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MicroRNA-based targeting of the Rho/ROCK pathway in therapeutic strategies after spinal cord injury

Tetsu Kimura*, Yuta Horikoshi

Spinal cord injury (SCI) is one of the leading causes of disability and is a devastating condition that requires long-term care, reduces social productivity, and gives an immense emotional burden on patients and their families. SCI frequently occurs due to traffic accidents, falls, slips, violence, sports, and medical accidents in today's society. The initial mechanical damage triggers a secondary injury cascade that induces more intractable damage (Silva et al., 2014). Secondary injury mechanisms have been postulated, including neuronal apoptosis, inflammation, oxidative stress, and excitotoxicity. The failure of axons and nerves to regenerate may contribute to the difficulty in recovering function after spinal cord injury. Therefore, suppressing axon growth inhibition or encouraging axon regeneration must be beneficial for the treatment of SCI. This article will discuss the involvement of microRNAs (miRNAs), a non-coding RNA that affects various physiological and pathological conditions, in the Rho/Rho-kinase (ROCK) pathway in SCI pathogenesis, especially in axon regeneration, and its therapeutic application.

Implications of Rho/ROCK pathway in SCI: RhoA is a small GTPase protein belonging to the Rho GTPase family, and Rho-associated coiled-coil protein kinase (ROCK) is a downstream effector of RhoA. The Rho/ROCK pathway is associated with various important cellular functions, including cycle progression, dendrite alignment, spine morphogenesis, growth cone development, axon guidance, neuronal survival, and neuronal death. Excessive Rho/ROCK activity is implicated in the pathogenesis of a variety of diseases, including subarachnoid hemorrhage, retinal disease, epilepsy, Parkinson's disease, Alzheimer's disease, ischemic stroke, and spinal cord injury, and Rho/ROCK pathway inhibition may be a potential therapeutic target for these diseases (Loirand, 2015).

A growing body of evidence suggests that the Rho/ROCK pathway impedes neural regeneration by axonal growth cone collapse after SCI. While axons in the peripheral nervous system can regenerate after injury, axons in the central nervous system (CNS) have limited regenerative capacity. When myelin, which consists of oligodendrocytes ensheathing axons, is injured, the CNS axons are exposed to myelin debris containing myelin-associated inhibitors. Myelin-associated inhibitors, such as Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp), are expressed in oligodendrocytes and transmit signals to neurons via the Nogo receptor (NgR), which activates the Rho/ROCK pathway, leading to axon growth inhibition (**Figure 1**; Fujita et al., 2014). Repulsive guidance molecule (RGM) is a protein with three homologs, RGMa, RGMb, and RGMc, inducing growth cone collapse. RGMa expression is increased after SCI, and axon regeneration is inhibited (Yamashita et al., 2007). The binding of RGMa to its receptor, neogenin, activates the RhoA/ROCK pathway and inhibits neurite outgrowth (Fujita et al., 2014). Reactive astrocytes form a glial scar, secrete inhibitory extracellular matrix molecules such as chondroitin sulfate proteoglycans into the lesion site, and activate the RhoA/ROCK pathway via protein tyrosine phosphatase, NgR, and leukocyte common antigen-related phosphatase to inhibit axon elongation.

As mentioned above, myelin-related molecules such as Nogo/MAG/OMgp, RGMs, and a glial scar-related extracellular matrix molecule such as chondroitin sulfate proteoglycans converge on the Rho/ROCK pathway, leading to inhibition of axon regeneration in SCI (Fujita et al., 2014; **Figure 1**). Indeed, inhibition of Rho has been reported to contribute to axon regeneration and neuroprotection after spinal cord injury. In animal experiments, β-elemene, Leucine-rich repeats, and Ig domain-containing 1-Fc, ibuprofen, small interfering RhoA, RhoA+FK506, fasudil, p21Clip1/WAF1F, Y27632, and VX-210 were found to have neuroprotective effects after SCI, in terms of axonal sprouting, nerve fiber regeneration, reduced formation of syrinx cavities, and white matter protection, leading to the recovery of motor function (Luo et al., 2021).

Suppression of Rho/ROCK pathway by miRNAs promotes neural recovery after SCI: MiRNAs are small non-coding RNA molecules that bind to the messenger RNAs (mRNAs) 3'UTR of target genes and induce mRNA degradation and inhibition of translation, thereby regulating various gene expressions such as neurogenesis, tumor metastasis, cell proliferation, apoptosis, and differentiation (Smirnova et al., 2005). In recent years, a growing number of studies have demonstrated the importance of miRNA involvement in promoting axon outgrowth and inhibiting neuronal apoptosis after SCI (Yan et al., 2012). Several recent studies have focused on the association between the Rho/ROCK pathway and miRNAs in axon regeneration after SCI (**Figure 1**).

Semaphorin-3A (Sema3A), a neuronal secreted repulsive guidance cue, induces neuronal growth cone collapse during the nervous system development. Sema3A binds to the receptor complex of PlexinA1 and Neuropilin-1 (NRP-1) and modulates the Rho/ROCK pathway. MiR-30b, which binds to Sema3A during induction of neurite outgrowth in retinal ganglion cells, was explored whether it promotes neurite outgrowth after SCI. Up-regulation of miR-30b suppressed sema3A expression and RhoA/ROCK activity via the PlexinA1/NRP-1 co-receptor, promoting neurite outgrowth of primary sensory neurons and restoration of spinal cord sensory conduction function (Wang et al., 2020a; **Figure 1**).

It has also been reported that miR-135a-5p stimulates axonal regeneration and inhibits

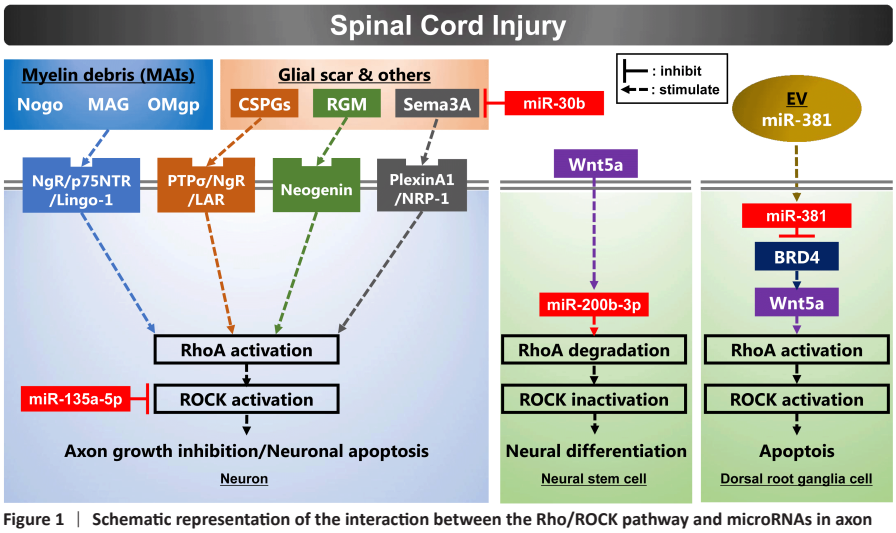

Figure 1 | Schematic representation of the interaction between the Rho/ROCK pathway and microRNAs in axon growth and apoptosis after spinal cord injury.
Spinal cord injury triggers multiple signaling pathways that upregulate Rho/ROCK pathways in neurons, resulting in axon growth inhibition and neuronal apoptosis. By inhibiting certain parts of these signaling pathways, miR-135a-5p and miR-30b promote axonal regeneration. Wnt5a promotes neural differentiation of neural stem cells by inhibiting the Rho/ROCK pathway via miR-200b-3p. MiR-381 delivered by EV suppresses BRD4/Wnt5a axis and leads to inhibition of apoptosis through the inactivation of the Rho/ROCK pathway. BRD4: Bromodomain-containing protein 4; CSPG: chondroitin sulfate proteoglycans; EV: extracellular vesicle; LAR: leukocyte common antigen-related phosphatase; MAG: myelin-associated glycoprotein; MAI: myelin-associated inhibitor; NgR: Nogo receptor; NRP-1: neuropilin-1; OMgp: oligodendrocyte-myelin glycoprotein; p75NTR: p75 neurotrophin receptor; PTPσ: protein tyrosine phosphatase; RGM: repulsive guidance molecule; ROCK: Rho-kinase; Sema3A: semaphorin-3A; Wnt5a: Wnt family member 5a.

apoptosis. ROCK is a target gene of miR-135a-5p, and the phosphate protein kinase B (AKT)/glycogen synthase 3β (GSK3β) pathway is one of the downstream signaling pathways of ROCK, whose activation regulates axon growth. Upregulation of miR-135a-5p suppressed the ROCK-AKT/GSK3β pathway, promoting axon regeneration during the functional recovery after SCI (Wang et al., 2020b; **Figure 1**). Genetic manipulation using this signaling pathway may be a candidate for clinical application in stem cell therapy after SCI.

Neural stem cell (NSC) transplantation may be one of the potential strategies to repair the neural circuit after SCI. However, the differentiation rate of NSCs into neurons is minimal, while most NSCs differentiate into astrocytes. The Wnt signaling pathway is crucial in many biological processes, including neural differentiation and regeneration. Li et al. (2020) demonstrated that Wnt5a induced NSC differentiation into neurons by suppressing RhoA expression to promote spinal cord repair after SCI. Furthermore, they indicated that upregulation of miR-200b-3p by Wnt5a was essential for the differentiation of transplanted NSCs into neurons by suppressing the Rho/ROCK pathway after SCI (**Figure 1**).

Prospects for miRNAs affecting the Rho/ROCK pathway in therapeutic strategies for spinal cord injury: Extracellular vesicles (EVs) are membrane-enclosed particles secreted by nearly all somatic cells, including those of the CNS. EVs may be valuable candidates for cell-free therapy because of their bioactivity, biocompatibility, and cell targeting capabilities, with less concern for immunogenicity and uncontrolled growth and differentiation in cell transplants (Dutta, 2021). As a potential therapeutic method, EVs are considered new vehicles for biologically active molecules such as mRNAs, miRNAs, and long noncoding RNAs. Jia reported that miR-381 delivered by the EVs derived from bone marrow mesenchymal stem cells (MSCs) inhibited neuron apoptosis and promoted the functional recovery after SCI by suppressing the bromodomain-containing protein 4/Wnt5a axis followed by the inactivation of the Rho/ROCK pathway (Jia et al., 2021). In their study, miR-381 encapsulated in EVs was administered intravenously and showed excellent efficacy, which is a straightforward method of administration and has great clinical significance. MSCs are a promising source of therapeutic EVs and have recently attracted attention as a target for developing new therapies in various diseases. Establishing strategies to enhance the CNS-specific regenerative potential of EV-based SCI therapeutics will require exploration of cell sources (such as neural progenitor/stem cells, primary cortical neurons, Schwann cells, MSCs), engineering of the producing cells microenvironment (such as supplementation with insulin-like growth factor 1, three-dimensional bioprinting), and loading of therapeutic cargo (such as genetic modification and overexpression, EV surface modification) (Dutta, 2021).

On the other hand, Li et al. (2020) reported that transplantation of Wnt5a-modified NSCs promotes tissue repair and functional recovery after SCI, as described above. They injected lentivirus-Wnt5a-transfected NSCs into the injury site of SCI. They observed that the transplanted NSCs mainly differentiated into neurons and promoted spinal cord repair, indicating a novel strategy to promote neuronal recovery after SCI. Thus, there may be several possible ways to deliver miRNAs to the lesion site after spinal cord injury. Further research is needed to establish a therapeutic strategy in clinical practice.

Conclusions: This article discussed the implications of the Rho/ROCK pathway in the pathophysiology, especially in axon regeneration, in SCI, and then the involvement of miRNAs affecting the Rho/ROCK pathway. The Rho/ROCK pathway is crucial because myelin-related molecules such as Nogo, MAG, OMgp, RGM, and glial scar-related extracellular matrix molecules called chondroitin sulfate proteoglycans converge, inhibiting axon regeneration. Furthermore, several miRNAs suppress the Rho/ROCK pathway and have the potency to induce axon regeneration and neural differentiation, leading to functional recovery. Recent studies have indicated the efficacy of intravenous administration of EVs containing miRNAs and direct injection of transfected NSCs into the lesion site. Further studies are expected to elucidate the most effective and safe treatment strategies targeting the Rho/ROCK pathway modulated by miRNAs after SCI.

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