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MicroRNAs as potential therapeutics for treating spinal cord injury

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Abstract
MicroRNAs are a class of recently discovered, small non-coding RNAs that have been shown to play essential roles in a vast majority of biological processes. Very little is known about the role of microRNAs during spinal cord injury. This review summarizes the changes in expression levels of microRNAs after spinal cord injury. These aberrant changes suggest that microRNAs play an important role in inflammation, oxidative stress, apoptosis, glial scar formation and axonal regeneration. Given their small size and specificity of action, microRNAs could be potential therapeutics for treating spinal cord injury in the future. There are rapidly developing techniques for manipulating microRNA levels in animals; we review different chemical modification and delivery strategies. These may provide platforms for designing efficient microRNA delivery protocols for use in the clinic.

Key Words
microRNAs; spinal cord injury; reactive astrogliosis; axonal regeneration; antagomir; anti-miR; neural regeneration; reviews

Abbreviations
SCI, spinal cord injury; miRNA, microRNA; siRNA, small interfering RNA; BMP, bone morphogenetic protein; LNA, locked nucleic acid

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INTRODUCTION

Spinal cord injury (SCI) caused by traumatic factors such as traffic accidents often results in abnormality or loss of motor and sensory functions, such as dysfunction of cardiovascular and respiratory systems, which can be life-threatening. The processes that take place following SCI can be classified on the basis of time into acute, sub-acute and chronic phases, each of which is accompanied by sets of complex pathophysiological reactions including inflammatory response, oxidative stress, glial scar formation and axonal regeneration[1]. Primary injury occurs as a result of a direct mechanical insult, which induces hemorrhage, edema and ischemia of the local tissue as well as massive glutamate release from neurons[2]. Initiated by the primary injury, secondary injury is a chain reaction that occurs thereafter and leads to a progressive increase in the amount of neural injury over a time period of days to months. Inflammatory response and oxidative stress are two major components of secondary injury. The resultant high level of inflammatory cytokines and strong oxidizing reagents often damages the surrounding tissue and mediates injury-associated cell death[3-6]. Upon injury, endogenous spinal astrocytes become hypertrophic days after SCI *via* a

process called reactive astrogliosis[7-9]. Secreted extracellular matrix glycoproteins such as chondroitin sulfate proteoglycan, reactive astrocytes, and abundant other cell types eventually form a compact tissue structure, the glial scar[10]. Glial scarring is a self-defensive reaction of the injured central nervous system, but the major components of the glial scar exhibit inhibitory properties toward neurite outgrowth and act as a physical and molecular barrier to axonal regeneration[7-8, 10-15]. In some non-mammalian species such as zebrafish, the central nervous system possesses regenerative capacity after injury. In mammals, however, transected axons fail to regrow in the spinal cord. This inability is largely due to the inhibitory environment around the injury site and possibly altered intrinsic cellular signaling pathways[12, 16-18].

MicroRNAs (miRNAs) are a class of small non-coding RNAs, which mainly regulate gene expression after transcription. They have been ignored largely due to limitations of technique that prevented their detection until recently. They are usually around 22 nucleotides (nt) in length, and the major mechanism of gene regulation is to bind target mRNAs and target them for degradation or inhibit their translation[19]. Like other genes in the genome, miRNA genes are transcribed into primary miRNA transcripts (pri-miRNAs) by RNA polymerases *pol* II and *pol* III. The pri-miRNAs are cleaved to generate 60–70 nt stem loop intermediates known as the miRNA precursors (pre-miRNAs)[19] by the RNase III enzyme Drosha[20] and the double-stranded RNA-binding domain protein DiGeorge critical region 8[21-22]. In *Drosophila,* the homolog protein is called Pasha[23]. The pre-miRNAs are actively transported from nucleus to cytoplasm by Ran-GTPase and exportin-5, and further cleaved into 20–22 nt mature miRNAs by the RNase III enzyme Dicer. The mature miRNAs are then loaded into the RNA-induced silencing complex, where they exert their function of silencing gene expression. Because miRNAs bind 3'-untranslated regions of target genes through imperfect complementarity, algorithms developed to predict miRNA targets are helpful, but not always correct. Experimental validations are needed to prove these predictions. MiRNAs have been identified as crucial regulators in developmental timing, cell proliferation, cell differentiation and death, hematopoiesis, neurodevelopment and neural regeneration[24-30]. However, the role of miRNAs in SCI has just begun to be realized, adding another layer of mechanistic complexity to this pathophysiological process. In addition, miRNAs provide a novel class of therapeutic targets for treatment of SCI, especially because of their small size.

MiRNA EXPRESSION AND POTENTIAL FUNCTIONS IN SCI

There are few reports in the literature describing the potential role of miRNAs in SCI, most of which have used expression analysis (Table 1). These studies analyzed miRNAs in different SCI models, time frames of SCI progression and species ranging from rodents to zebrafish[31-37].

Table 1 Altered miRNA expression following spinal cord injury (SCI) (miRNAs showing consistently altered expression after SCI are labeled in bold)

miRNAs showing significantly altered expression after SCI		Targeted SCI processes	SCI model	Reference
Up at 4 hours, 1 and 7 days	**223,** 206, 290, 214, 20b-5p, 17, **146a,** 20a, 672, 203, **21,** 378, 199a-3p, 15b, 221, 1, 146b, 31, 374, 152, 92a, 106b, 93, 333, 674-5p, 872, 92b, 145, 98, 30a*	Inflammation, apoptosis, oxidative stress	Rat contusive SCI (10 g, 12.5 mm) at T_{9-10}	[31]
Down at 4 hours, 1 and 7 days	30c, 34a, 338*, 30b-5p, 30d, 384-5p, 219-2-3p, 543*, 325-3p, 138, 379*, **495, 129*,** 323, 137, 219-5p			
Up at 4 hours, down at 1 and 7 days	128, 100, 487b, 127, 434, 107, 103, 99a, 154, 181a, **124, 133b,** 133a, 451			
Up at 12 hours	1, 133a, **133b, 223,** 451	Inflammation, apoptosis	Mouse compression SCI at T_{11-12}	[32-33]
Down at 12 hours	**124a, 129-3p,** 342, **495,** 541			
Up at 10 and 31 days	let-7a, 16 (no change after exercise); **21** (increased only after exercise at 10 days)	Apoptosis	Rat complete transection at T_{10}	[34]
Down at 10 and 31 days	15b (down at 31 days, decreased only after exercise at 10 days)			
Up at 4 and 14 days	223, 21, 146a	Cell cycle, inflammation, apoptosis, cell motility	Rat contusive SCI (10 g, 12.5 mm) at T_{12-13}	[35]
Down at 4 and 14 days	1, **124, 129-1, 129-2**			
Up at 24 hours and 7 days	**133b**	Axonal regeneration	Zebrafish complete transection	[36]
Up in BMPR1a CKO	21	Reactive gliosis	Mouse compression SCI at T_{11}	[37]

MiRNA expression profiling after SCI

Liu *et al* [31] published the first report on miRNA expression analysis after SCI at the whole genome scale. They applied a moderate injury (10 g weight drop from 12.5 mm height) in a rat contusive SCI model and collected miRNA samples at 4 hours, 1 and 7 days after injury. MiRNA microarray analysis showed that expression of 60 miRNAs changed significantly. Among them were three classes: (1) 30 miRNAs that constantly increased at 4 hours, 1 day and 7 days; (2) 16 miRNAs that constantly decreased; and (3) 14 miRNAs that increased at 4 hours and decreased at 1 and 7 days after SCI (Table 1). Analysis using gene target prediction algorithms allowed the authors to suggest that these miRNAs with drastic expression changes within a short time window after contusive SCI mainly target genes related to inflammation, oxidative stress and apoptosis. Although the exact functions of miRNAs during SCI progression need to be identified and validated in further functional studies, the fact that the expression levels of numerous miRNAs changed after SCI indicates that miRNAs may play significant roles in responding to injury. Using the same moderate rat contusive SCI model, Strickland *et al* [35] extended the time point of analysis to 14 days after SCI. MiRNA microarray analysis showed that expression of 36 miRNAs was changed dramatically at 4 and 14 days after injury. Among these, four miRNAs showed increased expression, while 32 miRNAs showed decreased expression (Table 1). In addition to quantitative reverse transcriptase-PCR (qRT-PCR) validation of the expression changes of selected miRNAs, the authors carried out *in situ* hybridization experiments to show that miR-124 and miR-129, expression of which was decreased after injury, are mainly expressed in large neuron-like cells within the spinal cord grey matter, suggesting that their decreased expression may be a result of neuronal cell death upon SCI. However, miR-1, expression of which was also decreased after injury, was detected in neural bundles and small cell bodies, while miR-21 is mostly found in spinal cord grey matter and is strongly co-expressed with nestin. Although expression of miR-21 drops 14 days after injury, it increases dramatically 4 days after injury. As pointed out by the authors, their preliminary data show that miR-21 is very important in regulating proliferation and apoptosis in neural progenitor cells[38]. Therefore, it is predicted that miR-21 would be expressed in neural progenitor cells of endogenous spinal cord grey matter and that it might regulate proliferation of these cells in response to injury.

MiR-223 in inflammation after SCI

Nakanishi *et al* [32] also detected miRNA expression level changes at 12 hours after SCI using miRNA microarrays. However, they used a mouse compression SCI model in their study. Statistical analysis showed that five miRNAs were up-regulated significantly, while another five were down-regulated, consistent with the results of a previous study[35]. Nakanishi *et al* [32] observed that the neuronal miRNA miR-124 was mainly expressed in spinal cord gray matter and that its expression was decreased at 12 hours and until 7 days after SCI, correlating well with the period of injury-induced neuronal cell death. However, the unique finding of this report is the expression and potential function of miR-223 in SCI. Up-regulated miRNA miR-223 was found in cells with a diameter of around 10 μm, and this miRNA aggregated near the injury site. Based on their size and location in the spinal cord, the authors postulated that these cells were invading neutrophils and confirmed this conclusion in their subsequent report to show that over 60% of miR-223-positive cells co-express Gr-1 protein (a neutrophil marker)[33]. It was suggested that miR-233 might be involved in regulating secretion of inflammatory factors such as interleukin-1, -6 and TNF-α near the injury site. qRT-PCR analysis revealed two peaks of miR-223 expression, 12 hours and 3 days after injury. The former corresponds to invasion of neutrophils, while the latter may represent macrophage infiltration. The authors further strengthened their conclusion by showing the presence of a known positive regulator of miR-223, CCAAT/enhancer binding protein α, and the absence of a known negative regulator, NFI-A, around the injury epicenter by immunofluorescence staining.

MiRNA expression changes after post-SCI exercise

Timing of altered miRNA expression around the injury site suggests their potential role in mediating inflammation, infiltration of hematogenous cells, the endogenous progenitor response to the insult and neuronal cell death after SCI. During the later stages of SCI, secondary damage will spread to nearby regions of the spinal cord causing more cell death[39]. Liu *et al* [34] used a rat transection SCI model and extended the time point of analysis to 31 days after SCI; they collected tissue samples from L_{4-6} instead of the injury site. In addition, forced cycling exercise was performed on rats after SCI to test for possible activity-dependent plasticity of miRNA expression. qRT-PCR results showed that let-7a and miR-16 were expressed more at 10 and 31 days after injury and were not affected by exercise. At 10 days after injury, however, trained rats expressed more miR-21 and less miR-15b than the untrained group. Expression of phosphatase and tensin homologue and programmed cell death protein 4, miR-21 target genes, decreased with exercise, while the anti-apoptotic gene Bcl-2, a common target of miR-15b and miR-16, showed increased expression with cycling exercise. In line with this notion, the authors demonstrated down-regulated

expression of caspase-7 and caspase-9 at the mRNA level and of caspase-7 at the protein level. This result implied that rehabilitation-like activity after SCI might prevent cell death through regulating apoptosis-associated miRNAs, although the effect of post-injury exercise seemed to be transient because no significant changes in miRNA expression were observed at 31 days after injury.

MiR-21 in glial scar formation after SCI

From the studies mentioned above, miRNAs with altered expression levels upon injury primarily target genes related to acute SCI pathophysiological processes such as inflammatory reaction, oxidative stress and apoptosis. This may be due to the fact that, in most cases, sample collection was done within a few hours to a few days after injury. Unlike these studies, Sahni *et al* [37] examined glial scar formation after contusive SCI of mice and identified miR-21 as a possible effecter of the bone morphogenetic protein (BMP) signaling pathway in regulating glial fibrillary acidic protein expression and astrocyte morphology. Using mouse genetics, the authors showed that conditional knockout of BMP receptor 1a (BMPR1a) leads to lower levels of reactive gliosis, higher levels of inflammatory cell invasion and a lower density of surviving axons, while knockout of BMPR1b promotes reactive gliosis and therefore reduces injury volume. Interestingly, the miR-21 expression level in BMPR1a knockout astrocytes is much higher than that in wild-type astrocytes, while overexpression of miR-21 in cultured wild-type astrocytes dramatically reduced the levels of glial fibrillary acidic protein and caused cell shrinkage. However, glial fibrillary acidic protein is not a predicted target of miR-21, suggesting that an indirect regulatory mechanism may exist. Nevertheless, this is one of the first studies addressing functional aspects of miRNAs in SCI leading to the concept of manipulating miRNAs following SCI for potential therapeutic purposes.

MiR-133b in axonal regeneration after SCI

Another functional study of miRNAs following SCI was performed in zebrafish. Yu *et al* [36] investigated the role of miR-133b in axonal regeneration after complete transection of the spinal cord. Unlike mammals, zebrafish and some other non-mammalian species possess the ability to regenerate the central nervous system after injury. It was first demonstrated that, upon SCI, miR-133b was up-regulated dramatically in the nucleus of the medial longitudinal fascicle of the brainstem, while the expression of miR-133b remained constant in other regions. The nucleus of the medial longitudinal fascicle contains neurons that project axons into the spinal cord and mediate the swimming activity of zebrafish; the increase in miR-133b level in these neurons suggests a functional role[38]. To directly decipher the potential function of miR-133b in axonal regeneration, the authors carried out a loss of function study in which an antisense morpholino against miR-133b was applied to the transection site. The reduction in the level of miR-133b in the nucleus of the medial longitudinal fascicle was accompanied by decreased swimming activity of the fish and the number of regenerated axons crossing the injury site as determined by retrograde tracing. Therefore, the authors concluded that elevated levels of miR-133b in the nucleus of the medial longitudinal fascicle of zebrafish are essential for their locomotor recovery and axonal regeneration. They provided further evidence to suggest that RhoA, a predicted target gene of miR-133b in zebrafish, might be involved in this regeneration process. Interestingly, the miR-133b expression level is increased transiently a few hours after SCI in rats, but decreased at 1 day and 7 days[31-32]. An interesting question would be to determine if a forced high level of miR-133b after SCI could encourage axonal regeneration in rats and humans. As the authors pointed out, this hypothesis needs to be tested by further experiments and will facilitate the translation of an miRNA-related therapy into the clinic. Nevertheless, this is one of the first reports testing miRNA function following SCI, especially neural regeneration.

DESIGNING MiRNA-BASED MOLECULES AS THERAPEUTICS FOR SCI

Effective therapy for SCI patients is lacking. The only Food and Drug Administration-approved treatment for SCI in the clinic is a high dose of methylprednisolone given acutely after injury. This treatment preserves spinal cord tissue and motor function through neuroprotective mechanisms. However, long-term side effects such as osteoporosis, eye problems, muscle weakness and dizziness as a result of this treatment have discouraged usage by many patients[40-41]. On the other hand, the discovery of drastically altered miRNA expression after SCI not only reveals novel mechanisms underlying this traumatic progress, but also offers opportunities for potential therapeutic interventions. Manipulating miRNA levels as a means of gene therapy has certain advantages. First, miRNAs are only about 22-nt long; they diffuse into tissues and are absorbed by cells relatively easily compared with DNA plasmid constructs. Second, a single miRNA can regulate the expression of a set of genes that share a miRNA-binding sequence on their 3'-untranslated regions. Therefore, miRNAs may have a bigger impact and greater effectiveness than

gene therapy. Last, many miRNAs show tissue-specific expression patterns. For example, miR-124 is mainly expressed in neurons[42-43], while miR-1d and miR-133 show muscle specificity[42, 44]. This is a property that can be utilized to reduce side effects in non-targeted tissues[45]. Theoretic principles of miRNA treatment for SCI could be either to overexpress "good" miRNAs, such as anti-apoptotic miR-21 or to reduce "bad" miRNAs such as apoptotic miR-15b by antisense inhibition[34].

The development of miRNA delivery technology is a rapidly growing field given the high expectation for these molecules as therapeutics. Chemical modifications on small RNA molecules have been developed to raise their binding affinity and stability[46-47]. For example, Krutzfeldt *et al* [48] successfully antagonized mouse miR-16, miR-122, miR-192 and miR-194 by intravenously injecting synthetic antisense inhibitors with chemical modifications, called "antagomirs". Cholesterol linkage at the 3' end and 2'-O-methyl modification of RNA molecules significantly increased their stability and efficacy compared with unmodified or partially modified ones. Subsequently, Elmén *et al* [49] antagonized liver specific miRNA, miR-122, in both mouse and African green monkey, and reduced blood cholesterol level efficiently using synthetic locked-nucleic-acid-modified oligonucleotide (LNA-antimiR). The modification of LNA-antimiR resulted in tighter double strand binding and, thus, higher efficacy at a low dosage compared with antagomirs. This might be the reason why the authors did not observe LNA-antimiR-associated toxicity in their study. Another smart way to reduce miRNA levels is to stably express a so-called "decoy miRNA target" *via* a lentiviral system. The decoy miRNA target, which is integrated into the genome and strongly expressed, contains binding sites of specific miRNAs and reduces their functions by competing with their natural binding targets[50]. Remarkably, using a decoy miRNA target strategy, Naldini group has made a stable miR-223 functional knockout mouse line, which phenocopies the knockout of the miR-223 gene[46]. More recently, a variety of both viral and non-viral delivery methodologies have been reported to perturb miRNA expression *in vivo* (Table 2) targeting tissues ranging from liver[48] and lung[51-53] to brain[54-57], and biological processes including tumorigenesis[51, 55, 58-61], angiogenesis[62-64], neurogenesis[57] and synaptic plasticity[65].

There are currently no reports on miRNA treatment for SCI in mammals. However, siRNAs targeting a variety of different genes have been shown to reduce neuropathic pain after SCI[76-86]. The delivery of siRNAs is mainly done *via* viral and non-viral technologies. Lentiviral systems can deliver siRNAs efficiently into spinal cord tissue, but virally induced toxicity is a concern, especially in future clinical settings.

Table 2 Therapeutic designs for perturbing microRNA (miRNA) levels *in vivo*

Chemical modifications or delivery	Targeted miRNAs	Biological process or disease	Reference
Cholesterol-linked, 2'-OMe ("antagomirs")	16, 122, 192, 194	Cholesterol biosynthesis	[48]
Locked-nucleic-acid (LNA-antimiR)	122	Cholesterol biosynthesis	[49, 66]
	21	Lung inflammation	[53]
	33	Cholesterol homeostasis	[67]
	29	Aneurysm formation	[68]
	21	Systemic lupus erythematosus	[69]
	142-3p	Endotoxin-induced mortality	[70]
	miRNA families	Breast tumors	[71]
Lentiviral	223	Hematopoiesis	[50]
	15a/16	Chronic lymphocytic leukemia	[59]
	616	Prostate cancer	[60]
	221, 222	Tumorigenesis	[61]
	16,424	Angiogenesis	[64]
	124, 7d, 181	Synaptogenesis	[65, 72]
Retroviral	150	Mononuclear cell mobilization	[73]
	101	Prostate cancer	[74]
	33	Cholesterol homeostasis	[75]
	132	Neurogenesis	[57]
Adenoviral	134	Dendritogenesis	[56]
	34a	Medulloblastoma	[58]
Transfection reagents	let-7	Lung tumors	[51]
	133b	Lung cancer	[52]
Nanoparticles	124-a	Brain targeting	[54]
	296	Angiogenesis	[62]

Certain transfection reagents can overcome the relatively low efficiency of non-viral siRNA delivery methods. For example, Luo *et al* [87] have shown that a synthetic siRNA targeting delta opioid receptor mixed with transfection reagent *i-Fect*^{TM} and intrathecally administered to the lumbar spinal cord of rats, reduced target protein expression and blocked drug-induced antinociception in a dose-dependent manner. The relatively low effective dose of this siRNA/*i-Fect*^{TM} mixture demonstrated its improved delivery efficiency and caused no apparent toxicity. All these delivery techniques for siRNAs can be readily adopted to miRNAs because of their similar sizes and shared cellular process machinery. In some cases, compared with siRNAs, miRNAs may be a more ideal tool for gene silencing with lower cellular toxicity[88].

CONCLUSION AND PERSPECTIVE

MiRNAs, important gene expression regulators, play essential roles in many biological processes. However, miRNA function during the pathophysiological process of SCI is largely unknown. We summarized the findings of

recent publications, mostly involving expression analyses after SCI, implicating the potential functions of these small non-coding RNA molecules in many post-injury processes including inflammation, apoptosis, glial scar formation and axonal regeneration (Figure 1).

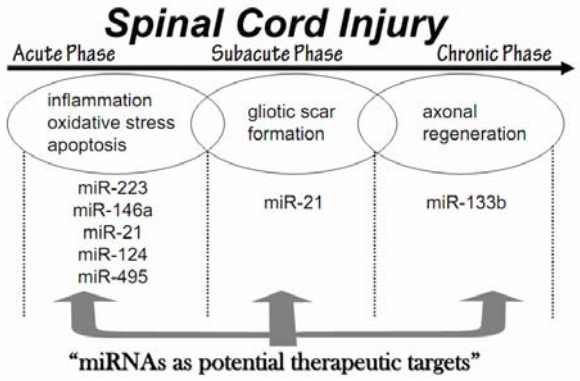

Figure 1 Potential miRNA-based interventions at different stages following spinal cord injury (SCI).

Distinct miRNAs show changes at different stages after SCI, including during inflammation, oxidative stress, apoptosis, glial scar formation and axonal regeneration. These changes imply that miRNAs may be novel targets for treating SCI.

In addition, the latest advances in chemical modification technology continue to generate more stable and efficient antisense modified oligonucleotides to functionally alter deregulated miRNAs *in vivo*. MiRNAs possess great potential to become a new generation of therapeutic drugs, but there are still potential problems such as high dose-associated side effects and toxicity when applied *in vivo*, and unpredictable off-target effects of individual miRNAs. Nevertheless, we believe that with the fast development of science and technology, miRNA-based therapeutic interventions will surely benefit SCI patients in the near future.

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