to modulate the phenotype of Schwann cells, suggesting that miR-182 is involved in nerve injury (Yu *et al.*, 2012). MicroRNA-338 and microRNA-21 had been indicated to associate with the development of neural tissues after injury (Wang *et al.* 2016). In addition, miR-137 and miR-491 regulated dopamine transporter to mediate aberrant regulation of dopaminergic neurotransmission (Jia *et al.*, 2016). A recent work conducted by (Bremer *et al.*, 2010) demonstrated that 16 high expression of miRNAs may participate in myelination; additionally, some miRNAs were required for Schwann cell ablation of the enzyme Dicer1 (Bremer *et al.*, 2010). Li *et al.* reported that inhibition of let-7 enhanced Schwann cell migration and axon growth by targeting nerve growth factor *in vivo* (Li *et al.*, 2015). Analogously, the overexpression miR-221/miR-222 was found Schwann cell in answer to peripheral nerve injury, while

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miR-221/222 silencing decreased proliferation and migration of Schwann cell *in vitro* (Yu *et al.*, 2012). In summary, emerging evidence suggests that dysregulation of miRNAs may associate with neurological disorders, which was involved in the inflammation, oxidation, and apoptosis (Wang *et al.*, 2012; Liu *et al.*, 2009). Above these studies have implied that miRNAs are one of the important regulators in gene expression for appropriate neural functions. However, the mechanisms of miR-494-3p remain unclear under nerve injury conditions.

In this study, we established the Sprague Dawley rats model treated surgical injury to inspect the expression of Kruppel like factor7 (KLF7) mRNA and miR-494-3p *in vivo* and investigate their association *in vitro*. This study may contribute to providing potential treatment targets for peripheral nerve injury therapeutic in the future.

Materials and Methods

Animal surgery

For the establishment of the peripheral nerve injury model *in vivo*, all animals (Sprague Dawley, SD) used in this study were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). SD rats fed on standard pellet chow and water in an incubator with a 12 h light/dark cycle. SD rats were treated sciatic nerve crush injury by following previous practices (Pepper *et al.*, 2017). Briefly, under sterile surgical conditions, rats were deeply sedated using inhaled isoflurane anesthesia; subsequently, sharp transection at 5 mm above the sciatic notch was performed with forceps to form nerve crush injury. Rats were sacrificed (on 14 d) for measuring the expression of miR-494-3p and KLF7. **The animal experiment was performed according to the protocol, which was approved by the Animal Care Committee of The Affiliated Mindong Hospital of Fujian Medical University.**

Cell culture

Schwann cells RSC96 (ATCC, Manassas, VA, USA) were maintained in F-12 (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS: GIBCO BRL) in an environment with 5% CO_2 and 95% air at 37°C.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA isolated from RSC96 cells with TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) referring to instructions. RNA reverse transcription was exercised by using All-in-One miRNA cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) and Prime Script RT Reagent kit (Thermo Fisher Scientific). After that, the SYBR Green PCR Kit (Thermo Fisher Scientific) was used to test the levels of miR-494-3p and KLF7 under the ABI Step One Real-time PCR System (Thermo Fisher Scientific). The relative expression of miR-494-3p and KLF7 were analyzed normalized to endogenous small nuclear RNA U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta Ct}$ method, respectively.

The special primers were purchased from Sangon (Shanghai, China) and listed: miR-494-3p (F, 5'-GAAACAT ACACGGGAAACC-3'); R, 5'-AAAGAGGTTTCCCGTGTA TG -3');

KLF7 (F, 5'-TTTCCTGGCAGTCATCTGCAC-3'; R, 5'-GGGTCTGTTTGTTTGTCAGTCTGTC-3');

GAPDH (F, 5'-CCTCTCTCTAATCAGCCCTCTG-3'; R, 5'- AGAAGGCTGGGGCTCATTTG-3');

U6 (F, 5'-CTCGCTTCGGCAGCACA-3'; R, 5'-AACG CTTCA CGAATTTGCGT-3').

Western blot

RSC96 cells or tissue samples were lysed with RIPA lysis buffer (Thermo Fisher Scientific) on ice for 30 min. The protein in the supernatant was quantified by using a BCA protein assay kit (Beyotime, Shanghai, China). After denaturation, equal proteins were segregated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then delivered onto polyvinylidene difluoride membranes (Beyotime). Then the blots were incubated with primary antibodies Bax (1:800 dilution; Ambion, Foster City, CA, USA), Bcl-2 (1:800 dilution; Ambion), C-caspase 3 (1:800 dilution; Ambion), KLF7 (1:800 dilution; Millipore, Billerica, MA, USA), ERK (1:800 dilution; Ambion), p-ERK (1:800 dilution; Ambion), JNK (1:800 dilution; Ambion), p-JNK (1:800 dilution; Ambion), or GAPDH (1:1200 dilution; Millipore) at 4°C. After 24 h, and the membranes were interacted with HRP-conjugated secondary antibody (1:2000 dilution; Ambion) for 2 h after washed twice times. Blots were visualized via commercial enhanced chemiluminescence chromogenic substrate (Beyotime).

Vector construction and transfection

MiR-494-3p mimic (miR-494-3p) and control miR-NC, miR-494-3p inhibitor (anti-miR-494-3p) and control (anti-NC), specific small interfering RNA (siRNA) against KLF7 (si-KLF7) and negative control (si-RNA), KLF7 overexpression vector (KLF7) and empty vector (vector) were obtained from Beyotime. Lipofectamine 2000 reagent (Thermo Fisher Scientific) was used to infect RSC96 cells with 50 nM of synthetic oligonucleotides or 1 μg of vectors by referring instructions. After transfection for 48 h, cells were collected for further analysis.

Cell viability

Cell-Light^{TM} EdU DNA Cell Proliferation Kit (Beyotime) was applied for measuring cell viability value. RSC96 were placed into 96-well plates (5×10^3 cells/well) and grew at 37°C with 5% CO_2 overnight, and then incubated with 50 μM of EdU. After fixation, cells interacted with a Hoechst33342 reaction cocktail for another 30 min. All steps were executed at room temperature. We counted the number of EdU-labeled nuclei under the fluorescence microscope (Bio-Rad, Hercules, CA, USA).

Cell apoptosis

The cell apoptosis rate was measured with Annexin V-FITC/PI kit (Solarbio, Beijing, China) under flow cytometry. Briefly, post-transfected for 24 h, RSC96 cells were harvested and stained with 5 μL Annexin V-FITC and PI for 10min in the dark. Next, flow cytometry was performed to test the apoptotic rates of RSC96.

Dual-luciferase reporter assay

The putative relationship between miR-494-3p and KLF7 was predicted by TargetScan. The 3'UTR sequences of KLF7 containing binding sites of miR-494-3p were amplified by PCR inserted into pGL3-basic vectors (Realgene, Nanjing, China), named as KLF7 WT 3'-UTR. KLF7 MUT 3'-UTR was constructed by Quik-Change II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). RSC96 cells co-transfected with 40 nM miR-494-3p or miR-NC and 100 ng corresponding luciferase reporter vector using Lipofectamine2000 (Thermo Fisher Scientific). After the post-transfection 48 h, luciferase activity was examined.

Statistical analysis

In this study, we used SPSS (IBM, Chicago, IL, USA) for statistical analysis. The data were shown as mean ± SD from three independent experiments. The comparisons between the two groups were conducted using Student's *t*-test, while multiple group comparisons were assessed using a one-way analysis of variance (ANOVA). If $p < 0.05$, the difference was regarded as statistically significant.

Results

MiR-494-3p was downregulated while KLF7 was upregulated after sciatic nerve injury

To begin with, Sprague Dawley rats with sciatic nerve injury model was established to explore the potential roles of miR-494-3p and KLF7 *in vivo*. The time-dependent differential expression of miR-494-3p was measured by RT-qPCR assay. The results implied that the abundance of miR-494-3p was decreased first and then rose, reaching a minimum at 4 d (Fig. 1A). Conversely, KLF7 mRNA levels were gradually increased until 7 d but downregulated at 14 d (Fig. 1B). In addition, the KLF7 protein level change result was in parallel with the mRNA level (Fig. 1C). These data suggest that miR-494-3p and KLF7 were involved with peripheral nerve injury in Sprague Dawley rats.

Inhibition of miR-494-3p promoted cell proliferation and suppressed apoptosis of Schwann cells

As shown in Figs. 2A and 2B, transfection experiences were successful and confirmed that the expression of miR-494-3p was effectively increased in the miR-494-3p group when compared with that in the miR-NC group. In turn, anti-miR-494-3p led to a decrease of miR-494-3p in Schwann cells. To assess the biological effects of miR-494-3p on the Schwann cells, EdU based proliferation assays were conducted. The results suggested that the proliferation of Schwann cells transfected with miR-494-3p was inhibited compared to that in the miR-NC controls, while a higher level of cell viability was observed in anti-miR-494-3p cells than in the anti-NC group (Figs. 2C and 2D). Moreover, overexpression of miR-494-3p facilitated Schwann cells apoptosis, while miR-494-3p knockdown inhibited apoptosis by flow cytometry assays (Figs. 2E and 2F). Finally, western blot analysis was used to assess protein levels of Bcl-2, Bax, and C-caspase 3 in Schwann cells. Consistent with apoptosis results, overexpression of miR-494-3p repressed Bcl-2, but enhanced Bax and C-caspase 3 expression in Schwann cells, while miR-494-3p silencing led to opposite effect on the expression of Bcl-2, Bax, and C-caspase 3 (Figs. 2G–2J). Collectively, miR-494-3p knockdown promoted cell proliferation and suppressed apoptosis of Schwann cells.

KLF7 was target of miR-494-3p

Since KLF7 and miR-494-3p were aberrantly expressed *in vivo*, we hypothesized that KLF7 might be targeted by miR-494-3p. As shown in Fig. 3A, TargetScan online provided the binding sites between KLF7 and miR-494-3p. To validate this prediction, we constructed the KLF7-WT/MUT luciferase reporter vector and then insured them into Schwann cells with miR-494-3p or miR-NC. Luciferase reporter analysis revealed that the luciferase activity was remarkably inhibited in Schwann cells transfected with KLF7-WT and miR-494-3p compared with that in the miR-NC group, while similar results were not found in the KLF7-MUT group (Fig. 3B). Moreover, overexpression of miR-494-3p resulted in an obvious reduced of KLF7 expression, no matter mRNA or

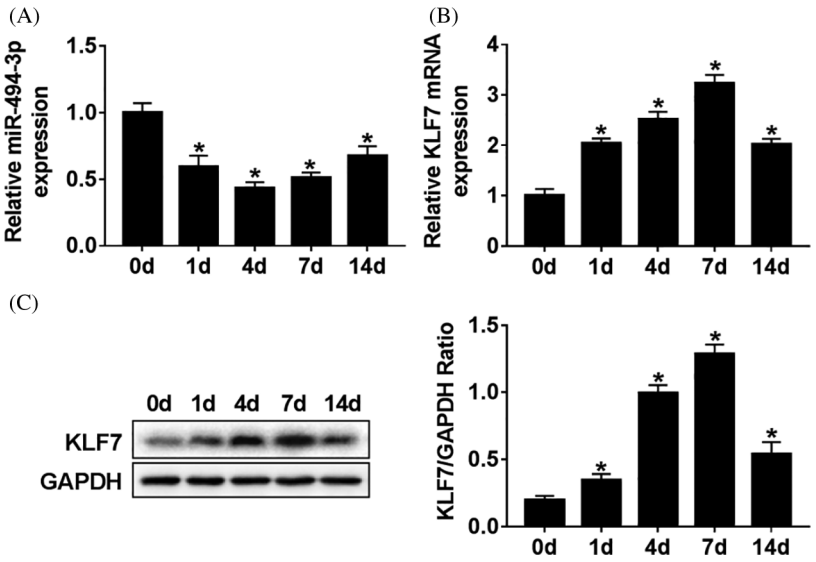

FIGURE 1. The expressions level of miR-494-3p and KLF7 in vivo.
(A, B and C) The expression levels of miR-494-3p and KLF7 were measured in SD rats at 0, 1, 4, 7, and 14 days after sciatic nerve injury by RT-qPCR and western blot assay. *$p < 0.05$.

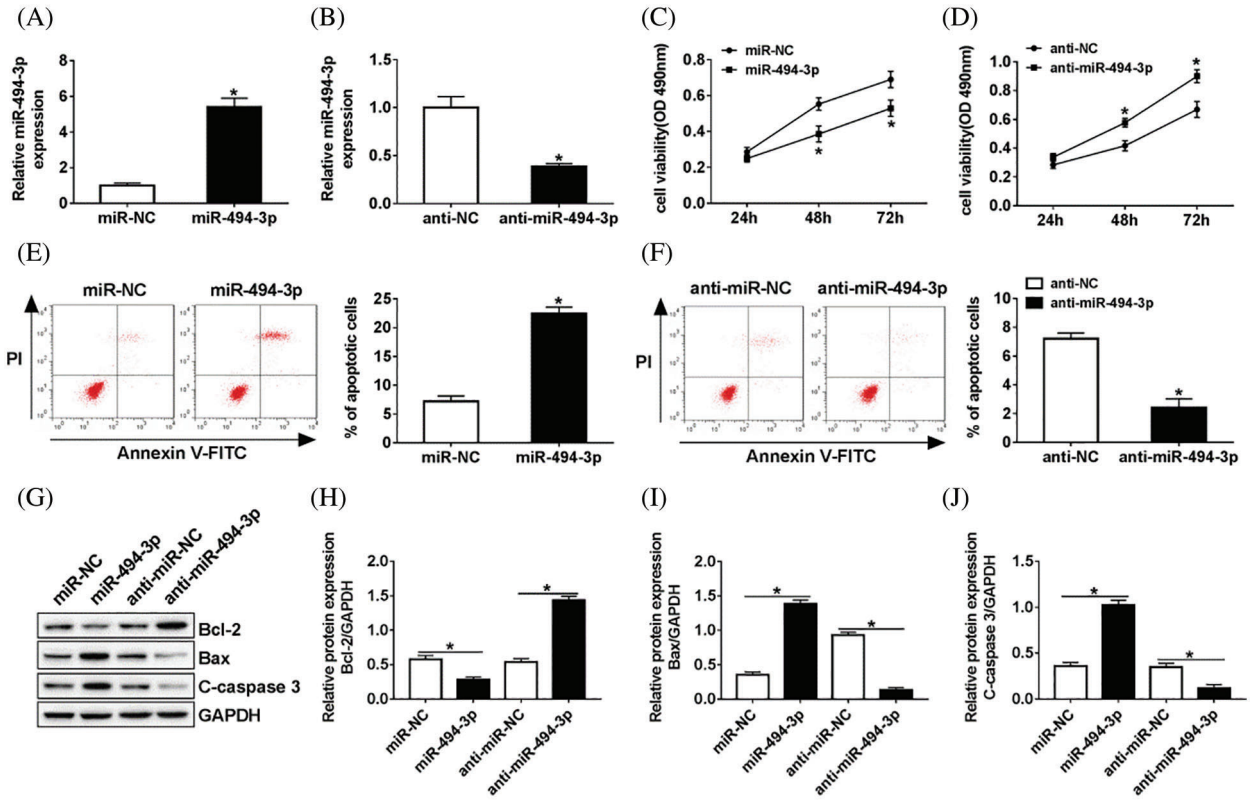

FIGURE 2. Efficacy of miR-494-3p in Schwann cells *in vitro*.
Schwann cells were transfected with miR-494-3p or anti-miR-494-3p as well as respective control miR-NC or anti-NC. (A and B) The expression level of miR-494-3p was measured in transfected Schwann cells by RT-qPCR assay. (C and D) Cell viability was detected utilizing Cell-LightTM EdU DNA Cell Proliferation Kit. (E and F) Flow cytometry assay was used to assess apoptosis in Schwann cells after transfection. (G, H, I and J) Western blot analysis revealed protein levels of Bcl-2, Bax, and C-caspase 3 in Schwann cells. *$p < 0.05$.

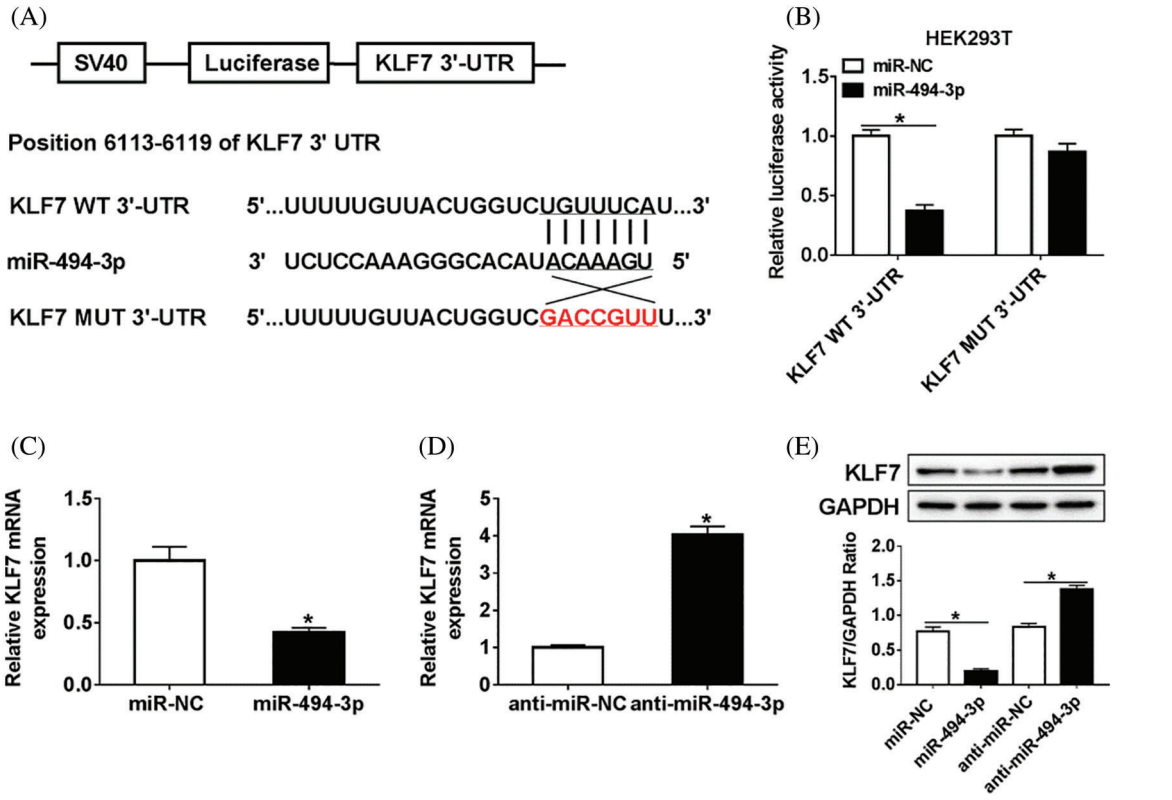

FIGURE 3. The association between miR-494-3p and KLF7 in Schwann cells.
(A) The putative binding sites of KLF7 3'UTR and miR-494-3p, as well as mutation sites, were displayed. (B) Luciferase activity was estimated in Schwann cells co-transfected with KLF7-WT/MUT and miR-494-3p/miR-NC. (C, D and E) The expression level of KLF7 was calculated in Schwann cells transfected with NC, miR-494-3p, anti-NC, or anti-miR-494-3p with RT-qPCR and western blot assay. *$p < 0.05$.

protein (Figs. 3C–3E). All data indicated that miR-494-3p suppressed KLF7 expression in Schwann cells.

KLF7 silencing inhibited proliferation and induced apoptosis of Schwann cells

To further explore the molecular mechanism of KLF7 regulating the biological processes, the loss functional experience was established. As present in Figs. 4A and 4B, KLF7 was decreased in Schwann cells treated with si-KLF7#1 or si-KLF7#2 when compared with the si-NC group. Meanwhile, cell viability was measured by Cell-Light^{TM} EdU DNA Cell Proliferation Kit, and the results showed that the KLF7 knockdown impeded the proliferation of Schwann cells (Fig. 4C). In addition, flow cytometry analysis implied that the apoptosis rate of Schwann cells transfected with si-KLF7#1 and si-KLF7#2 was dramatically increased compared with control groups (Fig. 4D). The protein levels of Bcl-2, Bax, and C-caspase 3 were measured in cells transfected with si-KLF7#1, si-KLF7#2, or si-NC. The results of western blot analysis displayed that low-expression of KLF7 triggered a remark promotion in Bax and C-caspase 3 expression, while induced a reduction in Bcl-2 expression, suggesting that silencing of KLF7downregulated Bcl-2 and promoted Bax and C-caspase 3 expression to anti-apoptosis (Figs. 4E–4H). These data suggested that KLF7 has a vital role in Schwann cell growth and apoptosis.

Overexpression of KLF7 reversed effects of miR-494-3p upregulation on proliferation and apoptosis in Schwann cells

Subsequently, the relationship between miR-494-3p and KLF7 in Schwann cells was analyzed. RT-qPCR and western blot assay were performed to assess the KLF7 level in Schwann cells transfected with miR-NC, miR-494-3p, miR-494-3p+Vector, or miR-494-3p+KLF7. The data showed that high expression of miR-494-3p induced the inhibitory effects on KLF7 expression, whereas KLF7 overexpression reversed these effects (Figs. 5A and 5B). Furthermore, the upregulation of KLF7 abolished the reduction of cell viability and the increase of apoptosis in Schwann cells caused by miR-494-3p overexpression (Figs. 5C and 5D). Similarly, apoptosis-related protein was examined by western blot and implied that miR-494-3p overexpression repressed

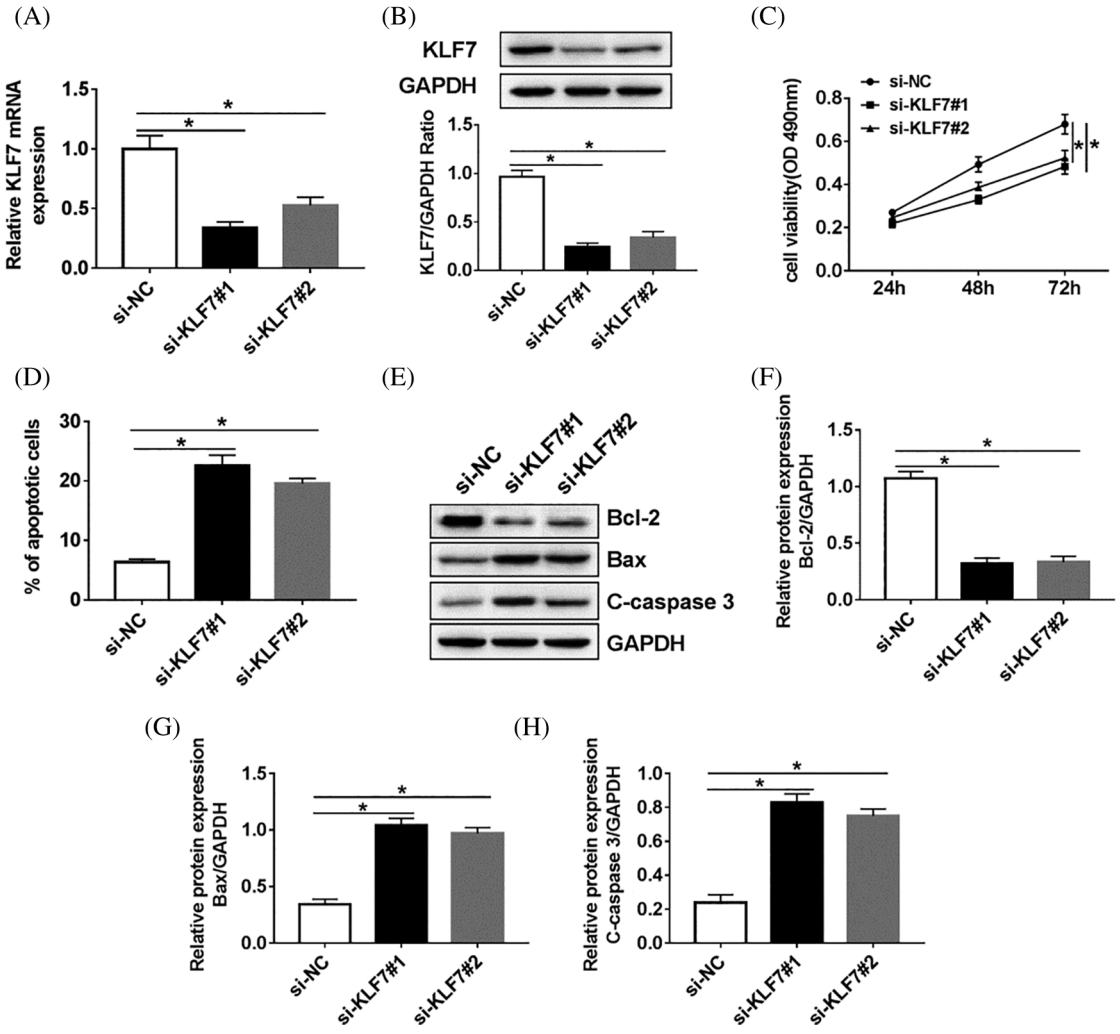

FIGURE 4. The effect of KLF7 silencing on proliferation and apoptosis of Schwann cells.
Schwann cells were transfected with si-KLF7 (si-KLF7#1 and si-KLF7#2) or si-NC. (A and B) The mRNA and protein levels of KLF7 were assessed using RT-qPCR and western blot assays. (C and D) Transfected Schwann cells viability and apoptosis were measured by Cell-Light^{TM} EdU DNA Cell Proliferation Kit and flow cytometry assays, respectively. (E, F, G and H) Protein levels of Bcl-2, Bax, and C-caspase 3 were determined by western blot in Schwann cells. $^{*}p < 0.05$.

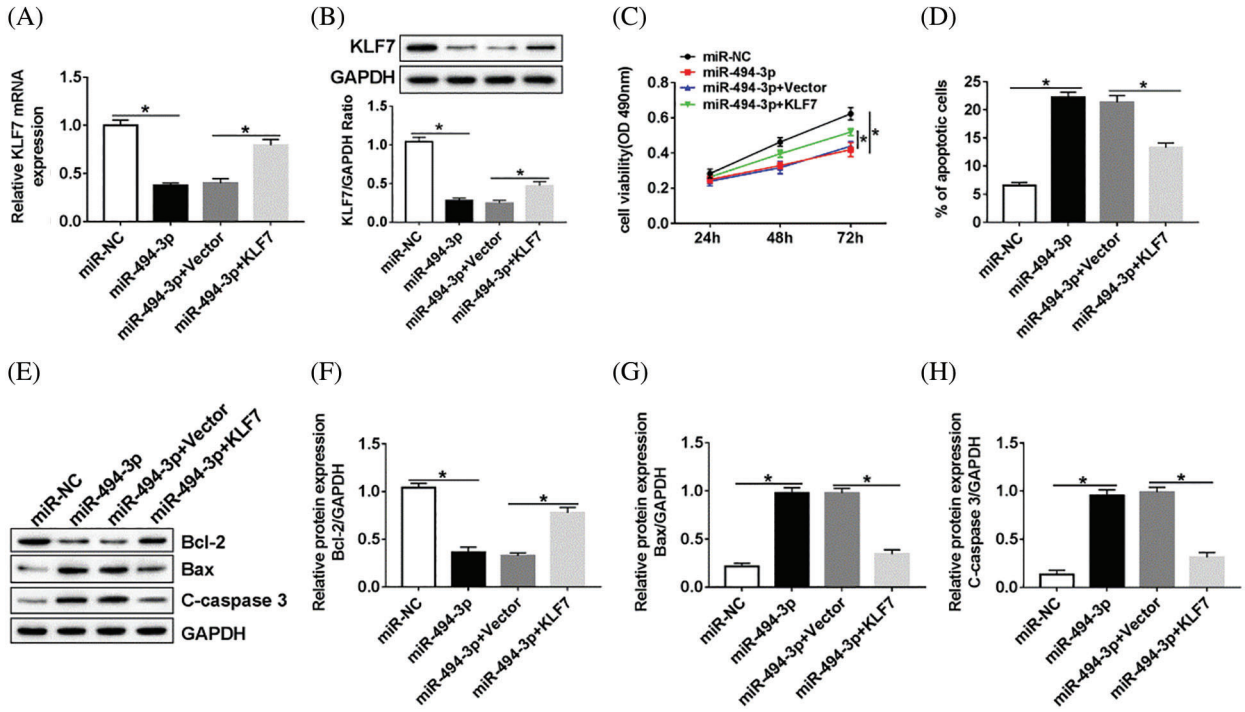

FIGURE 5. MiR-494-3p regulated cell proliferation and apoptosis through targeting KLF7.
Schwann cells were transfected with miR-NC, miR-494-3p, miR-494-3p+Vector, or miR-494-3p+KLF7. (A and B) The expression of KLF7 was detected in Schwann cells after transfection by RT-qPCR and western blot. (C and D) Proliferation Kit and flow cytometry analyses were performed to examine cell viability and apoptosis, correspondingly. (E, F, G and H) The protein levels of Bcl-2, Bax, and C-caspase 3 in Schwann cells were determined by western blot analysis. *$p < 0.05$.

Bcl-2 and promoted the expression of Bax and C-caspase 3, while the gain of KLF7 overturned upregulation of miR-142-5p mediated-effects on protein levels of Bcl-2, Bax, and C-caspase 3 (Figs. 5E–5H). Given the critical role of the ERK pathway during the sciatic nerve injury, we measured the expression levels of ERK pathway-related proteins in Schwann cells. As displayed in Supplementary Fig. 1, the ratio of ERK/p-ERK and JNK/p-JNK was increased in Schwann cells after transfection with KLF7, suggesting overexpression of KLF7 activating ERK pathway. Taken together, miR-142-5p directly targeted KLF7 to regulate proliferation and apoptosis of Schwann cells.

Discussion

A previous study has shown that miR-494-3p was downregulated in rat with sciatic nerve injury (Qian *et al.*, 2018), but whether miR-494-3p plays a regulator role in the repair of peripheral nerve injury has not been defined. (Yi *et al.*, 2016) tested miR-30c expression in Sprague Dawley rats that underwent sciatic nerve transection within 28 d (Yi *et al.*, 2016); similarly, in the present study, we also established the Sprague Dawley model with peripheral nerve injury. Following sciatic nerve injury (within 14 d), miR-494-3p was downregulated, reaching a minimum on day 4, and was then upregulated to near normal level, while the KLF7 level was gradually increased until 7 d, but downregulated at 14 d. These results confirmed that the abnormal expression of miR-494-3p and KLF7 might play essential roles in peripheral nerve injury progression. However, the molecular events during peripheral nerve injury and regeneration remain not fully clear. (Zhan *et al.*, 2017) firstly implied that miR-494 was upregulated in retinoblastoma, and miR-494 might contribute to the development of various cancer types (Zhan *et al.*, 2017). Recent work illustrated that overexpression of miR-494 aggravated of oxidative stress *in vivo* and induced neuronal death by inhibiting DJ-1 (Xiong *et al.*, 2014). Furthermore, miR-494 led to A549 lung cancer cells' premature aging (Ohdaira *et al.*, 2012). Not surprisingly, Li *et al.* further implied that miR-494 impeded cell proliferation and invasion via targeting SRY-related high mobility group-Box gene 9 (Li *et al.*, 2015). These findings indicated that miR-494 might exert a suppression effect on cell proliferation by targeting different genes. Consistent with (Ventura and Jacks 2009) results, miRNAs functioning was depending on particular targets in different tissues (Ventura and Jacks, 2009). Our results showed that overexpression of miR-494 decreased cell viability and induced a significant increase of apoptosis; additionally, miR-494-3p knockdown obviously enhanced cell proliferation and suppressed death. Moreover, functional analysis implied that miR-494 triggered an obvious arrest in gap 2/mitosis in cholangiocarcinoma cells to modulate the cell cycle progression (Yamanaka *et al.*, 2012). Moreover, miR-494-3p promoted tumor survival and metastasis in human glioblastoma cells by regulating proliferation and apoptosis through the PTEN/AKT signaling pathway (Li *et al.*, 2015). However, it is unknown about the role of miR-494 during peripheral nerve injury. It has been demonstrated that KLF7 is principally involved in numerous processes of the nervous systems and can promote axon regeneration in the corticospinal tract

(Blackmore *et al.*, 2012; Laub *et al.*, 2006). The association between miR-494 and KLF7 is worth further studied. KLF7 has been recognized as a regulator of axon outgrowth and regeneration (Lei *et al.*, 2006). KLF7 was suggested to facilitate the recovery after injured nerves through the formation of synapses with motor neurons and axonal plasticity (Li *et al.*, 2017). Furthermore, previous research revealed the important role of the ERK pathway in myelination (Veldman *et al.*, 2007; Napoli *et al.*, 2012). The activation of the ERK pathway contributed to the dedifferentiation of Schwann cells and nerve repair (Cervellini *et al.*, 2018). Not surprisingly, the ERK pathway was activated in Schwann by KLF7 overexpression through phosphorylation of ERK and JNK, implying the neuroprotective properties of KLF7. Overexpression of KLF7 protected nerve cells from sciatic nerve injury by improving Schwann cells survival and axonal regeneration of the peripheral nerve and induced myelination fiber's regeneration function (Wang *et al.*, 2007).

Collectively, our data suggested that KLF7 was regulated by miR-494-3p in Schwann cells. Besides, restoration of KLF7 reversed the effects of miR-494-3p overexpression in Schwann cells, suggesting that miR-494-3p has a key role in the proliferation and apoptosis of Schwann cells via targeting KLF7. Collectively, our results clarified that miR-494 played a protective role in peripheral nerve injury, which will contribute to finding novel potential therapeutic targets for peripheral nerve injury in the future.

Conclusion

Overall, our study suggested that miR-494-3p silencing promoted cell proliferation and inhibited apoptosis of Schwann cells by negatively targeting KLF7 *in vitro*. In addition, the upregulation of KLF7 abolished the effects of miR-494-3p overexpression on proliferation and apoptosis of Schwann cells.

Availability of Data and Materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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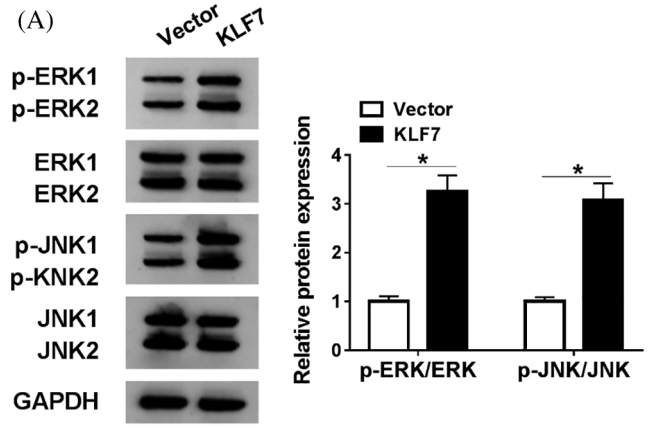

SUPPLEMENTARY FIGURE 1. The expression of ERK pathway-related protein in Schwann cells.
(A) The protein levels of ERK, p-ERK, JNK, and p-JNK were determined by western blot analysis in Schwann cells transfected with KLF7 or vector. *$p < 0.05$.