News Release

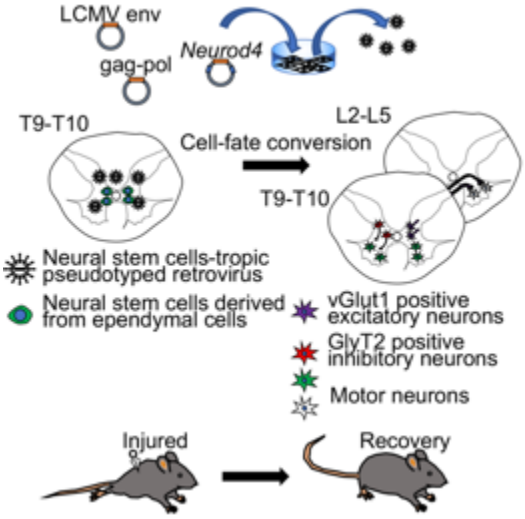


Figure 1

Title

Neurod4 **converts endogenous neural stem cells to neurons with synaptic formation after spinal cord injury**

Key Points

- ***Neurod4***, **which is predominantly expressed in injured** ***Xenopus laevis*** **tadpole, is a promising basic helix-loop-helix transcription factor to exert neuroregeneration**
- **A pseudotyped retroviral vector with the neurotropic lymphocytic choriomeningitis virus envelope can preferentially introduce** ***Neurod4*** **into activated neural stem cells after spinal cord injury**
- ***Neurod4*** **converts endogenous neural stem cells to neurons after spinal cord injury and the new excitatory and inhibitory synaptic formation leads to functional recovery**

Summary

The transcriptome analysis of injured *Xenopus laevis* tadpole suggested that *Neurod4L.S.*, a basic helix-loop-helix transcription factor, was the most promising transcription factor to exert neuroregeneration after spinal cord injury (SCI) in mammals. We generated a pseudotyped retroviral vector with the neurotropic lymphocytic choriomeningitis virus (LCMV) envelope to deliver murine *Neurod4* to mice undergoing SCI. SCI induced ependymal cells to neural stem cells in the central canal. The LCMV vector preferentially introduced *Neurod4* into activated neural stem cells, which converted to neurons with axonal regrowth and suppressed the scar-forming glial lineage. *Neurod4*-induced inhibitory neurons predominantly projected to the subsynaptic domains of motor neurons at the epicenter, and *Neurod4*-induced excitatory neurons predominantly projected to subsynaptic domains of motor neurons caudal to the injury site suggesting the formation of functional synapses. Thus, *Neurod4* is a potential therapeutic factor that can improve anatomical and functional recovery after SCI (Figure 1).

Research Background

Treating traumatic spinal cord injury (SCI) is difficult, and individuals often have permanent and severe disabilities. These disabilities partially result from the human body's limited ability to repair and regenerate neural tissue in the spinal cord. However, after SCI, the ependymal cells lining the central canal of the spinal cord can dedifferentiate between the acute and subacute phases. Indeed, the ependymal cells can revert into NSCs, and redifferentiate into glia and neurons. Therefore, we hypothesized that nerve regeneration may be achievable if endogenous injury-induced dedifferentiated NSCs can be reprogrammed into neurons.

The African clawed frog (*Xenopus* laevis) *(X. laevis)* has an unusually high capacity for neuroregeneration and is an indispensable animal model for regenerative research. During the larval stage, *X. laevis* undergoes a near-complete recovery after SCI. In this study, we screened *X. laevis* larvae for potential transgenes that, based on a comprehensive assessment of expression profiles, would exert a regenerative effect in mammals. Our transcriptomic analysis revealed that *Neurod4* is a potential neuroregenerative transcription factor. Moreover, we used a pseudotyped retroviral vector to introduce the *Neurod4* gene into the dedifferentiated NSCs, which were derived from ependymal cells after SCI, and aimed to switch their lineage towards neurons and thereby improve neural function.

Research Results

Comparison of bHLH gene expression post SCI in Xenopus laevis tadpoles and mice

Basic helix-loop-helix (bHLH) transcription factors are functionally critical proteins that regulate cell proliferation; cell differentiation; cell lineage determination; the formation of muscle, neurons, gut, and blood; sex determination; and other essential developmental and genetic processes. Therefore, we analyzed the whole transcriptome data of 107 bHLH transcriptional factor genes after SCI in *X. laevis*. We found that the upregulation of *Neurod4* mRNA was pronounced at 2 DPI of the regeneration stage. We also conducted a quantification of mRNA expression levels for candidate genes (*Neurod4, Neurod1, Atoh1, Neurog2, Ascl1*) (*n=3* mice per group), which are known as critical genes for nerve regeneration in the mouse SCI model. Among the candidate genes, *Neurod4* showed no increase in expression after SCI compared to the Sham, therefore it was assumed that the exogenous introduction of *Neurod4* most likely had a complementary effect to promote nerve regeneration. We further confirmed that the acute-phase expression of *Neurod4* in *X. laevis* tadpole at 1 and 2 DPIs at the regenerative stage was substantially superior to injured mice at 1 and 3 DPIs among the candidate genes **(Figure 1)**.

Preferential transduction of pseudotyped-retrovirus with envelope of Lymphocytic choriomeningitis virus tropic to neural stem cells

Retroviruses only infect dividing cells such as activated neural stem cells and progenitor cells, and do not infect nondividing cells such as neurons. Lymphocytic choriomeningitis virus

(LCMV) is a neurotropic RNA virus, which preferentially infects neural stem and progenitor cells. We generated a pseudotyped retroviral vector with envelope glycoproteins derived from LCMV. The vector was designed to transduce replicating NSCs and therein transfer a transgene. Three days before inducing an SCI, we injected the LCMV pseudovirus with an aqueous green fluorescence protein into the cisterna magna. Indeed, most of BrdU-positive dividing cells around the central canal (CC) displayed AcGFP1 at 3 and 5 DPI. Furthermore, Nestin-positive cells steadily increase after SCI **(Figure 3)**.

Differentiation of activated neural stem cells into NeuN- and DCX-positive neurons by introducing Neurod4 into mice

We investigated whether introducing murine *Neurod4* into activated NSCs after SCI would result in neuronal regeneration. At 5 DPI, there was an obvious increase in the number of DCX-positive cells among *Neurod4*-introduced cells (AcGFP1-positive). Moreover, from 7 DPI to 42 DPI, we observed a progressive increase in the number of NeuN-positive cells among *Neurod4*-introduced (tauAcGFP1-positive) cells **(Figure 4)**. Thus, SCI may induce ependymal cells to dedifferentiate into activated NSCs and introducing *Neurod4* into these cells may facilitate their differentiation into neurons.

Promotion of the differentiation of excitatory and inhibitory neurons by Neurod4 expression in activated neural stem cells

We visualized *Slc17a6* (vGlut2, an excitatory neuronal marker) and *Slc6a5* (glycine transporter 2 [GlyT2], an inhibitory neuronal marker) using in situ hybridization (ISH) and ChAT using immunohistochemistry. The expression of *Slc17a6* and *Slc6a5* were elevated in the *Neurod4*-introduced group. Moreover, we observed ChAT-positive motor neurons derived from *Neurod4*-introduced cells were dispersed around the central canal **(Figure 5)**. Thus, *Neurod4*-expressing NSCs may differentiate into excitatory neurons, inhibitory neurons, or motor neurons, which may form neuronal networks and contribute to functional improvement after SCI.

Neurod4-induced excitatory and inhibitory neurons form functional synapses

We constructed a LCMV pseudoretroviral vector with *Neurod4* and *Syp-AcGFP1* transgene. We observed that *Neurod4*-induced excitatory (vGlut2-positive) neurons projected predominantly to the PSD-95-positive subsynaptic domains of the motor neurons at levels L2–L5 of the spinal cord from excitatory neurons at the epicenter. Conversely, *Neurod4*-induced inhibitory (GlyT2-positive) neurons predominantly projected to the GlyR-positive subsynaptic domains of the motor neurons at the epicenter from inhibitory neurons at the epicenter **(Figure 6)**.

Suppression of GFAP-positive astrocytes and improvement of spinal cord regeneration by Neurod4 expression after spinal cord injury

Since glial scars impair the regrowth of axons and inhibit neuronal regeneration, we investigated the formation of astrogliosis. Control astrocyte underwent a typical change of

hypertrophy, process extension at 7 DPI, while *Neurod4*-introduced astrocyte didn't. Furthermore, the number of astrocytes decreased in *Neurod4*-introduced mice compared to control mice at 42 DPI. Thus, *Neurod4* may potentially reduce astrocyte differentiation by diverting ependymal cells towards a neuronal lineage instead of an astrocytic lineage.

To examine an axonal regrowth after SCI, we constructed axon tracer of red fluorescent protein and infected into the M1 cortex pyramidal neurons and labeled axons in the corticospinal tract. In the *Neurod4* group, the tissue clearing illustrated more axons visible at the epicenter while in the control, the axons stopped at rostral to the lesion, and observed a red signal in the corticospinal tract beyond the injury site **(Figure 7)**. Therefore, *Neurod4* may promote axonal regrowth by suppressing glial scar formation.

Synaptic formation and functional recovery by Neurod4 after SCI

We visualized the corticospinal tract connection between the M1 cortex and *Neurod4*-induced neurons at epicenter or motor neurons at levels L2-L5 of the spinal cord. We injected axon tracer of red fluorescent protein into the M1 cortex. At the epicenter, excitatory synapses were found around the AcGFP1-positive cells only in the *Neurod4*-introduced group. Moreover, the *Neurod4* group had a substantial number of presynaptic marker of red fluorescent protein with motor neurons and postsynaptic marker in the motor neurons at levels L2-L5 of the spinal cord. The number of newly-formed synapses in the ventral horn were markedly increased in the *Neurod4* group, compared to the control group. Thus, synapse formations at the motor neurons were achieved beyond the injury site in the *Neurod4* group.

To further demonstrate functional rescue, we assessed hindlimb locomotor function by using the Basso Mouse Scale (BMS). For 6 weeks post-SCI, mice were evaluated for locomotor recovery. The *Neurod4*-introdeced mice showed a significant improvement in locomotor function, compared to the control mice **(Figure 8)**.

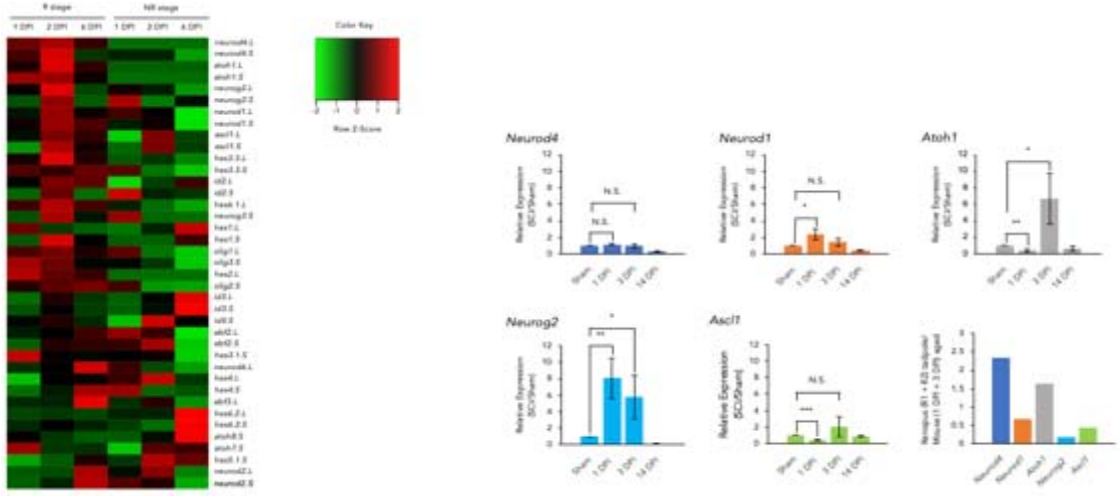


Figure 2 Comparison of the candidate gene expressions in the acute phase between *Xenopus laevis* tadpoles and aged mice after spinal cord injury

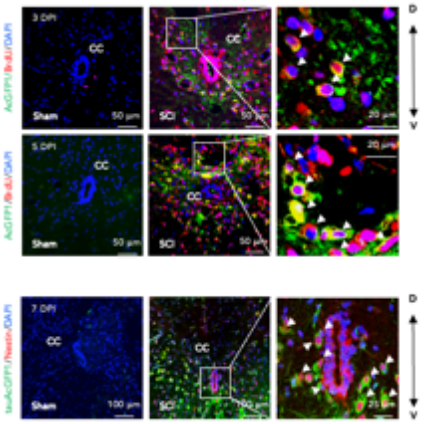


Figure 2　Preferential transduction of pseudotyped retrovirus with envelope of Lymphocytic choriomeningitis virus tropic to neural stem cells

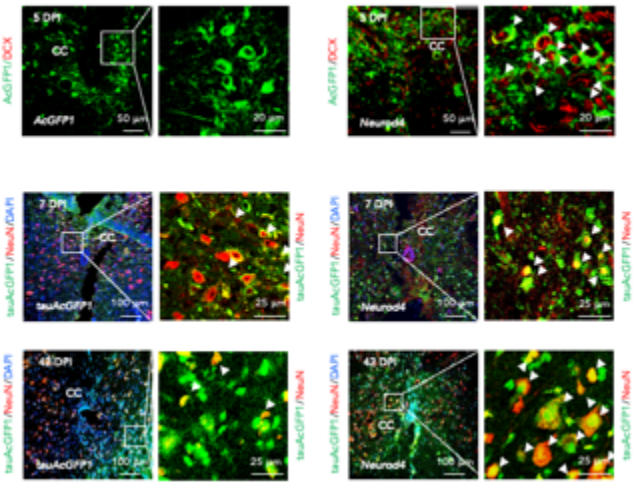


Figure 3　Differentiation of activated neural stem cells into NeuN- and DCX-positive neurons by introducing *Neurod4* into mice

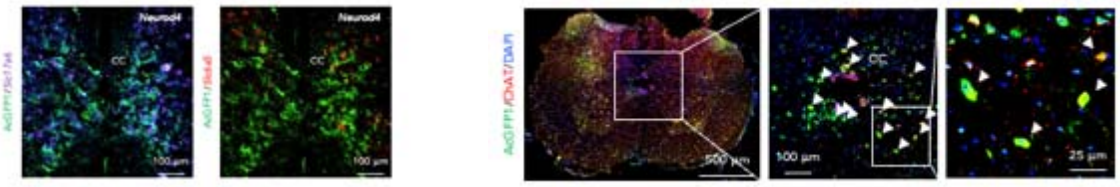


Figure 4　Promotion of the differentiation of excitatory and inhibitory neurons by *Neurod4* expression in activated neural stem cells

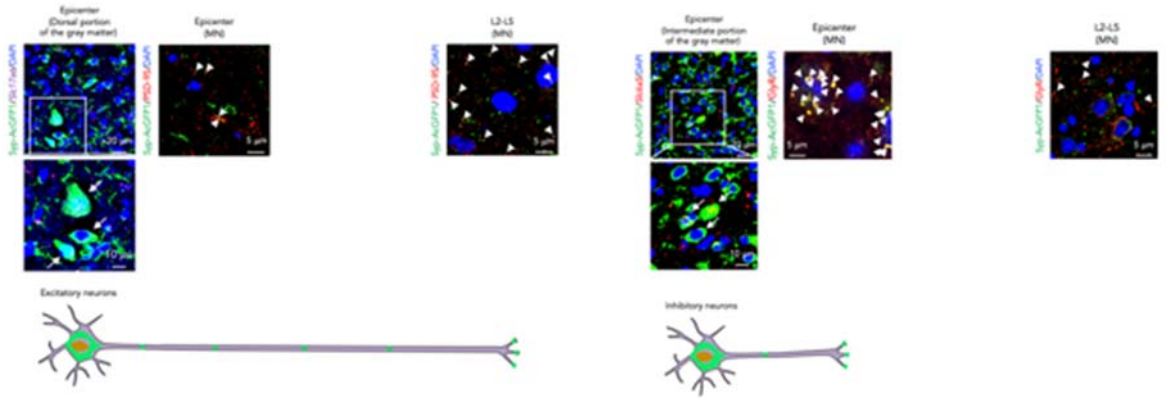


Figure 5　Projections into motor neurons of *Neurod4*-introduced excitatory and inhibitory neurons

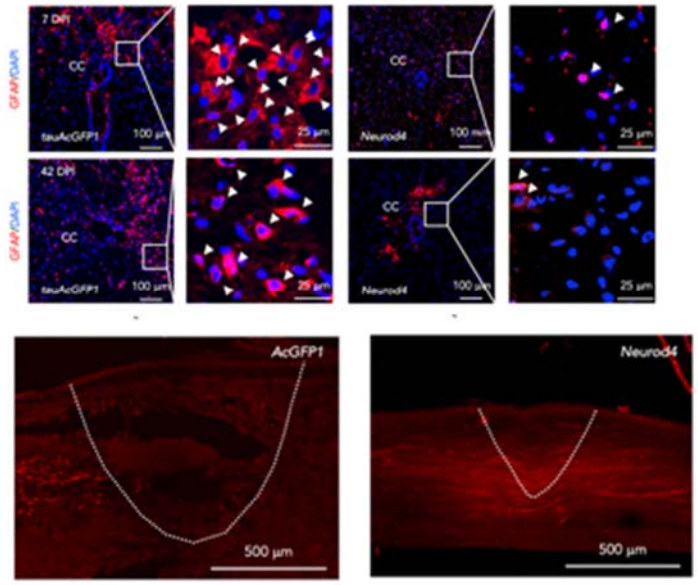
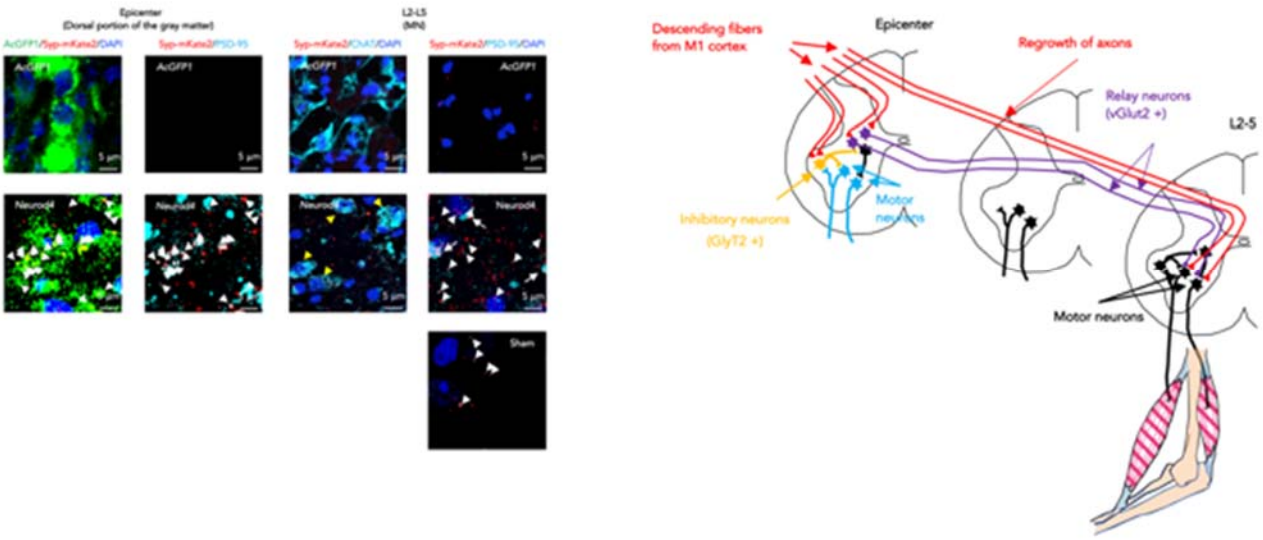


Figure 6　Glial scar suppression and axonal tracing from projection neurons of M1 cortex beyond injured region of spinal cord after recovery



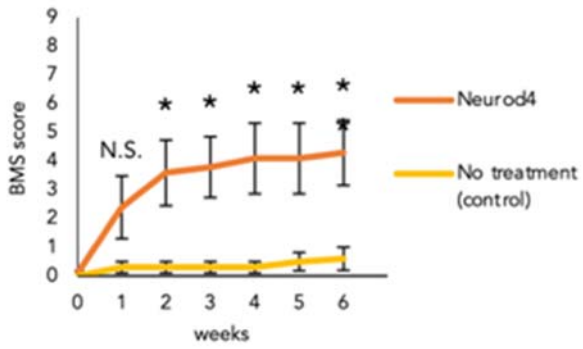


Figure 7　Synaptic formation and functional recovery by *Neurod4* after SCI

Research Summary and Future Perspective

In the present study, we demonstrated that introducing *Neurod4* as a transgene into activated neural stem cells after SCI facilitates differentiation of endogenous NSCs into 3 or more types of neurons and the production of relay neurons, which project to motor neurons of the hindlimbs. As a secondary effect, glial scar formation was suppressed after the subacute phase of SCI. This effect allowed an environment that was conducive for axons to elongate beyond the injury site and form synapses to motor neurons and thereby improve motor function in the hindlimbs. Although further studies on some of these changes are necessary, *Neurod4* has proven to be a potential therapeutic factor that could potentially regulate nerve regeneration and the induction of a neuroregenerative gene into endogenous ependymal-derived NSCs is a potential treatment method for SCI.

Publication

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