Wnt5a Affects Schwann Cell Proliferation and Regeneration via Wnt/c-Jun and PTEN Signaling Pathway

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To the Editor: Peripheral nerve injury has been a common clinical disease, and delayed treatment may ultimately lead to long-term physical dysfunction. The repair of peripheral nerve injury has become a major challenge in clinical practice. Different from the central nervous system, the peripheral nervous system has the capacity to regenerate after injury. However, the peripheral nerve regeneration after injury is a slow process, and more than 3 months is usually needed for the axons regenerating to the distal target organs or tissues, when the distal nerve stump and skeletal muscle usually become atrophy and the regenerated axons lost the ability to remodel in next few months. Therefore, accelerating the recovery of the peripheral nerve and the connection between central nervous system and distal nerves after injury is important for the improvement of postinjury nerve recovery. Available studies have revealed that the Wallerian degeneration occurs at the lesioned site after peripheral nerve injury. The proximal stump can sprout to connect the distal stump. The proliferation of the Schwann cells is critical for the repair and regeneration of peripheral nerves after injury. In recent years, increasing studies focus on the role of various signaling pathways in the peripheral nerve regeneration, one of which is the Wnt signaling pathway.

The Wnt signaling pathway is an important pathway based on the discovery of the wingless gene found by Sharma in 1973 and the int-1 gene found by Nusse in 1982. Wnt signaling pathway has several distinct intracellular pathways including the canonical pathway (Wnt/β-catenin pathway) and noncanonical pathways (Wnt/Ca^{2+} pathway, Wnt/PCP pathway, and Wnt/c-Jun pathway). Wnt1, Wnt2, Wnt3, Wnt3a, Wnt8, and Wnt8b are involved in the canonical pathway, while Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, and Wnt11 play important roles in the noncanonical pathways. In recent years, a variety of studies have confirmed the important role of canonical Wnt signaling in the postinjury nerve regeneration. The canonical Wnt signaling was able to control the axial growth of Schwann cells during postinjury nerve repair. However, the role of noncanonical Wnt signaling in the postinjury peripheral nerve regeneration is needed to be further elucidated.[1]

Wnt5a can bind to the transmembrane receptors to transduce extracellular signals into intracellular signals and induce a signaling cascade, which then regulates cell growth and differentiation. Previous studies have shown that nerve growth factors can promote the sprouting of proximal nerve stump to the distal stump by regulating the expression of Wnt5a in sympathetic neurons. Wnt5a expression is significant during axonal growth to distal stump.[2] Furthermore, the sympathetic neurons of *Wnt5a* knockout (*Wnt5a*$^{-/-}$) mice show slower axonal sprouting and axonal growth after nerve growth factor treatment; the abnormal formation of sympathetic nerves and reduced innervation of tissues are also observed in *Wnt5a*$^{-/-}$ mice, which eventually result in cell death during the development.[3]

On the basis of the important role of Wnt5a in the nerve regeneration, we aimed to explore the effect of Wnt5a on Schwann cells by means of lentiviral vector technology which induces interference with *Wnt5a* gene *in vitro*.

According to the sequence of *Wnt5a* mRNA, an RNAi was designed: 5'-GGACCACATGCAGTACATT-3'. The GV248 vector was used. The viral vector GV248-Wnt5a-RNAi was established. The restriction sites AgeI and EcoRI were introduced. Double-stranded DNA was synthesized after primer annealing. The double-enzyme digestion vector and the annealed double-stranded DNA were transformed into competent cell with T4 DNA ligase. Positive colonies were identified by polymerase chain reaction (PCR). Plasmids were extracted after sequencing, qualified, and then used in the following experiments. 293T cells were transfected. After transfection for 4 days, the fluorescent cells in each virus dilution were observed under a fluorescence microscope. Transducing units (TUs) were calculated as follows: Virus titer = fluorescence cell number/virus stock quantity. RSC96 cells were divided into blank group, negative control group, and GV248-Wnt5a-RNAi group. The multiplicity of infection (MOI) was 10 and enhanced infection solution (ENi. S) was used. Cells were observed under a fluorescence microscope at 72 h and photographed. Then, cells were collected for subsequent experiments. Total cellular protein was extracted from RSC96 cells. Western blot (WB) analysis was used to detect the expression of Wnt5a. Cell proliferation was evaluated by Cell Counting Kit-8 (CCK-8) assay. Total RNA

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was isolated from RSC96 cells. Quantitative real-time (qrt)-PCR was used to determine the mRNA expression of *Wnt5a*, *PTEN*, *CTNNB1*, *MAG*, *CCND1*, *c-Jun*, and glyceraldehyde-3-phosphate dehydrogenase. All the data are analyzed with Student's *t*-test. $P < 0.05$ was considered statistically significant.

In our research, RSC96 cells were observed under a light microscope after culture for 0, 3, 5, and 7 days. These cells were spindle shaped, triangular, or polygonal. The nuclei were large and located at the center or periphery of cells. The *Wnt5a* mRNA expression in blank group was 8.390 ± 0.165, which was high and met the requirement for the knockdown experiment.

We successfully constructed *Wnt5a* RNAi lentiviral vector. The positive clone was 534 base-pair (bp) after being connected to the vector-based short hairpin RNA, while the negative clone without connection was 500 bp. Sequencing showed that the synthesized *Wnt5a* short hairpin RNA (shRNA) was inserted correctly. The recombinant lentiviral plasmid was used to transfect 293T cells for 24 h, and the fluorescent cells were observed by fluorescence microscopy. The medium was clear and transparent, and the final lentivirus titer was 1×10^9 TU/ml. The RSC96 cells were seeded into 12-well plates at 3–5 × 10^4/ml. When the cell confluence reached 20%, 1 μl of *Wnt5a* gene carried lentivirus and negative control virus (titer: 1×10^9) was used to infect RSC96 cells. The infection condition was ENi. S and MOI was 10. Culture medium was refreshed 16 h later and the expression of green fluorescent protein was observed 72 h later. The cell morphology showed well and cell infection efficiency was high. The infected RSC96 cells could be used for subsequent experiments. The expression of *Wnt5a* mRNA in infected RSC96 cells was detected by qrt-PCR. The infection was repeated for 3 times. *Wnt5a* mRNA expression in blank group was 0.683 ± 0.007, 0.776 ± 0.008, and 0.756 ± 0.008, that of negative control group was 1.001 ± 0.064, 1.002 ± 0.080, and 1.000 ± 0.038, while that of GV248-Wnt5a-RNAi lentivirus infection group was 0.062 ± 0.006, 0.069 ± 0.008, and 0.042 ± 0.003, respectively. Compared with the negative control group, the *Wnt5a* mRNA expression in GV248-Wnt5a-RNAi group significantly reduced at different time ($P < 0.01$). The *Wnt5a* mRNA expression was reduced by 94.22%. WB analysis confirmed that Wnt5a protein level was significantly lower in GV248-Wnt5a-RNAi lentivirus infection group [Figure 1].

At 1, 2, 3, 4, and 5 days, the A_{450nm} was 0.239 ± 0.001, 0.669 ± 0.010, 1.030 ± 0.022, 1.515 ± 0.015, and 2.044 ± 0.042, respectively, in negative control group and 0.236 ± 0.001 ($P < 0.05$), 0.521 ± 0.014 ($P < 0.01$), 0.759 ± 0.027 ($P < 0.01$), 1.217 ± 0.018 ($P < 0.01$), and 1.792 ± 0.006 ($P < 0.01$), respectively, in GV248-Wnt5a-RNAi group. These results showed that transfection with GV248-Wnt5a-RNAi significantly inhibited cell growth. The mRNA expression of *PTEN*, *CTNNB1*, *MAG*, *CCND1*, and *c-Jun* in the Wnt signaling pathway was further detected by qrt-PCR. The mRNA expression of *PTEN*, *CTNNB1*, *MAG*, *CCND1*, and *c-Jun* was 0.993 ± 0.032, 1.014 ± 0.053, 1.023 ± 0.216, 1.040 ± 0.071, and 1.121 ± 0.146, respectively, in negative control group and 1.778 ± 0.033, 0.536 ± 0.098, 2.573 ± 0.126, 0.886 ± 0.046, and 0.520 ± 0.036, respectively, in GV248-Wnt5a-RNAi group. A significant difference was observed in the mRNA expression of these genes between two groups ($P < 0.05$).

Peripheral nerve injury is a common disease and has been a focus in neuroscience researches. After peripheral nerve injury, Wallerian degeneration is a characteristic pathological process in nerve repair, during which there is a complex interaction between Schwann cells and various surrounding cells. When the necrotic axons and myelin rupture into pieces and are phagocytosed by macrophages, Schwann cells begin to proliferate and form a channel for the regeneration of axons into the distal stump. Once the regenerative nerve has successfully connected to the distal stump, the nervous system will remodel in order to restore the nerve function. Available studies have shown that various growth factors, growth factor receptors, and receptor-mediated signaling pathways may influence the biological processes of Schwann cells such as differentiation, proliferation, survival, migration, and metabolism. Therefore, shortening the remodeling time of nervous system and accelerating the peripheral nerve repair as much as possible will be beneficial for the recovery of neurological function.

In this study, the effect of Wnt5a on Schwann cells was investigated by *Wnt5a* silencing in RSC96 cells. First, an RNAi-specific sequence for rat Wnt5a gene was designed for the construction of a lentiviral vector expressing *Wnt5a* shRNA. PCR and sequencing showed that the *Wnt5a* shRNA sequence was consistent with that predesigned and inserted correctly into the vector. Moreover, the

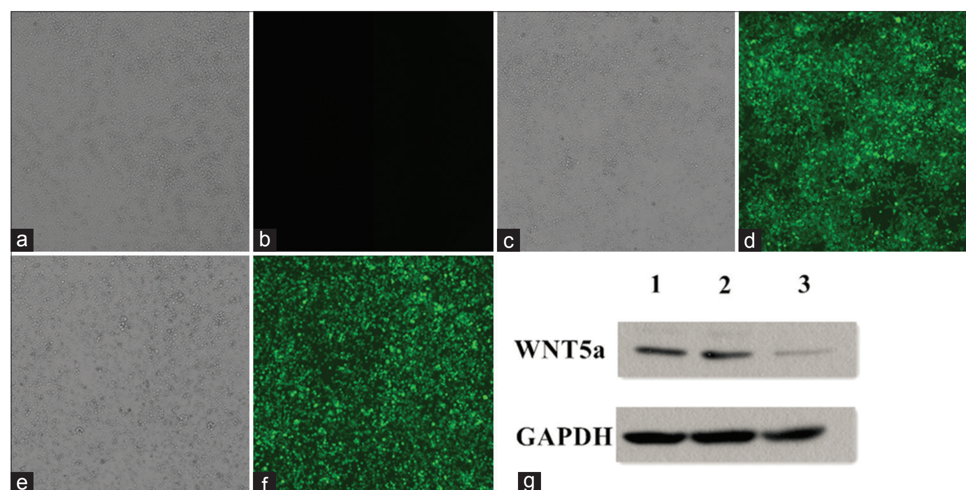

Figure 1: Microscope images of Wnt5a expression in RSC96 cells in different groups with or without GV248-Wnt5a-RNAi lentiviral vector transfection (×125). (a and b) Blank group. (c and d) Negative control group. (e and f) GV248-Wnt5a-RNAi group. (a, c, and e) Light microscope. (b, d, and f) Fluorescence microscope. (g) Western blot analysis. Lane 1: Mock group; Lane 2: Negative control group; Lane 3: GV248-Wnt5a-RNAi lentivirus infection group; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

lentiviral vector was able to express green fluorescent protein under a fluorescence microscope. The viral titer was 1×10^9 TU/ml, and the RSC96 cells showed a good growth after infection with this vector. Further analysis by qrt-PCR showed the *Wnt5a* mRNA expression significantly decreased by 94.22%. WB analysis confirmed that Wnt5a protein level was significantly lower. These suggested that RSC96 cells with reduced Wnt5a expression were obtained.

Peripheral nerve injury can cause axonal injury which leads to Wallerian degeneration, which then triggers Schwann cell proliferation and myelination. *CCND1* and *MAG* are the key genes controlling these processes. As reported, CCND1 is an important protein in the cell cycle and can promote the progression of cell cycle. MAG is a transmembrane protein of the immunoglobulin superfamily and plays a key role in the early stage of myelination and the maintenance of stable axonal myelin interaction. MAG has also been shown as a neurite outgrowth inhibitor *in vitro*, and the increased MAG expression may inhibit axonal germination and regeneration.[4] In this study, the expression of CCND1 significantly decreased after silencing of *Wnt5a* expression, whereas the expression of MAG markedly increased. Simultaneously, CCK-8 assay showed that the number of cells reduced significantly after silencing of *Wnt5a* expression as compared to the negative control group, suggesting that the cell proliferation decreased after silencing of *Wnt5a* expression. Thus, inhibition of *Wnt5a* gene may reduce the proliferation and regeneration of peripheral Schwann cells.

Wnt5a can not only regulate cell proliferation and differentiation by activating noncanonical Wnt signaling pathway but also cooperate with the canonical Wnt/β-catenin pathway under certain conditions. Previous studies have confirmed that the Wnt/PCP signaling pathway can be activated by Wnt5a through Fzd and Dvl.[5] The Rho family of GTPases (RhoA) and Rac proteins can be activated by Dvl and further affect the downstream Rho kinase (RocK) and JNK. They can bind to the N-terminal of transcription factors AP-1 and c-Jun, inducing the expression of downstream target genes to regulate the cytoskeleton rearrangement, cell polarity, and migration. Our results indicated that the expression of c-Jun significantly decreased after silencing of *Wnt5a* expression. In addition, the expression of PTEN in *Wnt5a* silenced Schwann cells also markedly increased, in contrast to downregulated *CTNNB1* gene expression. There is evidence showing that PTEN can regulate cell cycle to affect the cell growth and division. In addition, it also plays an important role in the peripheral nerve repair and remodeling after injury. Some studies have confirmed that silencing of *PTEN* expression could activate PI3K/AKT signaling pathway and thereafter attenuate inflammation in animal models, suggesting that PTEN may inhibit the anti-inflammatory effect.[6] β-catenin (CTNNB1) is the core regulator in the canonical Wnt signaling pathway, and its expression determines the activation of canonical Wnt signaling pathway. It has been found that Wnt/β-catenin regulates inflammatory response. Perry *et al*.[7] found that the Wnt/β-catenin pathway could interact with the PTEN/PI3K/AKT pathway in the self-renewal of stem cells. Therefore, we speculate that the cooperative role of Wnt5a in Wnt/β-catenin pathway may be related to the PTEN.

Taken together, Wnt5a expression was successfully silenced in Schwann cells with the lentivirus interference technique. In addition, silencing of *Wnt5a* expression was able to reduce the proliferation and regeneration of Schwann cells and then inhibit the axonal regeneration. From these results, we infer that *Wnt5a* gene might affect the peripheral nerve regeneration via Wnt/c-Jun and PTEN signaling. Therefore, our research provides insight into the study of the pathogenesis and treatment of peripheral nerve injury and provides a theoretical support for the treatment of peripheral nerve injury with *Wnt5a* gene as a target in clinical.

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Conflicts of interest

There are no conflicts of interest.

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