MicroRNA 486 is a potentially novel target for the treatment of spinal cord injury

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MicroRNAs have been shown to effectively regulate gene expression at the translational level. Recently, we identified novel microRNAs that were upregulated in a mouse model of spinal cord injury. Among those, we have focused on microRNA 486, which directly represses NeuroD6 expression through a conserved sequence in its untranslated region. We correlated the overexpression of microRNA 486 in motor neurons with a poor outcome due to progressive neurodegeneration and a pathophysiology that is mediated by reactive oxygen species. The expression of microRNA 486 was induced by reactive oxygen species that were produced by inflammatory factors, and reactive oxygen species were accumulated in response to the knockdown of NeuroD6, which enhances the downregulation of glutathione peroxidase 3 and thioredoxin-like 1 after traumatic spinal cord injury. NeuroD6 directly bound to regulatory regions of thioredoxin-like 1 and glutathione peroxidase 3 in motor neurons and activated their expression, which promoted reactive oxygen species scavenging. Moreover, knocking down microRNA 486 induced the expression of NeuroD6, which effectively ameliorated the spinal cord injury and allowed the mice to recover motor function. The infusion of exogenic NeuroD6 in spinal cord injury lesions effectively blocked apoptosis by reactivating thioredoxin-like 1 and glutathione peroxidase 3, which was accompanied by a recovery of motor function. Collectively, these findings have identified a novel microRNA in spinal cord injury lesions called microRNA 486, demonstrating a new role for NeuroD6 in neuroprotection, and suggest a potential therapeutic target for spinal cord injuries.

Keywords: miR 486; neuroprotection; spinal cord injury

Abbreviations: COX2 = cyclooxygenase 2; GFAP = glial fibrillary acidic protein; GPx3 = glutathione peroxidase 3; miR 486 = microRNA 486; NF160 = neurofilament 160; SCI = spinal cord injury; SEPN1 = selenoprotein type N1; TXNL1 = thioredoxin-like 1; TUNEL = transferase dUTP nick end labelling; TNF = tumour necrosis factor α
Introduction

MicroRNAs are a new class of small, non-coding, regulatory RNA molecules that are implicated in tissue degeneration and regeneration. MicroRNAs have emerged as important players in translational regulation and have been implicated in the control of functional cell fate (Ivey et al., 2008; Barroso-del et al., 2009; Takaya et al., 2009). Furthermore, an alteration in functional gene expression has been shown to play an important role in secondary injury progression in several degenerative diseases (van Rooij et al., 2006; Zhao et al., 2007; Bala et al., 2009); therefore, aberrant microRNA expression could become a novel therapeutic target in the treatment of various diseases, including cancer, cardiovascular disease and traumatic injuries. In disease models, microRNA signal mediators are attractive candidates as upstream controllers of secondary spinal cord injury (SCI) progression, since microRNAs can modulate the expression of specific sets of functional genes. Based on the specific interactions of microRNAs with their target genes, RNA-based technologies are potential therapeutic strategies (Martinez et al., 2002; Krutzfeldt et al., 2005; Jackson et al., 2006). In addition, a number of microRNAs play a key role in neurodevelopment and are likely to be important mediators of cell differentiation into specific tissues or organs (Kosik, 2006; Kim et al., 2007). For example, specific microRNAs directly guide the development of midbrain dopamine neurons in the embryonic brain (Kim et al., 2007).

Neurodegeneration is caused by a variety of factors that depend on the intrinsic and extrinsic properties of the underlying neurodegenerative disorder and is generally characterized by disturbed cellular homeostasis due to cytotoxicity of intermediate side products, including overloaded inflammatory cells and the secretion of inflammatory factors. During the progression of neurodegenerative disorders, microRNAs regulate gene expression; therefore, they provide new molecular information and represent a potential therapeutic tool that may be used to regulate functional neuronal cell death and survival. By using microRNAs, it may be possible to regulate specific functional cell fates and behaviours by controlling the levels of various messenger RNAs and functional proteins, which are critical for tissues or cells to function. Previous studies have suggested that microRNAs might be involved in neurodegeneration and have shown that the altered expression of microRNAs contributes to secondary injury after traumatic injury to the CNS, including the spinal cord (Saugstad, 2010). Taken together, these studies emphasize that overall gene regulation requires optimal control of both positive and negative regulators of tissue regeneration. New findings regarding microRNAs and their specific role in neurodegenerative diseases will be important in understanding the diversity of neuronal function. During the pathogenesis of neurodegenerative diseases, the microRNA paradigm is closely linked to the regulation of gene expression that affects neuronal survival and regeneration, and activation of specific signalling pathways.

In this study, we used a microarray analysis of microRNA expression in SCI lesioned tissue to identify an enriched microRNA, miR 486 and the subsequent downregulation of one of its functional target genes, neurogenic differentiation 6 (NeuroD6). We found that the expression of miR 486 was induced by reactive oxygen species that were produced by inflammatory factors and that reactive oxygen species accumulated in response to the interference of NeuroD6, which directly enhances the downregulation of glutathione peroxidase 3 (GPx3) and thioredoxin-like 1 (TXNLI) after traumatic injury to the spinal cord. NeuroD6 has been shown to play a neuroprotective role in reactive oxygen species-involved secondary injuries because it directly regulates reactive oxygen species scavenging genes by binding to their regulatory regions. In addition, the NeuroD6 protein promotes neuronal differentiation and survival by expression of GPx3 and TXNL1 in pathological microenvironments and stimulates the mitochondrial biomass to trigger anti-apoptotic responses that involve molecular chaperones (Uittenbogaard et al., 2010). In this study, we demonstrated that the knockdown of NeuroD6 expression in normal spinal cord tissue effectively enhances the cytotoxic effects of pathological microenvironments, which are overloaded with ED1- and myeloperoxidase-positive microglia and macrophages in addition to enhancing the levels of cytotoxic inflammatory cytokines, such as interleukin 6 (IL6), tumour necrosis factor α (TNFα), IL1β and cyclooxygenase 2 (COX2). Knocking down NeuroD6 expression was also associated with the induction of apoptosis in motor neurons, which was triggered by actively proliferating macrophages and was associated with the secretion of pro-inflammatory cytokines, specifically those that actively mediate the degeneration of both motor neurons and myelin. These sequential pathological events finally resulted in a secondary injury to the spinal cord for a long period after the traumatic primary injury. Besides initiating neuronal differentiation, NeuroD6 promotes neuronal survival by expressing anti-apoptotic regulators that preserve mitochondrial integrity. Traumatic injury to the spinal cord, small interfering NeuroD6 and miR 486 all caused a malfunction of the mitochondrial reactive oxygen species scavenging system, which in turn induced continued neuronal and myelin degeneration. This process was effectively enhanced by a deficiency in NeuroD6 expression, which was caused by an overload of miR 486 after traumatic injury in the spinal cord. In the pathological microenvironment that accompanies an SCI lesion, NeuroD6 normally functions along with mitochondrial reactive oxygen species scavenging machinery to enable neuroprotective and neuroregenerative activities.

Collectively, we identified the first instance of injury-inducing, overexpression of miR 486 in motor neurons in traumatic spinal cord lesions. We also demonstrated that miR 486 induces reactive oxygen species-mediated neurodegeneration and active inflammatory factor recruitment by suppressing NeuroD6, which suggests that miR 486 could be a potential target for therapeutic interventions following SCI.

Materials and methods

Induction of mouse spinal cord injury

Adult female ICR mice weighing 30 g (~6 weeks of age) were housed in a controlled environment and provided with standard animal chow and water. All experiments were carried out in compliance with Korean regulations regarding the protection of animals that are used...
for experimental and other scientific purposes. For functional study, the animals were subjected to traumatic SCI through a modified version of the protocol described by Kwon et al. (2002). Animals received sham injuries and contusion injury was carried out using a custom designed electromagnetic force driven impactor, which has been shown to provide consistent and reproducible dynamic contusion injury severity and behavioural outcome measures. Briefly, following an incision through the skin, subcutis and muscle, a T (thoracic) 11-L (lumbar) 1 laminectomy was performed. The contusion injuries were delivered to the T11 spinal cord following a L1 laminectomy. After the surgery was complete, the skin was sutured and the animals were kept warm and allowed to recover from the anaesthesia before being returned to their home cages. Most SCI mice exhibit flaccid paralysis of the lower extremities, although some experience spastic features of

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**Figure 1** The effect of miR 486 and anti-miR 486 expression in motor neurons and the inhibition of NeuroD6 expression in spinal cord tissue after traumatic injury. (A) Schematic flowchart of the experimental procedure for SCI treatment and evaluation. (B) Differential expression of the miR 486 target gene NeuroD6 in spinal cord lesions before and 7 days after the infusion of either miR 486 or scrambled RNA in SCI tissue (*P* = 0.05, *n* = 5). Error bars, ANOVA. (C) In situ hybridization analysis of miR 486 expression in SCI lesions before and 7 days after the infusion of either antisense miR 486 or scrambled RNA; and identification of fluorescein conjugated antisense miR 486 (antisense miR 486flu; Exiqon) after the infusion of antisense miR 486 in SCI tissue. Scale bars = 100 μm. (D) Co-localization of miR 486 and NeuroD6 within motor neurons (NF160-positive) (white arrowhead and red arrow in dotted circle), neuronal progenitor cells (Nestin-positive) and astrocytes (GFAP-positive) (white arrowhead showing single NeuroD6-positive cell) in injured lesions at thoracic 11 (T11) of spinal cord tissue. In antisense miR 486 infused injured spinal cord tissue, residual miR 486 was colocalized with NF160-positive motor neurons, and interfered miR 486 expression induces NeuroD6 expression in motor neurons. Scale bars = 20 μm. (E) Immunohistochemistry result reveals that NeuroD6 is expressed in NF160-positive motor neurons in a SCI lesion (T11) after antisense miR 486 has been infused. The arrowhead indicates a NeuroD6 expressing NF160-positive motor neuron. The results are expressed as the mean ± SEM. Scale bars = 20 μm. MBP = myelin basic protein.
Figure 2 Therapeutic application and functional efficacy of antisense miR 486 in the traumatically injured spinal cord. (A) The functional outcome of antisense miR 486 treatment in injured spinal cords was assessed by Basso mouse scale (BMS). Repeated measures ANOVA with fixed effects and a Student’s t-test revealed a significant improvement in locomotor recovery by Day 30 post-injury (*P < 0.05, **P < 0.01, ***P < 0.001). (B) Anti-miR 486 or NeuroD6 infusion into injured spinal cords resulted in improved functional recovery,
the lower extremities. In this study, we used the mice that exhibited flaccid paralysis of the lower extremities.

The in vitro and in vivo silencing of miR 486 and NeuroD6 with small interfering RNAs

To knockdown miR 486 or NeuroD6 expression, small interfering RNA duplexes (antisense miR 486 or antisense NeuroD6) synthesized specifically for each gene were purchased from Dharmacon RNA Technologies. Cultured neural progenitor cells were grown in 6-well plates to ~60% confluence and were then transiently transfected with 10 μM small interfering RNA (antisense) using DharmaFECT® small interfering RNA transfection reagents according to the manufacturer’s instructions. The cells were allowed to recover for 48 h before being used in an experiment. The cells were harvested after 24 h for RNA isolation. Silencer® Negative Control small interfering RNA (antisense, catalogue number 4611; Ambion, Inc.) was used as a control for non-specific gene silencing. The cells were transfected with the small interfering RNAs (antisense) with DharmaFECT® small interfering RNA transfection reagents. To knockdown NeuroD6 expression in neural progenitor cells, two complementary hairpin small interfering RNAs (antisense) were synthesized from template oligonucleotides, which contained the 21-nt target sequences of mouse NeuroD6, and the cells were transiently transfected with 50 nM small interfering RNA (antisense). Three separate NeuroD6 small interfering RNAs (antisense, Silencer® predesigned small interfering RNAs; Ambion) and scrambled small interfering RNAs (antisense) with the same nucleotide content were assessed. As determined by real-time polymerase chain reaction and western blot analysis, transfection of the three NeuroD6 small interfering RNAs (antisense) resulted in an 80–90% reduction in NeuroD6 when compared to unrelated control and scrambled small interfering RNAs. The small interfering RNA that provided the most efficient inhibition (90–95%) of NeuroD6 was used for the remainder of the experiments. To determine whether the growth of neural progenitor cells was inhibited by the knockdown of NeuroD6, we transfected NeuroD6 small interfering RNA (antisense) into neural progenitor cell and counted Trypan Blue dye-exclusive viable cells or 5-bromodeoxyuridine-positive cells over a period of 6 days. To knockdown miR 486 in vivo, the mice were anaesthetized and immobilized with a stereotaxic instrument. Experimental trials were first conducted to ascertain that the previously described surgical procedure caused a consistent, total transection of the corticospinal tract on both sides. One day after the operation, antisense miR 486 (anti-miR 486; 20 μM stock solution) was diluted with DharmaFECT® (Dharmacon Inc.), and a total of 5 μl (2.5 μl of DharmaFECT® and 2.5 μl of antisense miR 486 or scrambled RNA) was injected with a 25-g Hamilton syringe after 2 days of SCI. Each mouse was anaesthetized with avertin, its dorsal spinal cord was opened with a surgical knife, and the vertebral bone was removed with surgical scissors and rongeurs. Antisense miR 486 was injected at a final concentration of 10 μM. To evaluate the therapeutic efficacy of NeuroD6 in SCI, we directly administrated an expression vector that contained the NeuroD6 gene (0.3 μg DNA/5 μl of Lipofectamine®) into the lesioned area of the spinal cord (T11-L1) 3 days after either SCI induction or the infusion of miR 486 or small interfering NeuroD6. A total of 60 mice were randomly assigned to each group. The experimental protocol was approved by the local institutional ethics committee in compliance with the current guidelines of the National Institutes of Health. All efforts were made to minimize animal suffering. The experiments were performed in accordance with international guidelines for the use of laboratory animals.

Table 1 Summary of Basso mouse score evaluation for anti-miR 486 and NeuroD6-mediated SCI treatment

<table>
<thead>
<tr>
<th></th>
<th>Normal spinal cord</th>
<th>SCI</th>
<th>SCI/scram RNA</th>
<th>SCI/anti-miR 486</th>
<th>SCI/NeuroD6</th>
<th>P-value</th>
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<tr>
<td><strong>Basso mouse score</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Pre-SCI</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>NS</td>
</tr>
<tr>
<td>Week 1</td>
<td>8.6 ± 0.47</td>
<td>0.8 ± 0.42</td>
<td>1.3 ± 0.45</td>
<td>2.3 ± 1.04</td>
<td>2.4 ± 1.00</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Week 2</td>
<td>8.7 ± 0.45</td>
<td>2.1 ± 0.31</td>
<td>1.6 ± 0.53</td>
<td>4.6 ± 0.90</td>
<td>5.1 ± 0.73</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Week 3</td>
<td>9.0 ± 0.00</td>
<td>2.6 ± 0.34</td>
<td>2.8 ± 0.69</td>
<td>5.5 ± 0.72</td>
<td>5.2 ± 0.69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Week 4</td>
<td>8.6 ± 0.47</td>
<td>3.0 ± 0.54</td>
<td>3.1 ± 0.50</td>
<td>6.0 ± 0.77</td>
<td>5.4 ± 0.78</td>
<td>&lt;0.001</td>
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NS = not significant.
divided into several groups. The first set consisted of 30 SCI mice that received either antisense miR 486 (n = 10), NeuroD6 (n = 10) or scrambled RNA (negative control; n = 10), and the second set consisted of 30 mice with an untreated spinal cord that received small interfering NeuroD6 (n = 10), miR 486 (n = 10) or scrambled RNA (negative control; n = 10).

Measurement of functional recovery following spinal cord injury

All animals (controls and experimental groups) were placed on a restricted diet to motivate them to perform the various behavioural tests. The motor function of the hind limbs was evaluated with the Basso mouse scale for locomotion (Basso et al., 2006). Mice were classified into different categories of hind-limb motor function as follows: 0–2 (Basso mouse scale score): completely paralysed, 3–4: some ankle movement, 5–6: frequently coordinative stepping, 7–9: consistent coordinative stepping, 10: normal function. At least three blind observers scored all open field tests and were positioned across from each other to observe both sides of the mice. Each mouse was assigned a number on the Basso mouse scale every 3 days before and after injury induction. The statistical analysis was carried out using the StatView software package (SAS).

Statistical analysis

Data are presented as mean (± SD) for baseline values. Baseline and post-treatment variables were compared using a parametric t-test. Data were reported from five or more independent experiments. For in vivo experiments, statistically significant differences in functional recovery between groups were calculated by repeated measures of the ANOVA test two-way repeated-measures ANOVA with post hoc Tukey’s test for multiple comparisons. For behavioural evaluation (hind limb paralysis) of miR 486 infused or small interfering NeuroD6 induced normal animal, we analysed with a Fisher’s Exact Test.

Results

MiR 486 overexpression in motor neurons effectively induces reactive oxygen species-mediated neurodegeneration in spinal cord injury

To study the molecular and functional roles of miR 486 in SCI lesions (T11), we used miR 486 specific antisense oligomers (antisense miR 486) (Fig. 1A). We found that injured spinal cord tissue significantly upregulated miR 486 compared with normal spinal cord tissue 7 days after SCI induction or 6 days after interference of miR 486 expressions in SCI lesion (Fig. 1B and C).

In spinal cord lesions, one of the target genes of miR 486, NeuroD6, which is downregulated, was differentially expressed (Fig. 1B and Supplementary Fig. 1). Importantly, antisense miR 486 infusion effectively induces more or less increased NeuroD6 expression in injured spinal cord tissue (Fig. 1B and D). Moreover, Dicer, retinoic acid receptor γ (RxR), EphrinA1 and Enox1 were significantly downregulated after injury (Supplementary Fig. 1). After 7 days of traumatic SCI, we analysed miR 486 expression and localization in motor neurons. Our in situ analysis of miR 486 and the immunofluorescence detection of neurofilament 160 (NF160), nestin, myelin basic protein and glial fibrillary acidic protein (GFAP) in anti-miR 486 infused SCI lesions revealed that residual miR 486 is considerably co-localized with partially degenerated NF160-positive motor neurons that also expressed low levels of NeuroD6 in the nucleus (Fig. 1D). Moreover, we found that NF160-positive motor neurons were 2’7’-dichlorodihydrofluorescein diacetate (DCFDA)-positive and accumulated reactive oxygen species in the cytosol (Supplementary Fig. 2A). To verify the NeuroD6 expression in motor neurons, we identified immunohistochemical co-localization of NF160 and NeuroD6 in antisense miR 486 treated injured spinal cord tissue (Fig. 1E). NeuroD6 expression was detected at strong levels in the nucleus and cytosol of motor neurons in anti-miR 486 infused SCI lesions and normal spinal cord tissue (Fig. 1D and E). In addition, we found that ED1-positive macrophages also produced reactive oxygen species but never detected miR 486 expression in macrophages (Supplementary Fig. 2C). Finally, we focused on understanding the therapeutic efficacy and molecular functions of miR 486 in the neurodegeneration and pathophysiology in SCI.

Interference of miR 486 expression significantly improves motor function

Finally, we attempted to evaluate the therapeutic efficacy and molecular functions of miR 486 in the neurodegeneration and pathophysiology of SCI. We blindly evaluated motor function over a period of 30 days using the Basso mouse scale. Compared to animals with a SCI alone, knocking down miR 486 expression in SCI animals resulted in improved motor function. All of the animals that had undergone a SCI had severe deficits in motor function in the initial days post-injury when compared with uninjured animals (Fig. 2A and B; Table 1). Three days after SCI, knocking down miR 486 expression in SCI animals resulted in a gradual recovery from the paralytic phenotype, and the animals were able to consistently support their own weight during planar stepping even though their paws were predominantly in a rotated position during walking. In contrast, injured animals that were injected with scrambled small interfering RNA had limited joint locomotion (Fig. 2A and B; Table 1). We also presented significant differences

### Table 2: Behavioural analysis of miR 486 or small interfering NeuroD6 infused animal

<table>
<thead>
<tr>
<th>Group (n = 10)</th>
<th>Paralysis</th>
<th>Non-paralysis</th>
<th>Fisher P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
<td>10</td>
<td>0.0001</td>
</tr>
<tr>
<td>scramRNA/SC</td>
<td>0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>miR486/SC</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>siNeuroD6/SC</td>
<td>6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>SCI</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>SCI/scramRNA</td>
<td>7</td>
<td>3</td>
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</table>

The infusion of miR 486 or small interfering NeuroD6 into uninjured spinal cords induced traumatic SCI-like symptoms, including hind limb paralysis. P-values are two-tailed. siNeuroD6 = small interfering NeuroD6.
Knockdown of miR 486 rescues NeuroD6 expression and induces neuroprotection via upregulation of GPx3, TXNL1 and SEPN1

Knocking down miR 486 expression in SCI mice increased the expression of its target genes after 2 days of direct infusion of an antisense miR 486 and Lipofectamine® mixture (Supplementary Fig. 1). Transfecting a small interfering RNA against miR 486 not only effectively inhibited the expression of miR 486, which was downregulated by 88%, but also downregulated the expression of the proinflammatory factors IL1β, IL6 and TNFα (Fig. 3A). In addition, the expression of myeloperoxidase, eNOS (endothelial nitric oxide synthase) and iNOS (inducible nitric oxide synthase) was significantly decreased 7 days after miR 486 expression was knocked down (Fig. 3B). In contrast, the expression of the reactive oxygen species scavenging enzymes selenoprotein type N1 (SEPN1), TXNL1, GPx1 and GPx3 significantly increased and COX2 was downregulated 3 and 7 days after miR 486 expression was knocked down in SCI mice (Fig. 3C and D). Additionally, the number of ED1- and Iba1-positive inflammatory cells, macrophages and microglial cells was significantly decreased in SCI mice 7 days after miR 486 knockdown (Fig. 3E and Supplementary Fig. 2E). The immunohistochemical analysis of miR 486 knockdown in injured spinal cord tissue showed a significant increase in the expression of TXNL1, GPx3 and SEPN1 in spinal cord lesions, and NF160-positive motor neurons were preserved (Supplementary Fig. 2B). In addition, TXNL1, GPx3 and SEPN1 expressions were primarily localized to the cytosolic and nuclear regions of NF160-positive motor neurons (Supplementary Fig. 2B). In contrast, an increase in miR 486 expression in normal spinal cord tissue induces a downregulation of the expression of NeuroD6, GPx3, TXNL1 and SEPN1 in NF160-positive motor neurons (data not shown). In addition, the expression of GPx3, TXNL1 and SEPN1 was also detected in NF160-positive motor neurons in injured spinal cord tissues that were infused with antisense miR 486 (Supplementary Fig. 2B). Normal motor neurons also had an increased expression of NeuroD6, GPx3, TXNL1 and SEPN1 in the nuclear zone and in the cytosol at the single cell level (data not shown). Knocking down miR 486 expression resulted in increased NeuroD6 and heat shock protein 90 (HSP90) expression and also induces further activation of PI3K/Akt and P38/MAPK along with downregulated apoptotic cell death signals, Bax, cytochrome C, P53 and cleavage form of caspase-3, and increased the expression of Bcl2 in SCI animals (Table 4; Supplementary Figs 4 and 5).

In differentiated neuronal cultures, the knockdown of miR 486 expression significantly increased PI3K/Akt phosphorylation, downregulated p53 expression and increased the survival and growth of these cultured neuronal cells (Supplementary Fig. 5A–C). In contrast, exogenic miR 486 infusions significantly induced downregulation of NF160, TuJ and myelin basic protein expression in normal spinal cord tissue (Fig. 4A and B). Compared to SCI animals, miR 486-infused animals showed a very similar expression pattern of the redox generating factors ED1, myeloperoxidase, inducible nitric oxide synthase and endothelial nitric oxide synthase, with a high level of reactive oxygen species generation (DCFDA-positive; 88.7%) in both groups of animals (Fig. 4C and Supplementary Fig. 2D). Additionally, miR 486 infusion into normal spinal cords resulted in significantly increased ED1-positive and myeloperoxidase-positive cells to levels similar to those present in SCI lesions (Fig. 4C). Relative cell survival was significantly decreased after miR 486 infused normal spinal cord tissue and injured spinal cord tissue (22 and 13% at each) (Fig. 4D). Interference of miR 486 expression in injured spinal cord tissue showed that significantly increased cell survival (64%) of total

Table 3 The effect of anti-miR 486 on neural cell distribution in SCI lesions

<table>
<thead>
<tr>
<th></th>
<th>Normal spinal cord</th>
<th>SCI</th>
<th>SCI/scramRNA</th>
<th>SCI/anti-miR486</th>
<th>P-value</th>
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<tbody>
<tr>
<td>TuJ</td>
<td>4.97 ± 32.4</td>
<td>3.61 ± 24.1</td>
<td>3.46 ± 20.9</td>
<td>5.61 ± 34.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>NF160</td>
<td>5.72 ± 35.2</td>
<td>2.99 ± 19.6</td>
<td>3.12 ± 20.0</td>
<td>9.30 ± 53.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MBP</td>
<td>4.89 ± 28.8</td>
<td>0.59 ± 8.9</td>
<td>0.61 ± 8.5</td>
<td>1.01 ± 9.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>GFAP</td>
<td>6.52 ± 40.4</td>
<td>6.34 ± 39.8</td>
<td>6.79 ± 40.1</td>
<td>8.17 ± 52.0</td>
<td>&lt;0.05</td>
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P-values are ANOVA. MBP = myelin basic protein.

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cells in SCI tissue (Fig. 4D). Quantitative real-time polymerase chain reaction analysis of inflammation-related gene expression in normal spinal cord, miR 486-infused normal spinal cord, and miR 486- and antisense miR 486-infused normal spinal cord revealed a significant increase in the levels of proinflammatory factors, such as COX2, IL-1β, IL-6 and TNFα in miR 486-infused normal spinal cord and injured spinal cord tissue (Table 4). Additionally, we confirmed that macrophage (using cell line RAW 264.7) was not involved in miR 486-mediated NeuroD6 expression regulation even though RAW 264.7 expresses SEPN1, TXNL1 and GPx3 (Supplementary Fig. 3).

In vivo, mice with SCI and miR 486 infected normal spinal cords effectively induced high reactive oxygen species production and eventually resulted in cell growth attenuation and induced apoptotic cell death along with p-Pi3K/p-Akt downregulation (Supplementary Figs 2D and 5A–C). The expression level of heat shock protein 90 was relatively increased after antisense miR 486 infusion in injured spinal cord tissue along with downregulated P53 expression (Supplementary Fig. 4). The expression level of heat shock factor 1 (HSF1) was significantly decreased in injured SCI tissue and miR 486-infused normal spinal cord tissue. In contrast, HSF2 expression was not significantly changed in injured spinal cord and miR 486-infused normal spinal cord tissue (Supplementary Fig. 5D). The induction of miR 486 expression in neural progenitor cell cultures that were isolated from spinal cord tissue, effectively blocked the expression of NeuroD6 and the production of reactive oxygen species and proinflammatory factors. Additionally, cell survival was significantly decreased (data not shown), while apoptotic cell signalling was increased (Fig. 4E and F; Supplementary Fig. 2D). The transfection of either miR 486 or small interfering NeuroD6 significantly decreased motor neuron survival in these primary cultures. Compared with untreated or scrambled RNA-treated motor neurons that were used as controls, rescuing the expression of either anti-miR 486 treated cells or NeuroD6 expression in small
interfering NeuroD6-treated cells effectively returned cell survival levels to normal (Fig. 4G). Moreover, the DNA binding frequency of the regulatory region of NeuroD6 was increased following knockdown of miR 486 expression but significantly decreased in miR 486-overexpressing neural progenitor cells (Fig. 4H).

Additionally, we found that miR 486 treatment resulted in inefficient neurosphere formation and completely diminished neurogenic potency in neural progenitor cells (Supplementary Fig. 6A and B). Table 4 shows that relative expression of target gene of miR 486, NeuroD6 and inflammatory cytokines, redox scavengers and cell death and survival relating signal pathway molecules in miR 486 infused normal spinal cord, miR 486/anti-miR 486 treated normal spinal cord and anti-miR 486 infused injured spinal cord tissue (Table 4).

### Reactive oxygen species induces positive feedback for miR 486 expression and insufficient NeuroD6 triggers an antioxidant response through GPx3 and TXNL1 in SCI mice

In this study, the expression of NeuroD6 was correlated with oxidative stress, reactive oxygen species metabolism and the response of molecular chaperones, which are involved in gene expression (Fig. 5). Figure 5A shows the miR 486 binding region located in the 3'-untranslated region of the mouse NeuroD6 gene on chromosome 6 and the complementary homologous base sequence between the NeuroD6 and miR 486 genes. Knocking down miR 486 expression induced NeuroD6 to bind to regulatory regions of the GPx3 and TXNL1 genes (Fig. 5A and B). As observed in our gene expression analysis, knockdown of NeuroD6 expression in uninjured mouse spinal cord induced similar pathological phenomena to those seen in mice with SCI. Moreover, normal mice treated with small interfering NeuroD6 were completely paralysed 3 days after treatment (Table 2). The in vivo expression of NF160, β-tubulinIII and myelin basic protein in spinal cord tissue was downregulated after 48 h of small interfering NeuroD6 infusion (Fig. 5C). We assessed NeuroD6 expression by in situ hybridization in injured spinal cords as well as miR 486 and small interfering NeuroD6-infused spinal cords. NeuroD6 expression was significantly downregulated in miR 486 and small interfering NeuroD6-treated spinal cord tissue (Fig. 5C and Supplementary Fig. 7E). Reactive oxygen species significantly accumulated in SCI tissue as well as tissue from miR 486- and small interfering NeuroD6 infused spinal cords (Supplementary Fig. 7F). Knocking down NeuroD6 expression in uninjured spinal cords had a cytotoxic effect on motor neurons and enhanced the expression of TNFa, IL1β, IL6, COX2, iNOS and eNOS; this treatment also downregulated the expression of the reactive oxygen species scavenging machinery components SEPN1, TXNL2 and GPx3, which created a pathological microenvironment (Fig. 5D–F).
Figure 4 The potential role of miR 486 during secondary injury progression in the injured spinal cord. (A) Schematic flowchart of the experimental procedure used to knockdown miR 486 expression and to analyse miR 486 function. (B) The infusion of miR 486 effectively induced the downregulation of TuJ, NF160 and myelin basic protein (MBP) expression directly or indirectly in the normal spinal cord.
and Supplementary Fig. 7F). The expression of HSP90 and HSF was affected by NeuroD6 and was significantly decreased in small interfering NeuroD6-infused normal animals compared with controls (Supplementary Fig. 7A and B). Additionally, NeuroD6-mediated neural cell protection in both the spinal cord and in cultured neural progenitor cells was correlated with effective reactive oxygen species scavenging, phosphorylation of PI3K/Akt and P38/JNK, Bax/caspase-3 downregulation and normalized active oxygen species scavenging, phosphorylation of PI3K/Akt mediated neural cell protection in both the spinal cord and in controls (Supplementary Fig. 7A and B). Additionally, NeuroD6-interfering NeuroD6-infused normal animals compared with controls was affected by NeuroD6 and was significantly decreased in small interfering NeuroD6-infused spinal cord tissue overproduced reactive oxygen species (Supplementary Fig. 7F and 8A). Small interfering NeuroD6-mediated reactive oxygen species generation effectively induced apoptosis of cultured neural progenitor cells, which contributed to the diminished expression of reactive oxygen species scavenging enzymes (Supplementary Fig. 8B–D). In particular, the expression of ED1, COX2, iNOS and eNOS was increased, but the survival mediator, p-PI3K/p-Akt was downregulated. Additionally, the tissue had a pro-apoptotic signature with increased expression of p-P38, p-JNK, Bax, cytochrome C and caspase-3 (Supplementary Fig. 8E and G). The expression of HSP90 and C/EBP-α in cultured neural progenitor cells was decreased after NeuroD6 was knocked down (Supplementary Fig. 8H and I). The neurogenic potency of neural progenitor cells was significantly reduced following the knockdown of NeuroD6 expression (Supplementary Fig. 8I).

To determine whether NeuroD6 or reactive oxygen species regulated miR 486 expression, we analysed miR 486 expression levels before and after treatment with NeuroD6, H2O2, H2O2/NeuroD6 and H2O2/ascorbic acid (5 μg/ml) in differentiated neuronal cell cultures that were derived from the spinal cord. Interestingly, miR 486 expression increased up to 3-fold (320%) upon H2O2 treatment, and NeuroD6 or ascorbic acid pretreatment significantly reduced the H2O2-mediated miR 486 upregulation (194 and 132%, respectively) (Fig. 5H). Hydrogen peroxide-induced reactive oxygen species production was significantly inhibited by the expression of NeuroD6 (53%), SEPN1 (37%), TXNL1 (23%) and GPX3 (40%). Before neural progenitor cells were exposed to H2O2, pretreatment with NeuroD6 or ascorbic acid (5 μg/ml) upregulated SEPN1, TXNL1 and GPX3 expression substantially more than exposure to H2O2 alone (Fig. 5I–L). Moreover, exogenous NeuroD6 protected neuronal cells (NF 160-positive) from reactive oxygen species-mediated neurotoxicity (Fig. 5M). Figure 5N shows the signalling pathway for reactive oxygen species/miR 486-mediated motor neurodegeneration after traumatic injury to the spinal cord.

Achieving neuroprotection through the expression of exogenous NeuroD6 in mice with spinal cord injury

To examine the functional effect of NeuroD6 in SCI tissue, we analysed the direct regulation of redox scavenging gene expression, motor neuron protection and indirect secondary injury modulation (through a reactive oxygen species-controlling mechanism) for functional behaviour in miR 486- and small interfering NeuroD6-infused animals (Fig. 6A). Treatment with exogenic NeuroD6 ameliorated the traumatic damage that had been previously observed after miR 486 infusion into the spinal cord. Exogenous NeuroD6 expression significantly increased the expression of SEPN1, TXNL1 and GPX3 (Fig. 6B–E). When we evaluated the immunomodulatory function of infused NeuroD6 in each type of damaged spinal cord tissue, we noted a high ratio of NF160-positive to NeuroD6-positive surviving motor neurons compared to untreated SCI, miR 486 and small interfering NeuroD6-infused control tissues (Fig. 6F; Supplementary Fig. 9A and D). In contrast, neuronal cell protection against small interfering NeuroD6 or miR 486 infusion in normal spinal cord was increased. However, NeuroD6 infusion in the injured spinal cord resulted in a decrease in the GFAP-positive population in this tissue (Supplementary Fig. 9B and D). Four weeks after SCI, NeuroD6-infused animals recovered from paralysis, consistently supported their own weight during plantar stepping and had a predominantly rotated paw position during walking. In contrast, injured animals injected only with scrambled small interfering RNA had limited joint locomotion (Fig. 6G). Moreover, we observed a significant upregulation of several microRNAs (Mmu-miR 135a-1, Mmu-miR 135a-2, Mmu-miR 705, Mmu-miR 873, Mmu-miR 875, Mmu-miR 20a) at various time intervals after traumatic injury was induced in the mouse spinal cord (Supplementary Fig. 10A). Among those, miR 486 expression was significantly upregulated after traumatic SCI (Supplementary Fig. 10A). Supplementary Fig. 10B shows that various doses of miR 486, small interfering NeuroD6, anti-miR 486 and NeuroD6 effectively regulate miR 486 and NeuroD6 expression after SCI induction (Supplementary Fig. 10B). Supplementary Fig. 11 presents a global regulation pathway involving miR 486 and anti-miR 486 in SCI induced lesions.

**Figure 4 Continued**

(C) Western blot and immunohistochemical analysis revealed that miR 486 infusion into uninjured spinal cords significantly induced NeuroD6 downregulation and increased the number of ED- and myeloperoxidase-positive cells and reactive oxygen species derivatives (INOS and eNOS) to levels similar to those present in SCI lesions. (D) MiR 486 induced the attenuation of neural progenitor cell growth, reactive oxygen species production (E) and apoptosis (F) of cultured neural progenitor cells (TUNEL-positive cells). (*P < 0.01, **P < 0.05, n = 3). Student’s t-test. (G) The relative survival of cultured motor neurons before and after the infusion of miR 486 and small interfering NeuroD6 in the spinal cord. (*P < 0.01, **P < 0.05, n = 3). Student’s t-test. (H) The DNA binding frequency of NeuroD6 was increased after miR 486 expression was knocked down in neural progenitor cells but was significantly decreased in miR 486 treated cells compared with control (untreated) neural progenitor cells. (*P < 0.01, **P < 0.05, n = 3). Student’s t-test. CHIP = chromatin immunoprecipitation; NPC = neural progenitor cell.
Figure 5  Interference of NeuroD6 expression significantly induces a traumatic SCI-like phenotype in vivo. (A) The miR 486 binding region of the 3'-untranslated region of the mouse (Mmu) NeuroD6 gene on chromosome 6 and the complementary homologous base sequence between NeuroD6 and the miR 486 gene. (B) Compared to injured spinal cord tissue, NeuroD6 binding frequency to regulatory regions of the GPx3 and TXNL1 genes was significantly increased after knocking down of miR 486 expression in injured spinal cord tissue.
MicroRNAs regulate gene expression at the post-transcriptional level and are capable of controlling specific cellular and physiological processes that are associated with disease (Scott et al., 2005; Zhao et al., 2007); consequently, these non-coding microRNAs have newly discovered roles in the biology of several disease models. Conversely, functional genes that are fundamental for a specific cell type may selectively avoid microRNA targeting (anti-targets). Different tissues and physiological conditions are associated with specific patterns or alterations in microRNA expression, and tissue specific microRNA signatures have been found to be associated with specific diseases. Recently, we demonstrated that knocking down miR 486 expression in mouse SCI tissue effectively improved the recovery of hind limb function, as measured by an assessment of motor performance. Using a model of lower thoracic SCI that depends largely on the degeneration of both myelin and the axonal tracts of motor neurons (Sipski et al., 2004), we demonstrated that microRNA is deregulated in acute SCI and characterized the specific biological and molecular role of microRNA during the progression of secondary injury. These results suggested that multiple therapeutic directions were possible. Several therapeutic approaches have been used to alter the micro-environment of the injured spinal cord and/or to stimulate the endogenous repair system in an attempt to ameliorate secondary processes that are associated with disease (Scott et al., 2005; Zhao et al., 2007); consequently, these non-coding microRNAs have newly discovered roles in the biology of several disease models. Conversely, functional genes that are fundamental for a specific cell type may selectively avoid microRNA targeting (anti-targets). 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Several therapeutic approaches have been used to alter the micro-environment of the injured spinal cord and/or to stimulate the endogenous repair system in an attempt to ameliorate secondary injury (Xu et al., 2001; Hulsebosch, 2002; Han et al., 2010; Myers et al., 2011). Secondary injury includes the onset of traumatic damage and demyelination, which results in a gradual increase in neuronal cell death (Kobrine, 1975; Yu, 1994). The pathophysiological disturbances of the secondary injury also gradually increase oxidative stress and inflammatory reactions (Liu et al., 1996, 1999; Murphy and Smith, 2000; Crack and Taylor, 2005). Reactive oxygen species-mediated oxidative damage is primarily caused by imbalances in the generation of reactive oxygen species and the activity of protective mechanisms (Kluck et al., 1997; Lee et al., 2000; LaPlaca et al., 2001; Bushati and Cohen, 2007). Reactive oxygen species-mediated cell damage is normally controlled by endogenous antioxidant systems, which include glutathione, ascorbic acid and reactive oxygen species-scavenging-related enzymes (e.g. GPx). Recently, we found that traumatic injury induces miR 486 upregulation in the motor neurons found in spinal cord lesions. Notably, the final product of miR 486, reactive oxygen species, actively participated in a positive feedback system for miR 486 overproduction in the lesions (Fig. 5N). Moreover, miR 486 expression was significantly increased by the accumulation of reactive oxygen species in motor neurons in both injured spinal cords and cultured neural progenitor cells. Motor neurons in the normal spinal cord express the target of miR 486, NeuroD6, which plays a neuroprotective role against reactive oxygen species-mediated apoptotic neuronal cell death by inducing the expression of reactive oxygen species scavenging machinery, including GPx3, TXNL1 and SEPN1. Reactive oxygen species mediated miR 486 overexpression in damaged motor neurons resulted in both the progressive cytosolic accumulation of reactive oxygen species and the downregulation of NeuroD6, which directly controls the expression of GPx3 and TXNL1. In response to these changes, neurodegeneration in the lesion was accelerated after traumatic damage. Furthermore, accumulation of reactive oxygen species in neural cells gradually increased miR 486 expression and increased the extent of the secondary injury by actively recruiting macrophages to the lesion. Finally, traumatic injury resulted in the activation of the reactive oxygen species/miR 486-mediated positive feedback pathway, which led to incurable, accelerated neurodegeneration and indirectly induced demyelination and secondary injury. When we specifically knocked down miR 486 expression in an injured spinal cord, reactive oxygen species accumulation and inflammatory cytokine overexpression were both significantly reduced, and we observed motor neuron protection and functional recovery. The loss of miR 486 expression effectively protected motor neurons against the trauma that induced their primary destruction and also indirectly ameliorated secondary injury that derived from NeuroD6 controlling the expression of GPx3, SEPN1 and TXNL1 in spinal cord lesions. These antioxidants effectively scavenged reactive oxygen species, and the induction of miR 486 expression by reactive oxygen species was negatively regulated. The sequential events induced by knocking down miR 486 finally resulted in neuroprotection against reactive oxygen species mediated apoptotic motor neuron degeneration in SCI lesions. Recent studies have reported the increased expression of miR 21 and miR 24 following ischaemic preconditioning of the animal, which triggered functional potency by upregulating a HSP (HSP70) as well as endothelial and inducible nitric oxide synthase (eNOS and iNOS) (Chang and Mendell, 2007; Uittenbogaard et al., 2007; Yin et al., 2008, 2009; Dong et al., 2009).

**Figure 5 Continued**

(C) Knocking down NeuroD6 in normal spinal cords significantly induced motor neuron destruction and diminishes myelin protein levels 1 day after NeuroD6 infusion. (D) Compared to control tissue (scrambled RNA–treated normal spinal cord), knocking down NeuroD6 expression in uninjured spinal cord tissue enhanced the expression of the inflammatory factors ED1, COX2, INOS, eNOS (E) as well as IL-1β, IL-6 and TNFα (*P < 0.0004, n = 4). Error bars, ANOVA. (F) Compared to control tissue (scrambled RNA-treated normal spinal cord), knocking down NeuroD6 expression in normal spinal cords downregulated the expression of reactive oxygen species-scavenging machinery (*P < 0.002, n = 4). Error bars, ANOVA. (G) Compared to the normal spinal cord, both knockdown of NeuroD6 and miR 486 infusion effectively reduced ATP generation and increased caspase-3 activity in uninjured spinal cord tissue (*P < 0.0025, **P < 0.0001, n = 4). Error bars, ANOVA. (H and I) Hydrogen peroxide (H2O2) significantly induced miR 486 overexpression, and NeuroD6 effectively attenuated H2O2-mediated miR 486 expression in cultured spinal cord-derived neural progenitor cells (*P < 0.0025, n = 4). Student’s t-test. NeuroD6 expression was effectively blocked by H2O2 induced reactive oxygen species generation in neural progenitor cells. (J–M) SEPN1, TXNL1, GPx3 and NF160 expression levels were positively controlled by NeuroD6 overexpression, but H2O2 significantly downregulated the expression of SEPN1, TXNL1, GPx3 and NF160 in neural progenitor cells (*P < 0.05, n = 3). Student’s t-test. (N) A schematic flowchart of reactive oxygen species/miR 486-mediated neurodegeneration of motor neurons after traumatic injury in the spinal cord. The results are expressed as the mean ± SEM. ROS = reactive oxygen species.
Figure 6  Rescuing NeuroD6 expression in SCI tissue significantly increases the expression of reactive oxygen species scavenging enzymes and neuroregeneration of motor neurons. (A) A schematic flowchart of the experimental schedule for treatment with miR 486, antisense miR 486, small interfering NeuroD6 (siNeuroD6) and NeuroD6 and the subsequent functional evaluation. (B–E) Compared to the scrambled RNA/SCI control group, exogenous NeuroD6 expression in SCI mice as well as in miR 486 and small interfering NeuroD6 infused spinal cords induced the overexpression of NeuroD6, SEPN1, TXNL1 and GPx3 (*P < 0.001, n = 3). Error bars, ANOVA. (F) The amount of neuroregeneration after exogenous NeuroD6 expression in SCI tissue and in miR 486 and small interfering NeuroD6 infused spinal cord tissue was compared to the scrambled/SCI control group (*P < 0.01, n = 3). Error bars, ANOVA. (G) The functional efficacy of exogenous NeuroD6 treatment in injured spinal cords was measured by a Basso mouse scale (BMS) evaluation before and after miR 486 expressions was knocked down in SCI mice. An enlarged cell image is shown in the small box (*P < 0.05, **P < 0.01, ***P < 0.001). Error bars, ANOVA. BBB = blood-brain barrier; IHC = immunohistochemistry.
The major target of miR 486 in the motor neuron is NeuroD6, which has transcriptional networking roles in promoting neuronal survival and comprises part of a molecular switch that links neuronal differentiation and survival. Our study demonstrates that NeuroD6 plays a key role in antioxidant responses and prevents the generation of reactive oxygen species, both of which are crucial mediators of the pathophysiology of acute SCI. We demonstrate that the application of miR 486 induces SCI-like symptoms, which include the infiltration of microglial cells and macrophages that produce various reactive nitrogen species, inducing cytotoxic inflammatory cytokines. NeuroD6 can reduce these effects by its indirect antioxidant function and by modulating the downstream pathway that involves p-P38/p-JNK; therefore, the infusion of antisense miR 486 or exogenous NeuroD6 resulted in a lower level of intracellular reactive oxygen species, decreased the number of macrophages and microglia and upregulated reactive oxygen species scavenging machinery. Finally, NeuroD6 induces the inhibition of caspase 3. The primary protective effect of NeuroD6 in SCI therefore manifests as immunomodulation with the suppression of acute secondary neuronal apoptosis. Interestingly, miR 486, which also controls the expression of its target gene NeuroD6, has a critical role in primary injury blockage by directly binding to regulatory regions of TXNL1 and GPx3. NeuroD6 induced expression of GPx3 and TXNL1 in spinal cord lesions effectively scavenges overloaded reactive oxygen species and attenuates inflammatory immune cell propagation and infiltration in SCI tissue.

The results of this study demonstrate that the inhibition of miR 486 following SCI significantly decreases apoptosis and reduces functional deficits by upregulating NeuroD6 expression. Knocking down miR 486 or infusing NeuroD6 into SCI animals recovered hind limb reflexes more rapidly, and a higher percentage of these mice regained motor function compared to untreated SCI mice. Our finding that the loss of miR 486 expression effectively promotes motor function recovery in an animal model suggests novel drug targets for treating SCI in humans.

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Supplementary material
Supplementary material is available at Brain online.

References


