● RESEARCH ARTICLE

GSK3β inhibitor promotes myelination and mitigates muscle atrophy after peripheral nerve injury

Jian Weng, Yan-hua Wang, Ming Li, Dian-ying Zhang[*], Bao-guo Jiang[*]

Department of Orthopedics and Trauma, Peking University People's Hospital, Beijing, China

Graphical Abstract

GSK3β inhibitor is effective in the treatment of nerve demyelination and denervation muscle atrophy
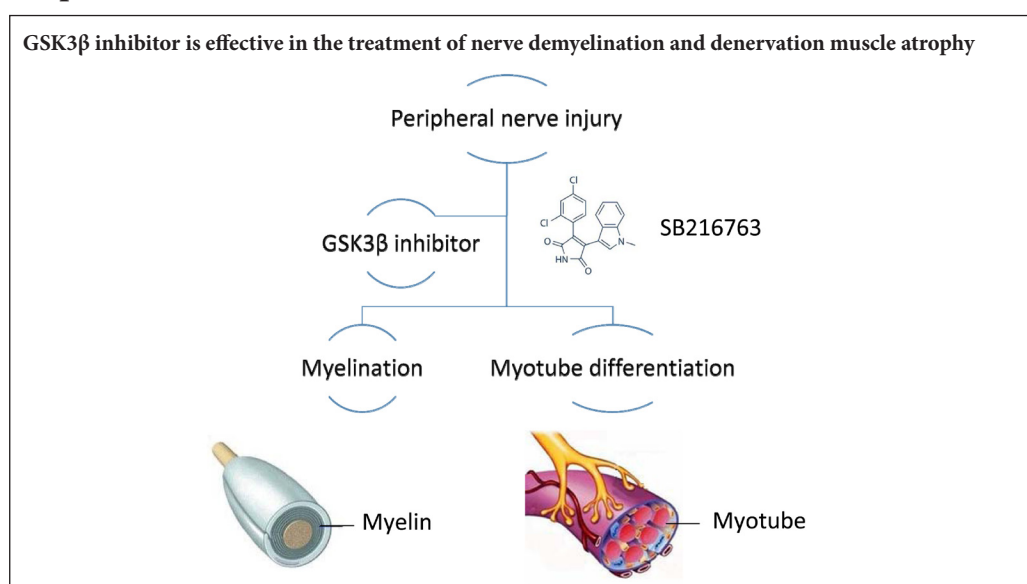
Correspondence to:
Dian-ying Zhang, Ph.D. or Bao-guo Jiang, Ph.D.,
zdy8016@163.com or jiangbaoguo@vip.sina.com.

orcid:
0000-0001-8436-5266 (Bao-guo Jiang)

Abstract

Delay of axon regeneration after peripheral nerve injury usually leads to progressive muscle atrophy and poor functional recovery. The Wnt/β-catenin signaling pathway is considered to be one of the main molecular mechanisms that lead to skeletal muscle atrophy in the elderly. We hold the hypothesis that the innervation of target muscle can be promoted by accelerating axon regeneration and decelerating muscle cell degeneration so as to improve functional recovery of skeletal muscle following peripheral nerve injury. This process may be associated with the Wnt/β-catenin signaling pathway. Our study designed *in vitro* cell models to simulate myelin regeneration and muscle atrophy. We investigated the effects of SB216763, a glycogen synthase kinase 3 beta inhibitor, on the two major murine cell lines RSC96 and C2C12 derived from Schwann cells and muscle satellite cells. The results showed that SB216763 stimulated the Schwann cell migration and myotube contraction. Quantitative polymerase chain reaction results demonstrated that myelin related genes, myelin associated glycoprotein and cyclin-D1, muscle related gene myogenin and endplate-associated gene nicotinic acetylcholine receptors levels were stimulated by SB216763. Immunocytochemical staining revealed that the expressions of β-catenin in the RSC96 and C2C12 cytosolic and nuclear compartments were increased in the SB216763-treated cells. These findings confirm that the glycogen synthase kinase 3 beta inhibitor, SB216763, promoted the myelination and myotube differentiation through the Wnt/β-catenin signaling pathway and contributed to nerve remyelination and reduced denervated muscle atrophy after peripheral nerve injury.

Key Words: nerve regeneration; glycogen synthase kinase 3 beta inhibitor; SB216763; myelination; myotube differentiation; denervated muscle atrophy; Wnt/β-catenin; Schwann cell; muscle cells; peripheral nerve injury; neural regeneration

Introduction

Peripheral nerve injury results in developmental atrophy of the target skeletal muscle and also poor functional recovery when surgery is delayed (Gigo-Benato et al., 2010; Gu et al., 2010; Willand et al., 2014; Zhang et al., 2017). The peripheral nervous system is different from the central nervous system as it has the capacity to regenerate after injury (David and Aguayo, 1985; Schmitt et al., 2003; Hall, 2005; Höke and Brushart, 2010). However, regeneration of the peripheral nerve after injury is a slow process (1–3 mm/d). Axons can take more than 3 months to regenerate into the distal target organs or tissues, meanwhile the distal nerve stump and skeletal muscle usually atrophy (Aydin et al., 2004). To improve the functional recovery of target muscle after periph-

eral nerve injury, it is essential to reduce the reinnervation time and reduce the atrophy of the denervated muscle (Moimas et al., 2013).

Previous studies have revealed that the Wallerian degeneration occurs at the lesion site after peripheral nerve injury (Wagner et al., 1998; Pesini et al., 1999; Wang et al., 2013; Xin et al., 2013). Promoting the migration and proliferation of the Schwann cells has benefits for the improvement of myelination and nerve regeneration (Le et al., 1988; Wang et al., 2016; Wen et al., 2017). Muscle regeneration also depends on resident satellite cells, which are located between the sarcolemma and basement membrane of muscle fibers (Montarras et al., 2005; Le and Rudnicki, 2007; Lepper et al., 2011). Restoration of myotube and myogenesis differentiation has been linked to a reduction of muscle atrophy (Sorci et al., 2003; Johnson et al., 2013; Lee et al., 2017). We hypothesize a therapy that has a positive effect on both Schwann cells and muscle cells, leading to a short reinnervation interval and good muscular function after peripheral nerve injury. In recent years, studies on the peripheral nerve injury have increasingly focused on the role of various signaling pathways, including the Wnt signaling pathway.

The discovery of the wingless gene found by Sharma in 1973 was the basis of the important Wnt signaling pathway (Sharma and Chopra, 1976). Since then other studies have shown that the Wnt/β-catenin signaling pathway has a direct role in myelin gene expression (Chew et al., 2011; Tawk et al., 2011; Meffre et al., 2015; Hichor et al., 2017). Wnt/β-catenin signals act as positive regulators during remyelination (Fancy et al., 2009; Makoukji et al., 2011). Recently, the Wnt/β-catenin signaling pathway has been considered the main molecular mechanism in age-related skeletal muscle atrophy (Rajasekaran et al., 2017). Wnt/β-catenin was involved in impaired muscle repair, such as loss of satellite cell number, muscle cell dysfunction, decreased myoblast proliferation and attenuated differentiation (Carlson et al., 2009). 3-(2,4-Dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763) is a typical inhibitor of glycogen synthase kinase 3 beta (GSK3β), which stimulates canonical Wnt/β-catenin signal (Li et al., 2012). Our previous study demonstrated that SB216763 can promote remyelination and myelin protein production (Chen et al., 2016). SB216763 reduces skeletal muscle atrophy (Litwiniuk et al., 2016). Pretreatment of muscle cell cultures with SB216763 prevented loss of myogenic differentiation and myogenesis induced by TNF-α (Verhees et al., 2013).

Considering the importance of the Wnt signaling pathway in nerve and muscle regeneration, we investigated the effect of the GSK3β inhibitor on both Schwann cells and muscle cells. Our *in vitro* model system mimics the progress of myelination and muscle atrophy, and evaluates the effect of SB216763 on RSC96 cells and C2C12 myotubes.

Materials and Methods
Cells culture and SB216763 treatment
Murine RSC96 Schwann cell and C2C12 myoblast cell lines, passages 1–3, were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in growth media: RSC96 cells with Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 units/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA); C2C12 cells with high-glucose DMEM (Sigma-Aldrich) containing 5 mM glucose, 10% fetal bovine serum and antibiotics., When cells reached 80–90% confluence, the medium was replaced by a differentiation medium (high-glucose DMEM containing 2% calf serum (Gibco) and antibiotics) to induce C2C12 cell myotube formation and differentiation. The differentiation medium was changed daily for 6 days. SB216763 (Apexbio, Houston, TX, USA) was prepared as a 20 mM stock in dimethyl sulfoxide. RSC96 and C2C12 cells were exposed to 10 µM of SB216763 for 48 hours as described previously (Chen et al., 2016). Following these treatments, cells were washed with phosphate-buffered saline (PBS; Gibco) to remove any free drug prior to the sequence experiments.

Scratch wound assay
RSC96 cells were plated in 6-well plates 24 hours before the scratch. A scratch length, approximately 1 mm, was made in the middle area of the plate using a 200 µL pipette tip. All wells were washed with PBS to remove cellular debris. Cells were incubated with growth media as mentioned above. The scratch was photographed at 0, 24 and 48 hours using an inverted fluorescence microscope equipped with microscope camera (BM CAM, Shanghai, China) and ToupView software (ToupTek, Hangzhou, China). Five photographs were taken of the scratched region of each group at each time point. Relative area of the gap was calculated as follows: Relative area in the gap of wound = (area in the gap at 24 or 48 hours/area in the gap at 0 hours) × 100%.

Index of C2C12 myotube contraction
On day 6 of differentiation, cells were stimulated with 100 µM carbachol (Apexbio). Five high-quality images of cells were taken sequentially at 1-minute intervals during carbachol stimulation (Niu et al., 2011). The difference in integrated optical density (IOD) between the first and subsequent images is mainly due to the myotube contraction. Thus, the average IOD of differential image represents the index of contraction. Calculated as below, index of contraction = $\{\Sigma(|\ IOD_5 - IOD_4| + |IOD_4 - IOD_3| + |IOD_3 - IOD_2| + |IOD_2 - IOD_1|)\}/4$. IOD_1 to IOD_5 stand for the mean IOD value of the first to fifth images. IOD was analyzed using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA).

Cell proliferation assay
RSC96 and C2C12 cells were seeded into 96-well plates at a density of 10,000 cells/well. After incubation for 48 hours with or without SB216763, cell viability was evaluated by Cell Counting Kit-8 (CCK-8) assay. Four hours before detection, 10 µL of CCK-8 solution (Sigma-Aldrich) was added into each well, followed by a further 1-hour incubation. The optical density (OD) values were measured at 450 nm with a
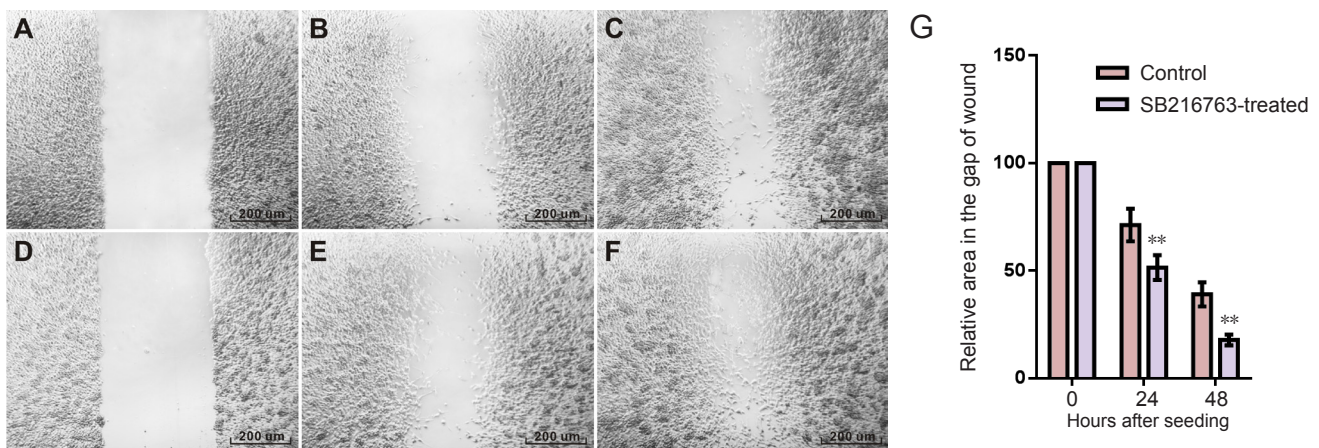
Figure 1 SB216763 promoted gap closure in RSC96 cells.
(A–C) Control cells at 0, 24 and 48 hours. (D–F) SB216763-treated cells at 0, 24 and 48 hours. Scale bars: 200 μm. (G) Relative area in the gap of wound at each time. At 24 hours after seeding, confluent RSC96 cells were treated with SB216763 to conduct the scratch assay. Relative area in the gap of wound was determined at 24 and 48 hours after scratching. As a result, SB216763 significantly promoted gap closure as compared with the control (**$P < 0.01$, *vs.* control group; mean ± SD, $n = 3$, Student's *t*-test). Three independent experiments were performed.

Table 1 Real-time fluorescent quantitative polymerase chain reaction primer sequences

Gene	Primer sequence	Product size (bp)
MAG	Forward: 5′-CCT GCC TCT GTT TTG GAT A-3′ Reverse: 5′-GTT CTT GGG GTA GGG ACT G-3′	187
CCND1	Forward: 5′-TGA CAC CAA TCT CCT CAA CGA C-3′ Reverse: 5′-GGA TGG CAC AAT CTC CCT CT-3′	112
nAChRs	Forward: 5′-CAA GAG CCA GAG AAA GCG GTC-3′ Reverse: 5′-ACA AAA CAA AAA GGC GAT GGC-3′	119
MyoG	Forward: 5′-CCA AGG TCT CCT GTG CTG ATG A-3′ Reverse: 5′-GGC AAA ACC ACA CAA TGC TTA G-3′	100
GAPDH	Forward: 5′-TTC AAC GGC ACA GTC AAG G-3′ Reverse: 5′-CTC AGC ACC AGC ATC ACC-3′	114

MAG: Myelin associated glycoprotein; *CCND1*: cyclin-D1; *nAChRs*: nicotinic acetylcholine receptors; *MyoG*: myogenin; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

microplate reader (Bio-Rad, Hercules, CA, USA).

Quantitative real-time polymerase chain reaction (PCR)
Total RNA was isolated from RSC96 and C2C12 cells using TRIzol reagent (Thermo Fisher, Waltham, MA, USA). cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The amplification was completed by two steps at each target gene annealing temperature. The specificity of the amplified product was determined according to the fusion curve, and the 2-ΔΔCt method was used to determine the mRNA expression. The primers used for PCR are given in **Table 1**.

Immunocytochemical staining
RSC96 and C2C12 cells with or without SB216763 treatment were washed three times with PBS and fixed for 15 minutes in 4% paraformaldehyde and 20 minutes in 0.5% Triton X-100 (Amresco, Solon, OH, USA), then deactivated with 3% H_2O_2 for 30 minutes. Subsequently, cell slices were blocked in PBS supplemented with 5% goat serum (Gibco)

at room temperature for 20 minutes. Cells were incubated with anti-β-catenin antibody (1:300; Abcam, Cambridge, MA, USA) at 37°C for 1 hour, washed in PBS and incubated with anti-mouse IgG-horseradish peroxidase secondary antibody (1:1,000; GTVision, Shanghai, China) for 30 minutes at room temperature. Following washing with PBS, horseradish peroxidase activity was detected using a diaminobenzidine substrate kit (Vector Labs, Burlingame, CA, USA) according to the manufacturer's instructions. Five frames per slide were analyzed. Images were collected with a Leica DFC-550 connected to an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) and analyzed using the software Leica Application Suite Advanced Fluorescence (Leica, Wetzlar, Germany). The ratio of nuclear localization cells was calculated as follows: The positive ratio was equal to the number of cells with positively stained nuclei/the number of cells with negatively stained nuclei × 100%.

Statistical analysis
Statistical analysis was performed using SPSS 21.0 for Windows software (IBM, Armonk, NY, USA). All data are presented as the mean ± SD and analyzed with Student's *t*-test. A value of *P* less than 0.05 was considered statistically significant.

Results
GSK3β inhibitor accelerated migration of RSC96 cells
One therapeutic aspect of a GSK-3 inhibitor for the treatment of peripheral nerve injury was the migration ability of Schwann cells as Wallerian degeneration progressed. To compare the motility of RSC96 cells with or without SB216763 treatment, scratch assay was used to detect cell migration in the area of a "wound", which was scratched into a confluent cell monolayer. The relative area of the gap of wound was measured by the advancement of cells over 24 and 48 hours. The scratch assay demonstrated that the relative area of the gap of wound in control group decreased to 71.2 ± 7.6% at 24 hours and 39.0 ± 5.6% at 48 hours, while
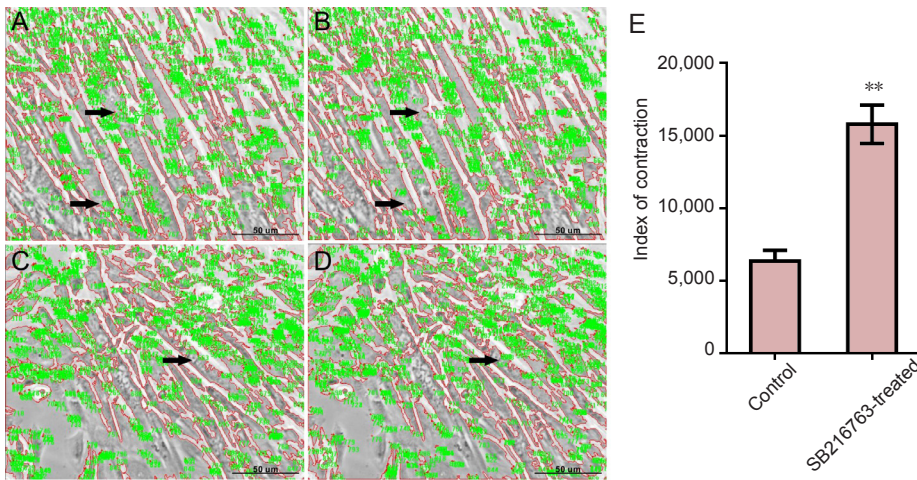
Figure 2 Significant differences in the contraction in C2C12 myotubes after carbachol treatment in each group.
(A, B) The first and second images of control cells. (C, D) The first and second images of SB216763-treated cells. Scale bars: 50 μm. C2C12 cells were differentiated into multinuclear myotubes by incubation with differentiation medium for 6 days. Images of contraction were taken sequentially at 1-minute intervals during carbachol exposure and images were observed as described in Materials and Methods sections (two of five images in each group were shown, black arrows reflected the integrated optical density changes of myotube). (E) Index of contraction: Higher index of SB216763-treated group reflected higher contractile activity (**$P < 0.01$, *vs.* control group; mean ± SD, $n = 5$, Student's *t*-test). Five independent experiments were performed.
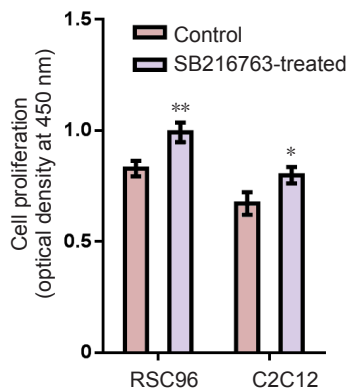

Figure 3 SB216763 promotes the proliferation of RSC96 and C2C12 cells (Cell Counting Kit-8 assay).
From the day after seeding, RSC96 and C2C12 cells were continuously exposed to SB216763 for 48 hours. There was significant difference in cell activity in the SB216763-treated group compared with the untreated-control group (*$P < 0.05$, **$P < 0.01$; mean ± SD, $n = 3$, Student's *t*-test). Three independent experiments were performed.
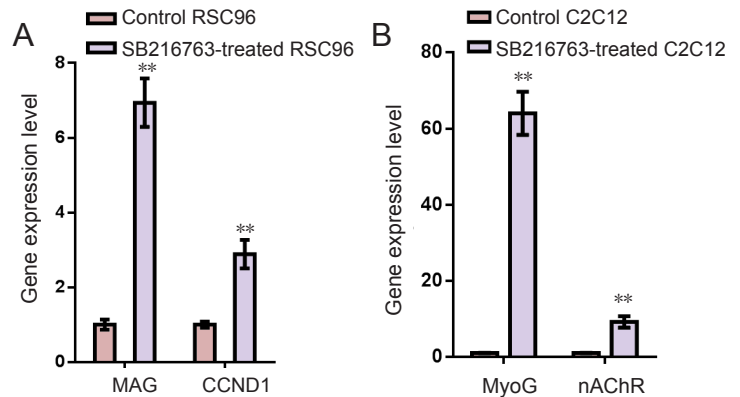

Figure 4 Quantitative polymerase chain reaction of mRNA expression in RSC96 and C2C12 cells in control and SB216763 groups.
Expression levels of myelin related gene MAG and CCND1, muscle related gene MyoG and endplate-associated gene nAChRs were all upregulated in the SB216763-treated cells compared with the control cells (**$P < 0.01$, *vs.* control group; mean ± SD, $n = 3$, Student's *t*-test). Three independent experiments were performed. MAG: Myelin associated glycoprotein; CCND1: cyclin-D1; MyoG: myogenin; nAChRs: nicotinic acetylcholine receptors.
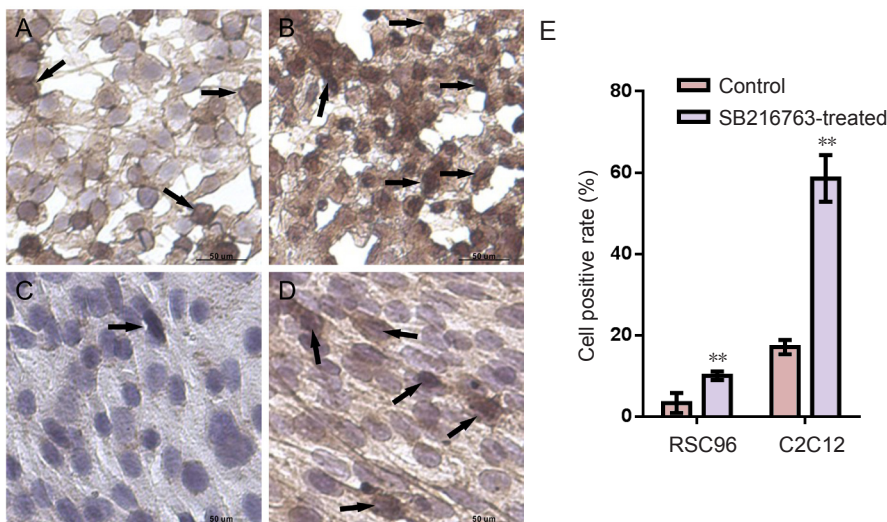

Figure 5 Immunocytochemical staining of β-catenin in RSC96 and C2C12 cells from SB216763-treated group and control group.
(A) Control RSC96 cells. (B) SB216763-treated RSC96 cells. (C) Control C2C12 cells. (D) SB216763-treated RSC96 cells. β-Catenin expression increased and nuclear localization was visible in the cells of the SB216763-treated group (black arrows). (E) The quantification of positive nuclear localization cells showed significant differences (**$P < 0.01$; mean ± SD, $n = 3$, Student's *t*-test). Three independent experiments were performed.

that in the SB216763-treated group decreased to 51.4 ± 5.8% at 24 hours and 17.8 ± 2.5% at 48 hours. The results showed that there were more cells in the gap and the area of the gap was smaller in the SB216763-treated group compared with the control group (*P* < 0.01; **Figure 1**).

GSK3β inhibitor stimulated contraction of C2C12 myotubes

Six days after differentiation, approximately 50% C2C12 cells had fused together to form the typical multinucleated myotubes. After exposure to 100 µM carbachol, vigorous contraction of C2C12 myotubes was readily observed. Examples at the first two times are shown in **Figure 2A–D**. Measurement of the index of contraction is described above, in the Methods. Analysis of the results revealed that the C2C12 myotubes in SB216763-treated group showed significantly higher contractile activity compared with the control group (**Figure 2E**).

GSK3β inhibitor induced proliferation of both Schwann cells and muscle cells

CCK-8 assay was used to determine the effects of 48 hours of treatment with SB216763 on the proliferation of RSC96 and C2C12 cells. The results showed that treatment with SB216763 significantly stimulated the growth of Schwann cells and muscle cells (**Figure 3**).

GSK3β inhibitor treatment resulted in regulation of related genes

The mRNA expression of myelin associated glycoprotein (MAG), cyclin-D1 (CCND1), myogenin (MyoG) and nicotinic acetylcholine receptors (nAChRs) were detected by quantitative PCR. The mRNA expression of both MAG and CCND1 was significantly greater in the SB216763-treated than the control RSC96 group. Similarly, the mRNA expression of MyoG and nAChRs was significantly higher (*P* < 0.01) in SB216763-treated than in the control C2C12 group (**Figure 4**).

GSK3β inhibitor promoted β-catenin expression and nuclear localization

Immunocytochemical staining was performed to detect β-catenin expression. Quantification of β-catenin nuclear localization was analyzed by counting the average positive cell ratio from five frames per slide. After SB216763 treatment, the overall β-catenin expression was greatly upregulated and the nuclear concentration of β-catenin was elevated in RSC96 cells. Beta-catenin expression level was higher and greater nuclear localization was shown in C2C12 cells of SB216763-treated group than in the control group (**Figure 5**). Our results demonstrated that the GSK3β inhibitor enhanced β-catenin expression and nuclear localization and possibly exerted its efficacy *via* Wnt/β-catenin signaling pathway.

Discussion

Peripheral nerve injury is a common disease and has been a focus in neuroscience research for decades. In a clinical setting of peripheral nerve damage, both nerve and muscles undergo degeneration processes. The identification of the molecular mechanism that fosters nerve regeneration, as well as to protect skeletal muscle from atrophy, is thus required. Enhancing the efficacy of remyelination is one of the main areas of research for the treatment of peripheral nerve injury (Torii and Yamauchi, 2016; Yi et al., 2017). Remyelination failure usually leads to irreversible axonal loss and functional impairment (Brück, 2005; Frohman et al., 2006). For successful axon myelin formation, it is critical to increase the proliferation of Schwann cells to support the injured axon. As reported, any delay of interaction between axon and distal target may lead to axonal degeneration, resulting in neuronal apoptosis (Ohnishi et al., 1985; Scheib and Höke, 2013). In turn, peripheral nerve injury could result in the atrophy of the target skeletal muscle. This denervation process induces some complex changes in the expression of genes involved in muscle atrophy (Durbeej et al., 2003). However, the mechanisms underlying the degeneration and regeneration of Schwann cells and muscle cells remain unclear. In our *in vitro* model, the GSK3β inhibitor, SB216763, plays a role in regulating both myelin formation and myogenesis. The preliminary data have shown that a GSK3β inhibitor may promote myelination and also reverse muscle cell atrophy through regulating the Wnt/β-catenin signaling pathway.

SB216763 directly inhibits GSK3β, leading to β-catenin stabilization and nuclear translocation, thereby activating the Wnt/β-catenin signaling pathway (Lenox and Wang, 2003). In the peripheral nervous system, GSK3β inhibitor treatment promotes remyelination of sciatic and facial nerves after crush (Makoukji et al., 2012). Previously we found that up-regulating Wnt/β-catenin signaling by SB216763 induced the clearance of myelin debris and the expression of myelin-related genes (Chen et al., 2016). Others have found that activation of Wnt signaling is important for muscle regeneration (Polesskaya et al., 2003; Brack et al., 2009). Wnt signaling also enhances the proliferation of muscle satellite cells (Otto et al., 2008).

In this study, two kinds of *in vitro* cellular models of peripheral nerve injury were used to study the effect of SB216763 on myelination and myotube differentiation. In the two cell lines, RSC96 and C2C12, tested, the beneficial effects of SB216763 on the progression of peripheral nerve repair are not limited to the local injured site. A previous study showed that migration of Schwann cell is essential to create a permissive environment to promote axon sprouting and also inhibit nerve scar formation (Chehrehasa et al., 2010). In our RSC96 model, SB216763 treatment also accelerated the migratory capacities of the cells. We obtained other comparable beneficial results in other assays. SB216763 treatment increased RSC96 cell proliferation, and enhanced myelin-related gene expression (MAG and CCND1). CCND1 is a β-catenin target gene for an important protein in the cell cycle and can promote the proliferation of cells (Zhao et al., 2016). MAG is a myelin-specific marker which plays a key role in the early stage of myelination and the maintenance

of stable axonal myelin interaction (Shim and Ming, 2010). These encouraging results revealed that the treatment with a GSK3β inhibitor can increase myelin formation.

Positive effects of SB216763 on C2C12 muscle cell line, *in vitro*, were also found. SB216763 stimulated C2C12 myotube contraction, which is caused by acetylcholine receptor activation. CCK-8 assay showed that SB216763 induced C2C12 cell proliferation. Quantitative PCR data indicated that SB216763 up-regulated the expression of MyoG and nAChRs of C2C12 cells. MyoG is a muscle-specific transcription factor that is essential for the development of skeletal muscle, and atrophy of denervated muscle could be prevented by high MyoG expression (Teraoka et al., 2012). nAChR acts as an activator of genes encoding endplate-associated proteins (Méjat et al., 2005). These data revealed that GSK3β inhibitor has the potential to restore muscular function as well as accelerate the myelination.

The mechanism by which GSK3β inhibitor exerts its efficacy was explored by immunocytochemical staining. Exposure to SB216763 increased the nuclear localization and expression of β-catenin, the landmark molecular event of the Wnt signaling pathway. All results indicated that SB216763, as a GSK3β inhibitor, may exert its positive effects on Schwann cells and muscle cells *via* the Wnt/β-catenin signaling pathway.

In summary, this *in vitro* study supports the hypothesis that this GSK3β inhibitor generates a permissive environment for promoting nerve regeneration and decelerating muscle atrophy in the peripheral nervous system. Our results suggest that a GSK3β inhibitor can be considered as an important therapeutic target in myelination and myotube differentiation. This research *in vitro* provides the basis for the hitherto absent *in vivo* animal model studies and further investigations into the underlying mechanism.

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