MEDICAL SCIENCE MONITOR

ANIMAL STUDY

e-ISSN 1643-3750
© Med Sci Monit, 2016; 22: 4516-4522
DOI: 10.12659/MSM.900956

Received: 2016.08.08
Accepted: 2016.09.20
Published: 2016.11.23

Calreticulin Promotes Proliferation and Migration But Inhibits Apoptosis in Schwann Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Source of support: Departmental sources

Background: Previous studies indicated that calreticulin (CRT) regulated various biological processes. This study was aimed to investigate the function of CRT in Schwann cells (SCs).

Material/Methods: SCs were separated from sciatic nerves of mice and were transfected with pcDNA3.1-CRT (pc-CRT), small interfering RNA targets CRT (siCRT), or their corresponding negative controls. The expression of CRT was determined by quantitative reverse transcription PCR (qRT-PCR) and Western blot analysis. Then, cell proliferation, migration, and apoptosis were measured by 3-(4, 5-dimethylhiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, modified 2-chamber migration assay, and flow cytometry, respectively. Finally, the phosphorylation levels of key kinases in the phosphatidylinositol-3-kinase (PI3K)/AKT and the extracellular signal-regulated kinase/ribosomal S6 kinase 2 (ERK/S6) pathways were detected by Western blot analysis.

Results: Overexpression of CRT remarkably increased viability ($P<0.05$, $P<0.01$ or $P<0.001$) and migration ($P<0.001$), but inhibited apoptosis ($P<0.05$). The CRT-knockdown showed the inverse impacts on viability ($P<0.05$ or $P<0.001$), migration ($P<0.001$), and apoptosis ($P<0.001$). Additionally, the phosphorylation levels of AKT (Thr^{308} and Ser^{473}), ERK, and S6 were all up-regulated in CRT-overexpressed cells ($P<0.001$), and were down-regulated in CRT-knockdown cells ($P<0.05$, $P<0.01$ or $P<0.001$).

Conclusions: Overexpression of CRT in SCs promoted cell proliferation and migration but suppressed cell apoptosis. The PI3K/AKT and ERK/S6 pathways might be involved in the functional effects of CRT on SCs.

MeSH Keywords: Apoptosis • Calreticulin • Cell Migration Inhibition • Cell Proliferation • Phosphatidylinositol 3-Kinases

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/900956

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Background

The peripheral nervous system (PNS) acts as the connection between tissues (organs) and the central nervous system (CNS) [1]. Peripheral nervous injury (PNI), resulting from inflicted nerve damage of mechanical or pathological mechanisms, disrupts the normal functions of sensory and motor neurons through damaging the integrity of axons and Schwann cells (SCs) [2]. When PNI takes place, the associated SCs dedifferentiate to a progenitor-like state, and then the progenitor-like SCs stretch out "tubes" helping the axons regrow from upstream of the damage [3]. Thus, SCs act as the major glia of the PNS and play a vital role in PN regeneration. In this study, we focused on SCs to explore the potential therapy for PNI.

Calreticulin (CRT) is a 46-kDa protein that is composed of 3 domains: the N-terminal domain is highly conserved between species, the P-domain is proline-rich, and the highly acidic C-terminal domain contains ER retrieval signals [4–6]. It is well recognized that CRT is located intracellularly, extracellularly, and on the cell surface [7]. The CRT is a high-capacity Ca^{2+}-binding molecular chaperone and has been reported as the pivotal regulator in many functions. Previous studies revealed that CRT participates in cell adhesion, proliferation, migration, and integrin-dependent Ca^{2+} signaling [8]. Other studies demonstrated that CRT plays essential roles in wound healing, resistance to anoikis [9], immune response [10], cellular phagocytic uptake, and expression of steroid-sensitive genes [7,11].

An increasing number of reports show that CRT plays an essential role in neurons, and CRT is necessary for neuronal development in mice [12]. A recent study demonstrated that CRT expression is elevated in nerve growth factor (NGF)-induced neuronal differentiation, and CRT is a crucial mediator of NGF signaling [13]. Furthermore, CRT has been reported to recruit microglia when CNS is damaged in the leech *Hirudo medicinalis* [14]. Therefore, the roles of CRT need to be investigated in SCs, which are the important components of the nervous system. Nonetheless, there are very few relevant reports.

In this study, SCs were transfected with plasmids or small interfering RNA (siRNA) to obtain CRT-overexpressed or CRT-knockdown cells. Then, we focused on the cell proliferation, migration, and apoptosis of CRT dysregulated cells we obtained to preliminarily investigate the regulation of CRT in SCs. Furthermore, the relevant phosphatidylinositol-3-kinase (PI3K)/AKT and extracellular signal-regulated kinase/ribosomal S6 kinase 2 (ERK/S6) pathways in the regulation were also studied. Our experiments uncovered the underlying molecular mechanisms of CRT regulation in SCs, which may provide insights into the clinical design of therapeutic methods.

Material and Methods

Primary SCs culture

Mice were purchased from the Experimental Animal Center of Nantong University. Every animal study was conducted in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by our local ethics committee. All efforts were made to minimize the number and suffering of mice involved in our study.

SCs were collected from sciatic nerves of P1 (postnatal day 1) mice according to the procedure described previously [15]. In brief, SCs were dissociated with 0.4% (w/v) collagenase (Sigma, USA) and 0.125% (w/v) trypsin (Sigma, USA) and then seeded onto 24-well plates (Primaria™, BD Bioscience, Stockholm, Sweden) with Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Dorset, UK) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen, CA, USA). After 24 h of incubation, 2 rounds of selection in 24 h using 10 μM cytosine β-D-arabinofuranoside (AraC) (Sigma-Aldrich, St. Louis, MO) were performed to remove fibroblast from SCs. Finally, SCs were passaged and cultured in DMEM containing 10% (v/v) FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Plasmids construction and siRNA synthesis

The full-length wild-type CRT gene amplified from plasmids (MG51682-CH, Sino Biological Inc., Beijing, China) was cloned into plasmids pcDNA3.1 (pcNC) (Invitrogen, CA, USA) to generate pcDNA3.1-CRT (pc-CRT) and confirmed by sequencing. The empty plasmid pcNC was transfected as a negative control. Specific siRNA against CRT (siCRT) targeting 5'-GGA GCA GUU UCU GGA CGG A-3' and a non-silencing siRNA (siNC) targeting 5'-TTC TCC GAA CGT GTC ACG T-3' were synthesized by GenePharma (Shanghai, China). The siNC was transfected as a negative control in comparison with siCRT. Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen, CA, USA) according to the manufacturer's instructions.

Cell viability assay analysis

The cell viability of SCs was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (Sigma, St. Louis, MO, USA) according to the manufacturer's protocol. Briefly, cells were seeded into 96-well plates at a density of 1×10^3 cells/well and cultured for 1–4 days. Afterward, 100 μL of MTT solution (0.5 mg/mL) was added and incubated at 37°C for 4 h. Following removal of the culture medium, 150 μL of dimethyl sulfoxide (DMSO, Sigma, St Louis, MO, USA) was added to the cells and the MTT color reaction was

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analyzed by use of an absorbance micro-plate reader (Thermo, Waltham, MA, USA) at 570 nm.

Apoptosis assay

An annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China) was used to analyze the cell apoptosis. Briefly, cells were first washed in phosphate-buffered saline (PBS) and fixed in 70% ethanol. Fixed cells were then rinsed twice with PBS and resuspended in binding buffer containing annexin V-FITC and PI in the presence of 50 μg/mL RNase A (Sigma-Aldrich, USA). After incubation at room temperature in the dark for 1 h, the cells were analyzed by flow cytometry (FACS Cantoll, Beckman Coulter, Fullerton, CA, USA) using FlowJo software.

Migration assay

The migration assay was examined by modified 2-chamber assay with a pore size of 8.0 μm. After trypsin treatment and PBS rinsing, SCs were suspended in 200 μL serum-free DMEM and were seeded on a polycarbonate membrane insert in a Transwell apparatus, and 600 μL complete medium was added to the lower compartment. After incubation at 37°C for 12 h, the insert was washed with PBS and all non-migrated cells were carefully scraped from the upper surface with a cotton swab. Migrated cells on the lower side of the insert were fixed with methanol and stained with crystal violet. SCs migration was determined by counting the number of stained cells with an inverted microscope (Olympus, Tokyo, Japan).

Quantitative reverse transcription PCR (qRT-PCR)

The expression of CRT mRNA in transfected SCs was detected by qRT-PCR. Total RNA was isolated from transfected cells using TRIzol reagent (Invitrogen, CA, USA) and DNase I (Promega, Madison, WI, USA) following the manufacture's protocol. Reverse transcription was performed using the MultiScribe RT kit (Applied Biosystems™, Invitrogen, CA, USA) and random hexamers or oligo (dT) according to the manufacturer's instructions. Primers (GenePharma, Shanghai, China) designed for CRT and GAPDH were as follows: CRT-forward 5'-CGG GGT ACC CGC CAC CAT GGC GAT GCT GCT ATC CGT GCC G-3', reverse 5'-CCG GAA TTC CAG CTC GTC CTT GGC CTG-3'; GAPDH-forward 5'-GCA CCG TCA AGG CTG AGA AC-3', reverse 5'-TGG TGA AGA CGC CAG TGG A-3'. Results were calculated using $2^{-\Delta\Delta Ct}$ method, and the relative mRNA expression of CRT was normalized to GAPDH [16].

Western blot

Proteins were extracted from transfected SCs in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Indianapolis, IN, USA). After that, the proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Then, equal amounts of sample protein were separated by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with 5% nonfat dry milk for 1 h at room temperature and incubated at 4°C overnight with antibodies against phosphorylated AKT Thr^{308} (p-AKT308, sc-16646), phosphorylated AKT Ser^{473} (p-AKT473, sc-101629), AKT (sc-8312), phosphorylated ERK (p-ERK, sc-16981-R), ERK (sc-292838), phosphorylated S6 (p-S6, sc-12898-R), S6 (sc-28773) (all from Santa Cruz, CA, USA), CRT (12238, Cell Signaling Technology, Danvers, MA, USA), or GAPDH (G9545, Sigma, USA), respectively. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1: 1000. Then, the PVDF membranes were washed and incubated with secondary antibody marked by horseradish peroxidase (HRP) for 1 h at room temperature. After rinsing, the PVDF membrane-carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 μL Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ software (Bio-Rad, Shanghai, China).

Statistical analysis

All experiments were repeated 3 times. The results are presented as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). The P-values were calculated using one-way analysis of variance (ANOVA).

Results

The difference in CRT expression in transfected SCs

To investigate the expression of CRT in transfected SCs, qRT-PCR and Western blotting were used to detect the mRNA and protein levels of CRT. Results of qRT-PCR in Figure 1A show that CRT mRNA expression was significantly increased in pc-CRT transfected cells ($P<0.001$), and was decreased in siCRT transfected cells ($P<0.01$). In line with the qRT-PCR results, Western blotting in Figure 1B shows that a higher CRT level was found in pc-CRT transfected cells in comparison with control, and a lower CRT level was found in siCRT-transfected cells in comparison with control. These results indicate that the CRT-overexpressed cells and CRT-knockdown cells are successfully achieved.

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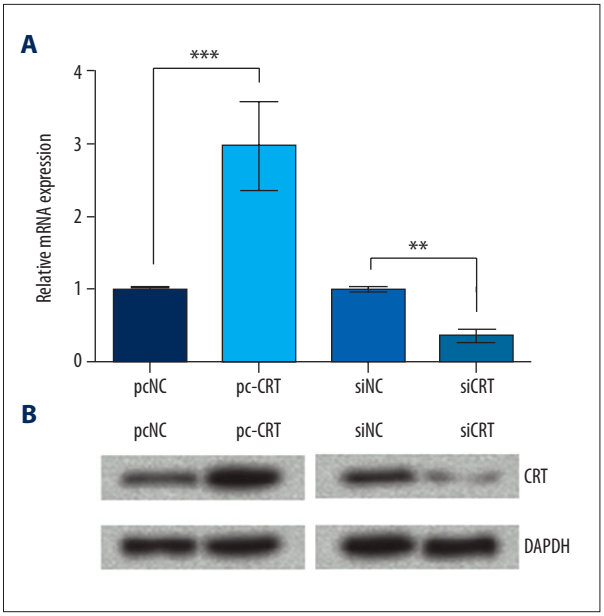

Figure 1. The difference in calreticulin (CRT) expression in transfected Schwann cells (SCs). SCs were transfected with pcDNA3.1 (pcNC), pcDNA3.1-CRT (pc-CRT), non-silencing small interfering RNA (siNC), or specific small interfering RNA against CRT (siCRT). After transfection, cells were harvested for quantitative real-time (qRT)-PCR (**A**) and Western blot analysis (**B**). Data presented are the mean of at least 3 independent experiments. Error bars indicate SD. ** *P*<0.01; *** *P*<0.001.

Overexpression of CRT induced SCs proliferation

To assess the effect of CRT on SCs, MTT assay was used to measure the cell viability. As shown in Figure 2, significant growth promotion was observed in SCs transfected with pc-CRT in comparison with the pcNC group. The cell viability was improved at 2 days (*P*<0.01), 3 days (*P*<0.05) and 4 days (*P*<0.001), respectively. The cell viability in the siCRT group was the total opposite; cell viability was suppressed at 2 days (*P*<0.05), 3 days (*P*<0.001), and 4 days (*P*<0.001), respectively. These results demonstrate that CRT exhibits remarkable promotion of cell proliferation.

Overexpression of CRT increased SCs migration

The influence of CRT on SCs migration was tested by the modified 2-chamber assay. Data in Figure 3 reveal that more cells in CRT-overexpressed SCs migrate to the bottom of the insert compared with the pcNC group (*P*<0.001). However, fewer cells in CRT-knockdown SCs were observed migrating to the bottom of the insert in comparison with the siNC group (*P*<0.001). These results suggest that CRT promotes SCs migration.

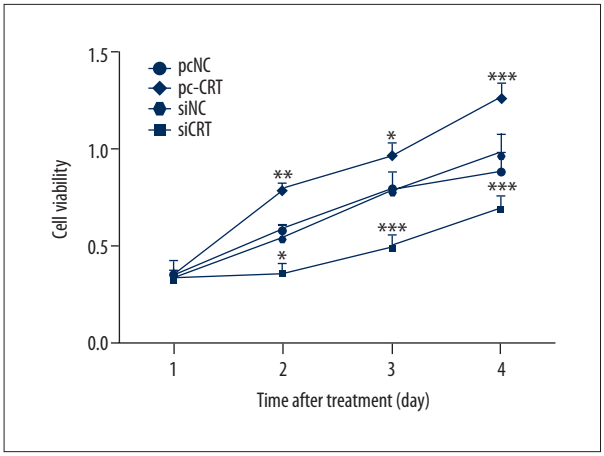

Figure 2. Effect of CRT on proliferation in SCs. SCs were transfected with pcNC, pc-CRT, siNC, or siCRT. Cells transfected with pcNC and siNC served as negative control for pc-CRT and siCRT transfected cells, respectively. Proliferation was measured by MTT assay. Data presented are the mean of 3 independent experiments. Error bars indicate SD. * *P*<0.05; ** *P*<0.01; *** *P*<0.001.

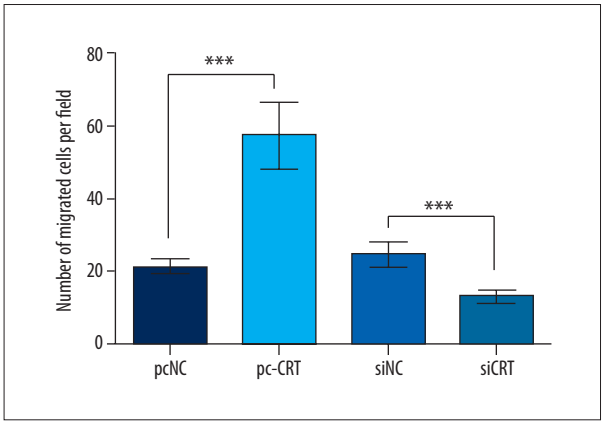

Figure 3. Influence of CRT on migration in SCs. SCs were transfected with pcNC, pc-CRT, siNC, or siCRT. Cells with pcNC and siNC served as negative control for pc-CRT and siCRT transfected cells, respectively. Cell migration was measured by a modified 2-chamber assay. Data presented are the mean of 3 independent experiments. Error bars indicate SD. *** *P*<0.001.

Overexpression of CRT inhibited SCs apoptosis

Flow cytometry was employed to measure the apoptosis in transfected SCs. As shown in Figure 4, the apoptotic rate of CRT-overexpressed SCs was significantly restrained (*P*<0.05). In SCs with CRT-knockdown, the apoptotic rate was significantly increased (*P*<0.001). These data illustrate that CRT effectively suppress the SCs apoptosis.

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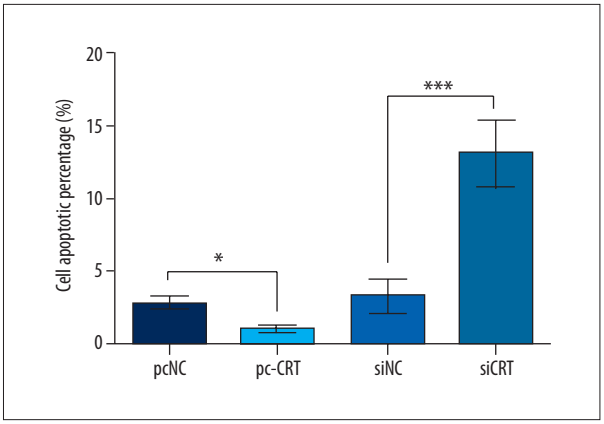

Figure 4. Promotion of apoptosis in CRT-knockdown SCs. SCs were transfected with pcNC, pc-CRT, siNC, or siCRT. Cells with pcNC and siNC served as negative control for pc-CRT and siCRT transfected cells, respectively. Cell apoptosis was measured by flow cytometry. Data presented are the mean of 3 independent experiments. Error bars indicate SD. *** $P<0.001$.

Overexpression of CRT activated the PI3K/AKT and ERK/S6 pathways

To reveal the underlying mechanisms of CRT-associated regulations, the phosphorylation of AKT (Thr^{308} and Ser^{473}) was examined. As shown in Figure 5, the phosphorylation levels of AKT (Thr^{308} and Ser^{473}) were markedly improved in CRT-overexpressed SCs compared with its control ($P<0.001$). As for phosphorylation levels of AKT in CRT-knockdown SCs, phosphorylation levels of Thr^{308} and Ser^{473} were suppressed to varying degrees in comparison with the negative control ($P<0.01$ or $P<0.05$). Because the ERK/S6 signaling pathway is located downstream of the pathway of PI3K/AKT, we also detected the phosphorylation levels of ERK and S6. The phosphorylation levels of both ERK and S6 were increased in CRT-overexpressed SCs compared with the negative control ($P<0.001$). As expected, the phosphorylation levels of ERK and S6 in CRT-knockdown SCs were decreased to varying degrees ($P<0.01$ or $P<0.001$). Moreover, there was no significant difference in the AKT, ERK, and S6 expression observed in all the groups. Thus, we demonstrated that CRT could activate PI3K/AKT and ERK/S6 pathways in SCs.

Discussion

Unlike the CNS, the PNS possesses a unique ability to regenerate [17]. Hence, we draw a conclusion that SCs play a central role in orchestrating the repair response. However, the regeneration of nerves in PN cannot recover to the initial conditions, and PNI still troubles 2.8% of trauma patients with severe PNI insults [18]. Thus, effective therapeutic methods for PNI should be investigated. In this study, we utilized gene transfection and silencing technology to first determine the critical function of CRT in SCs. Cell proliferation and migration were both promoted, whereas cell apoptosis was suppressed in CRT-overexpressed SCs. Totally opposite results were found in CRT-knockdown SCs. Furthermore, the possible relevant mechanism was also studied.

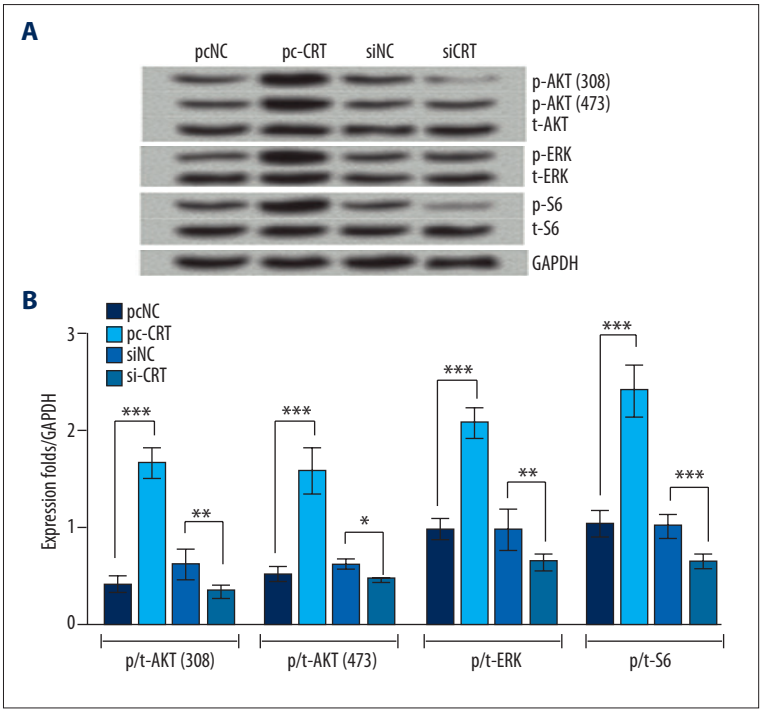

Figure 5. Overexpression of CRT in SCs activated the PI3K/AKT and ERK/S6 signaling pathways. SCs were transfected with pcNC, pc-CRT, siNC, or siCRT. Cells with pcNC and siNC served as negative control for pc-CRT and siCRT transfected cells, respectively. (**A**) expression levels of kinases in transfected cells. After transfection, total proteins extracted from transfected cells underwent Western blot analysis. (**B**) phosphorylation rates of different kinases in transfected cells. The band intensity was estimated by Image Lab™ software. The phosphorylation rate is expressed as the relative intensity of phosphorylated kinases/kinases and the final results were normalized by GAPDH. Data presented are the mean of 3 independent experiments. Error bars indicate SD. *** $P<0.001$.

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During PNI, SCs are reported to play a vital role in the detection of signals from damaged axons, which initiates injury response [19]. After that, SCs dedifferentiate and secrete growth-promoting trophic molecules that support axonal growth cone elongation [19]. Thus, we concluded that SCs viability might aid PN regeneration and the SCs apoptosis might reduce the regeneration. As the main glial cell, SCs must extensively migrate from nerve roots to the periphery to myelinate axons along their entire longitudinal axis in nerve development [20]. In remyelination after nerve injury, the migratory activity of SCs is also a critical aspect [21]. Thus, the SCs migration appears likely to aid regeneration of PN. On the other hand, previous studies used clinical translation of autologous SCs transplantation for the treatment of spinal cord injury [22], and cell therapy with skin-derived SCs was reported to be a potential treatment for PNI [23]. Therefore, it appears likely that cell transplantation with SCs, which possess higher proliferative and migratory abilities and lower apoptotic abilities, can massively accelerate the regeneration of injured nerves.

Previous studies have shown that the overexpression of CRT enhance gastric cancer cell proliferation [24]. Other studies demonstrated that overexpression of CRT enhanced wound healing by stimulating migration of human keratinocytes and fibroblasts [25,26], and CRT increased epithelial cell migration in both murine- and porcine-impaired animal models [27]. These reports highlight the promotion of CRT in cell proliferation and migration, which agrees with our findings in SCs. Recent studies demonstrated that depletion of CRT resulted in resistance to apoptosis [28,29]. However, Oyadomari et al. reported that overexpression of CRT actually protected pancreatic β-cells from nitric oxide-induced apoptosis [30]. This discrepancy might be due to the different cell types, stress stimuli, and experimental models used [31]. In our study, the overexpression of CRT was proved to inhibit apoptosis in SCs. These results show that the overexpression of CRT entirely promote SCs proliferation and migration and suppressed SCs apoptosis.

In various types of carcinomas, cell proliferation and apoptosis are regulated by the PI3K/AKT pathway [7]. The cell proliferation, survival and motility are complicated cellular processes, in which various growth factors and stimuli, as well as cytosolic and nuclear proteins, participated. The S6 was reported to be directly activated by ERK in response to the processes described above [32,33]. To explore the underling mechanism of the regulation inferred from our experiments, we analyzed the phosphorylation levels of AKT, ERK, and S6 that were involved in the PI3K/AKT and ERK/S6 pathways. The activation or inactivation of AKT has been reported to play an important role in CRT-associated apoptosis [31,34,35] and cell survival [36]. Additionally, it has been reported that CRT can serve as an upstream signal for the activation of the PI3K/AKT pathway [37], whereas Liu et al. reported that CRT overexpression inhibited AKT phosphorylation, showing that CRT-AKT axis regulation might be complex [6]. The results of our studies show that the phosphorylation level of AKT (Thr^{308} and Ser^{473}) is accelerated in CRT-overexpressed cells. Our subsequent experiments showed that the phosphorylation levels of ERK and S6 were enhanced in CRT-overexpressed cells. The current results show that both PI3K/AKT and ERK/S6 signaling are both activated in CRT-overexpressed SCs. Based on the above, we conclude that the effect of CRT on SCs proliferation, migration, and apoptosis might involve the PI3K/AKT and ERK/S6 pathways. These findings are consistent with the results obtained from some other cells discussed above and may provide a potential mechanism of CRT mediation in SCs. However, the details on how CRT affects the PI3K/AKT pathway is still an unknown and further research is needed.

Conclusions

We preliminarily investigated the effect of CRT on SCs. The CRT induced cell proliferation and migration, but suppressed apoptosis. These findings establish a foundation for further investigations on the function of CRT. Further mechanism of CRT regulation relevant with PI3K/AKT and ERK/S6 pathways may provide a direction for further studies. Our results shed light on unreported functions of CRT in SCs, which may provide an important theoretical basis for clinical treatment of PNI.

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