EFFECT OF DELAYED GENE THERAPY TREATMENT IN SPINAL CORD INJURY

BY

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ABSTRACT OF THE THESIS

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Gene therapies are being developed which seek to repair the spinal cord and give patients back their mobility by lentivirus-mediated expression of neurogenic transcription factors. Through targeted overexpression of key transcription factors, endogenous cells can be motivated to remodel the site of injury. Previously, our lab has identified a gene therapy in a mouse model of hemisection spinal cord injury (SCI) that, when injected immediately after injury, produces locomotor functional recovery by decreasing cell death, promoting proliferation, and activating neural stem cells. However, in clinical settings, therapeutic intervention would happen hours or even days after the injury. Thus, we aim to determine the effect of a delayed treatment for effective gene therapies of spinal cord injuries. Our hypothesis is that injecting the therapeutic agent some time after injury will still be effective to promote functional recovery. In this study, we used a lateral hemisection SCI mouse model and injected the lentiviral gene therapy one day after injury. The spinal cord tissue was harvested, stained, and analyzed at three different timepoints after the injection to determine the effects of the therapy on the site of injury.

Our results showed successful overexpression of the target transcription factor and consequently, decreased cell death in the subacute phase of injury, increased neural stem cell activation, and an increase in cholinergic neurons around the injury site. Our results show that the therapy successfully remodeled the site of injury and could potentially lead to functional recovery in a long-term study. Improving the SCI mouse model for testing gene therapies will better predict their efficacy in clinical settings.

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Chapter 1

Introduction

Injury to the spinal cord can devastate the motor function of an individual. Depending on the site of injury, the patient can experience paralysis below the waist, chest, or even complete paralysis below the neck. The most common causes of SCI are motor vehicle accidents, falls, acts of violence, and sports, all of which occur outside of the hospital without access to a potential therapeutic injection in the moments following injury. [1]

After injury, the focus is stabilizing the patient's vital signs and spinal column to prevent further injury. Once the patient is transported to the hospital, health professionals classify the extent of the injury using imaging and neurological examinations and decide how to proceed. Often, surgical intervention is necessary to remove broken bone fragments and restore alignment to reduce compression on the spinal cord. Current treatments focus on increasing the patient's quality of life through rehabilitation, medication to control pain, muscle spasticity, bladder and bowel, and assistive devices such as wheelchairs or electronic aids. However, there is no cure for spinal cord injuries (SCI). Therapies are being developed which seek to repair the spinal cord and give patients back their mobility. These therapies include cell transplants, stem cell therapies, and *in vivo* reprogramming. Ideally, therapeutic injections or cell transplantations should occur during the surgical intervention, since the spinal cord would be accessible. Therefore, when developing treatments, it is important to determine their effectiveness when injected some time after the initial injury occurs.

Preclinical studies rarely test the therapeutic window of drugs, but it is commonly thought that most drugs lose efficacy with increasing intervals between injury and first dose. Studies have been done to determine the therapeutic window for compounds aimed to treat traumatic brain injuries and have found a rapid loss of efficacy when dosed one hour after injury, even though positive results were seen when dosed 15 minutes after injury. [2] Another traumatic brain injury study, however, found that a small-molecule inhibitor of the integrated stress response pathway was able to reverse cognitive defects, even when administered 2 to 4 weeks after injury. [3] Since the therapeutic window for a drug is largely dependent on its mechanism of action, loss of efficacy varies.

The site of injury is a dynamic environment, as the body attempts to mitigate the damage. When there is trauma to the spinal cord, neuronal stem cells (NSCs) generate astrocytes and oligodendrocytes to begin damage repair. A glial scar forms as a result. In the acute injury phase, it limits the expansion of the lesion. In the chronic phase, however, it can inhibit axon growth and reestablishment of local circuitry across the site of injury. [4] Since new neurons can help to reduce glial scarring, treatments for SCI aim to increase functional recovery by increasing neurogenesis and decreasing astrogliosis.

In vivo reprogramming is a method that uses overexpression of transcription factors to convert differentiated somatic cells directly into another cell type *in vivo*. [5] Studies have successfully used *in vivo* reprogramming to convert astrocytes to neuroblasts in the brain, a key achievement in the search for treatments of SCI since glial scars are mainly composed of astrocytes. [6] Previously, our lab has tested different transcription factors to determine their therapeutic effect for SCI and found that overexpression of Genomic Screened Homeo Box 1 (Gsx1 or Gsh1) produces functional

recovery in SCI mouse models by promoting proliferation, activating neural precursor cells, and changing the balance of inhibitory and excitatory interneurons. Gsx1 is highly expressed in the embryonic central nervous system and involved in regulating differentiation of NSPCs into excitatory and inhibitory interneurons.[7, 8] Expression of Gsx1 is low or undetected in the adult spinal cord[7] and results from our previous study showed that reactivating this expression can induce neurogenesis after injury. The SCI mouse model to study this potential therapy involved injection of the lentiviral gene therapy immediately after producing a hemisection of the spinal cord. Although the results were promising, translating this model clinically would be nearly impossible due to the inevitable treatment delay in SCI.

In the field of SCI, little is known about therapeutic windows for therapies that are currently being developed. Cell transplantation studies normally perform the transplantation 7-9 days after injury, after ensuring that the animals have comparable injuries and locomotor scores.[9], [10] Although they have yielded positive results, these studies do not test the effects of these same stem cell transplants when conducted at different time points, such as directly after initial injury. *In vivo* reprogramming therapies affect the gene expression of cells at the area of injury when injected directly after injury. However, it is unclear whether the efficacy of gene therapy drugs is affected by increasing the time between injury and intervention. Therefore, the current model for testing gene therapy drugs needs to be modified to determine if they can still be effective when injected some time after injury occurs. We hypothesized that therapeutic intervention one day after injury promotes cell proliferation, neurogenesis, and functional recovery.

In this study, we employed a hemisection SCI in a mouse model, applied a gene therapy treatment one day after injury, and quantified its effects to determine if it is still efficacious. Improvement of the mouse animal model for testing gene therapies will allow scientists to better predict their effectiveness in clinical settings.

Chapter 2

Methods

2.1 Gene Therapy Production

Plasmid Amplification

Creating the lentiviral vector constructs required five different plasmids: a packaging vector, two envelope vectors, and two target vectors, one scrambled sequence that acted as the control and the other containing the transcription factor of interest (Gsx1).

Bacteria containing 5 different plasmids were amplified from glycerol stocks and plasmids were purified using a Plasmid DNA Midiprep Kit (Qiagen). Briefly, the bacteria were grown in suspension of LB and the antibiotic that they contain resistance to. After two days, the suspension was centrifuged, the supernatant was decanted, and the precipitated was mixed with a resuspension buffer. The bacteria were lysed for 5 minutes, until a third buffer was added to stop it. After centrifuging once more, the DNA was in the supernatant and needed to be purified. Using the columns and the buffers in the Midiprep Kit, the DNA was eluted and collected separately. Isopropanol was added to the DNA solution and then centrifuged to form a DNA pellet. After pellet air dried, it was resuspended in ppH2O and the concentration was measured using a Life Science UV/Vis Spectrophotometer (Beckman Coulter, DU 730).

Lentiviral Transfection

4.5 million HEK 293T cells were plated in two T150 flasks and allowed to grow overnight. H2O, CaCl2, the packaging vector, the two envelope vectors, and one of the

target vectors were added to Tube A. Tube B contained 2X HEPES buffer. Tube A was added to Tube B slowly, mixing well. The solution was then incubated at room temperature for 30 minutes. HEK 293T Growth Media was added to the mixture, mixed with pipette, and added to the flasks containing cells. Viral media was collected on day 2 and day 4 after transfection, and PEG 6000, NaCl, and PBS were added and mixed. The solution was stored overnight at 4°C. The following day, the tubes were centrifuged, and the pellet was resuspended in 176µL of Tris-HCL, pH 7.4. 10-20 µL aliquots were created and stored at -80°C.

Viral Titer Assay

To determine the titer of the viral therapy, a fluorescence tittering assay was used (Addgene). 75,000 HEK 293T cells were seeded into a 6-well plate and left to grow overnight. 10-fold serial dilutions of the lentiviral therapy in growth media with polybrene were added to each well. Cells were counted in one well to determine number of cells transduced. On day 4 after transfection, the cells were imaged, and the fluorescence was quantified. The viral titer was calculated using:

TU/mL = (Number of cells transduced x Percent fluorescent) / (Virus volume in mL)

2.2 Spinal Cord Injury Procedure

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee at Rutgers University. All animal work was conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Young adult mice (8-12 weeks) were randomly assigned to experimental and control groups (3-5 mice/group). The day before surgery, the dorsal fur was shaved off. The following day, the mice were anesthetized in an oxygen-enriched 5% isoflurane chamber for 3-4 minutes and subsequently kept under with 3% isoflurane and on a heating pad for the duration of the surgery to prevent hypothermia. The area was cleaned with betadine followed by 70% ethanol three times. An incision was made in the skin, facia, and muscle covering the apex of the curve on the mouse's back. A laminectomy of the T10 vertebral bone was performed to expose the spinal cord in the region of interest. A rongeur was used to remove sharp ends of bones when needed. Two drops of 0.125% bupivacaine were applied over the spinal cord. A hemisection was performed on the left side of the spinal cord by placing a scalpel perpendicular to the middle of the cord and cutting it in a circular motion. A piece of adipose tissue from the neck area was cut and placed in the exposed spinal cord. The muscles were sutured together, the skin was stapled, and subcutaneous injections of 0.5 mg/ml meloxicam and 10mg/ml cefazolin were administered. The mice recovered from anesthesia on a heating pad until awake.

2.3 Therapeutic Intervention

The next day the mice were placed under anesthesia in the same conditions as previously stated to inject the therapy. The staples and sutures were removed, and two drops of 0.125% bupivacaine were applied over the exposed cord. Using a 26s-gauge 10μL syringe (Hamilton, 84877), 1-2 μL of the lentiviral therapy, containing the target vector or the scrambled sequence control, were injected about 1-3 mm rostral and caudal to the injured site. The muscles were sutured, the skin was stapled, subcutaneous

injections of meloxicam and cefazolin were administered, and the mice recovered from anesthesia on a heating pad. Meloxicam subcutaneous injections were administered to the animals for three days post-operation.

2.4 Tissue Processing and Immunohistochemistry

At designated time points after injection, animals were euthanized and perfused with 1X phosphate buffered saline (PBS) followed by 4% (w/v) paraformaldehyde (PFA). The spinal cord section encompassing the injury and injection sites was harvested and fixed in 4% PFA for 1 day. The spinal cord section was washed three times with 1X PBS and then put in 30% (w/v) sucrose in PBS solution until the tissue sank to the bottom of the tube, about 1-2 days. The tissues were then embedded in cryopreservation solution (Tissue Tek OCT Compound) and placed in -80°C.

12μm thick sagittal sections of frozen spinal cord tissue were cut using a cryotome (Thermo Shandon Cryostat Cryotome) and air dried at room temperature. Tissue sections were stored in -80°C until staining. 30 minutes prior to beginning staining procedure, tissue sections were moved to room temperature to thaw. Methanol was used for antigen retrieval for 10 minutes at room temperature. The sections were there blocked and permeabilized for 1 hour at room temperature in a blocking buffer (10% donkey serum, 0.1% Triton-100, 0.1% Tween 20), and incubated with primary antibody overnight at 4°C. The following day, sections were washed with 1X PBS and then incubated with the corresponding secondary antibodies for one hour at room temperature. After washing with PBS, they were also stained with DAPI and then dried. Cytoseal20 mounting media was added followed by coverslips. The primary antibodies used were

Gsx1 (Sigma), Ki67 (BD Pharmingen 550609), Nestin (Millipore MAB353), Caspase3 (Cell Signaling 9661), CD68 (Millipore MAB1435), DCX (Santa Cruz SC8066), GABA (Sigma A2052), vGlut2 (Millipore AB2251-I), and ChAT (Sigma SAB2500236). **Figure 1** summarizes the design of experiment, from the initial SCI to the immunohistochemical analysis.

2.5 Imaging, Quantification, and Statistical Analysis

Fluorescent imaging was done with a ZEISS Axio Scope.A1 microscope. Automatic cell counting using thresholding was used to count the total number of cells. Manual cell counting was also performed to quantify co-expression of DAPI, the reporter gene in the lentiviral therapy, and gene markers. Data were analyzed using GraphPad Prism version 5.0 software for Microsoft Windows and are presented as mean ± standard error of the mean (SEM). Student's t-test were performed for direct comparisons. For groups of three or more, one-way ANOVA followed by Tukey post-hoc test was used. P-values less than 0.05 were considered statistically significant.

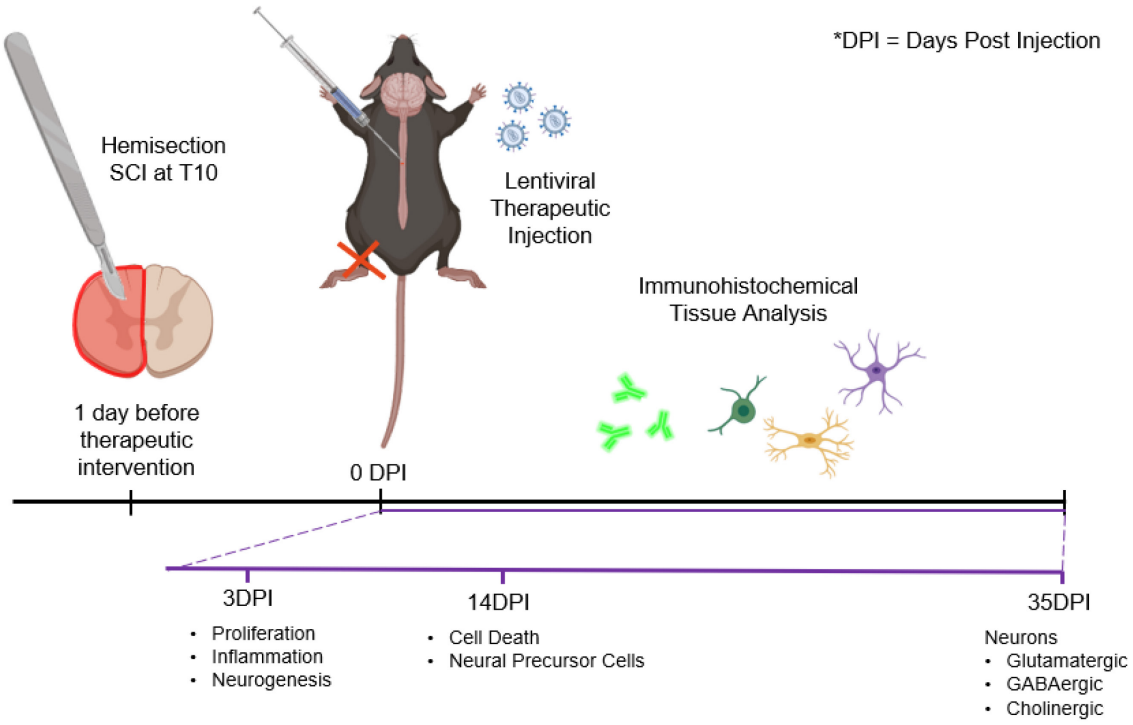


Figure 1: Schematic drawing of experimental design. 8-12 week old mice receive hemisection SCI at T10. One day later, mice are injected with lentiviral gene therapy rostral and caudal to the injury. At 3DPI, 14DPI, and 35DPI, the mice were sacrificed, and immunohistochemistry was performed to determine the cellular/molecular effects of the delayed treatment at the site of injury.

Chapter 3

Results

3.1 Lentivirus transduced into cells at the site of injection and successfully induced overexpression of target gene

One day after initial hemisection SCI, we injected animals with a lentiviral therapy meant to produce overexpression of the transcription factor, Gsx1 in affected cells. Both the control group and experimental group exhibited cells with red fluorescence near the injury site and the two adjacent injection sites due to the RFP gene incorporated into the plasmids of the lentivirus. This is an indication that the control and experimental therapies were injected successfully. **Figure 2A** shows the RFP signal localized around the injury site at multiple time points. The quantification of the percentage of cells with RFP signal out of all the cells in the experimental group's images at the three endpoints (3, 14, and 35DPI) is shown in **Figure 2B**. The percentage shows a marked reduction over time, the greatest being at 3DPI (34.90%±1.31; n=3), followed by 14DPI (21.11%±2.24; n=3) and 35DPI (15.25 %±1.21; n=3).

We immunostained for Gsx1 to verify that the therapy was able to induce overexpression of the transcription factor compared to the control group. **Figure 2C** shows a significant increase in Gsx1 signal in the experimental group (Gsx1-Lenti) compared to the control group (Ctrl-Lenti). We quantified the percent of Gsx1+/RFP+ cells out of all the RFP+ cells. **Figure 2D** shows a significant increase (66.46%±6.41; n=3) over the Control-Lenti group (8.21%±4.67; n=3) in these co-labeled cells, indicating that the lentiviral therapy was successful in inducing overexpression of Gsx1.

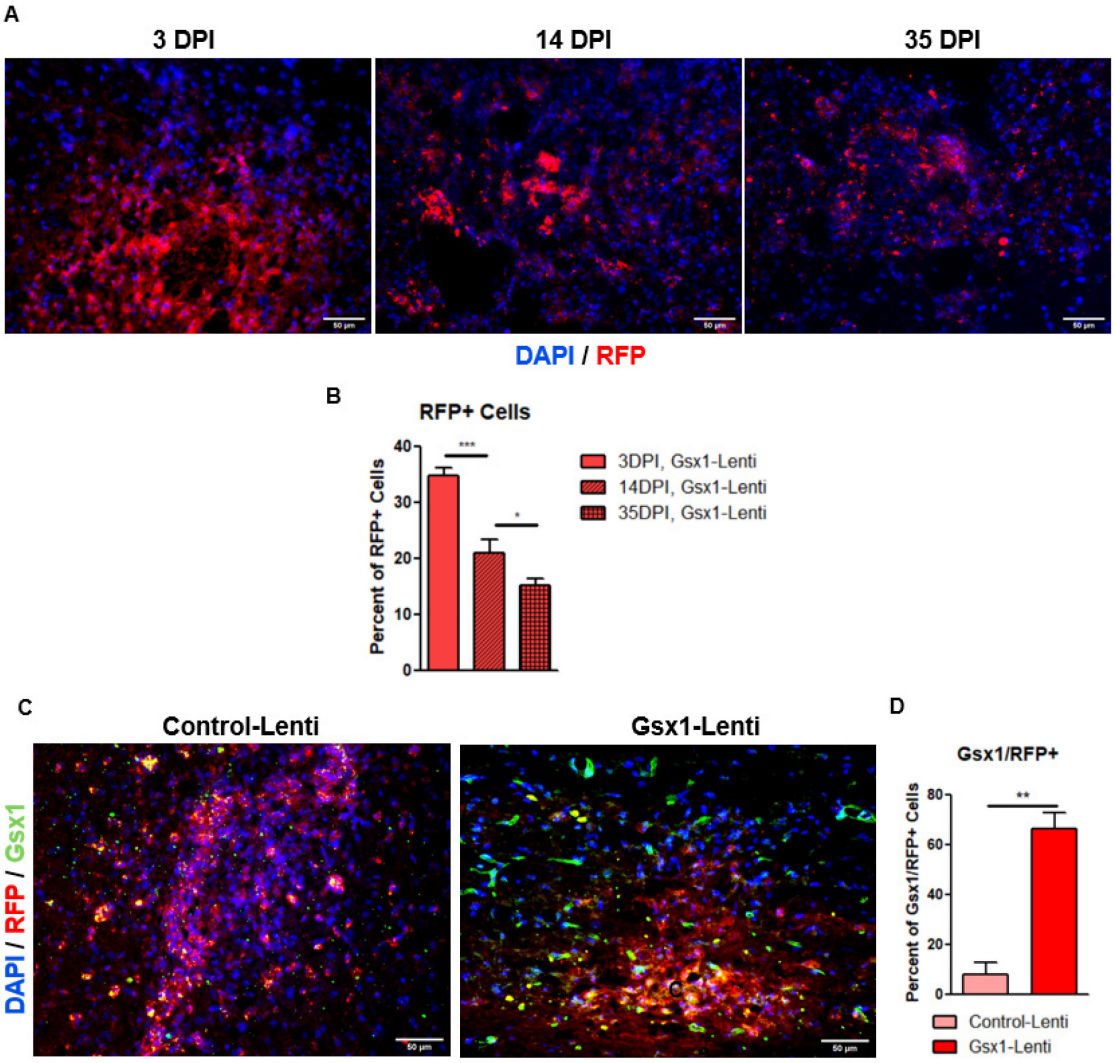


Figure 2: Lentivirus mediated Gsx1 expression in cells at the site of injury. (A) Representative immunofluorescence images of spinal cord sagittal section and (B) quantification of cells with viral reporter RFP expression at 3, 14 and 35 DPI in Gsx1-Lenti group. Mean + SEM; n = 3. One-way ANOVA. *** = $P < 0.001$, * = $P < 0.5$. (C) Representative images and (D) quantification of virally transduced cells with Gsx1 expression at 3 DPI. Mean + SEM; n = 3. Students' t-test. ** = $P < 0.01$.

3.2 Lentiviral transduction did not affect inflammation or proliferation at the site of injury

After verifying that the lentivirus successfully caused overexpression of the intended transcription factor, we began to quantify its effects through staining for specific markers. Cd68 is the macrophage marker, used to indicate inflammation, a common occurrence shortly after injury. The neuroinflammatory response includes activation of microglia and eventual glial scar formation. [4] As previously mentioned, glial scars, a product of the body's response to injury, can be detrimental after SCIs because they block the path of the descending outputs and sensory inputs between the brain and the rest of the body. **Figure 3A** illustrates marker gene expression for inflammation at 3DPI was observed in both the Control-Lenti and Gsx1-Lenti groups, indicating both groups sustained an injury. The difference in marker expression between the Gsx1-Lenti group (31.51%±2.87; n=3) and the Control-Lenti group (35.76%±2.75; n=3) are statistically insignificant. (**Figure 3B**) These results indicate that delayed treatment was unable to reduce inflammation at the site of injury.

Ki67 was used determine the effect of Gsx1 overexpression on cell proliferation when the therapy was injected one day after injury. **Figure 3C** shows representative images of the injection site immunostained for Ki67. Proliferation in the Gsx1-Lenti group was quantified (33.08%±0.70; n=3) and found to be insignificantly different to the Control-Lenti group (34.44%±4.56; n=3). (**Figure 3D**)

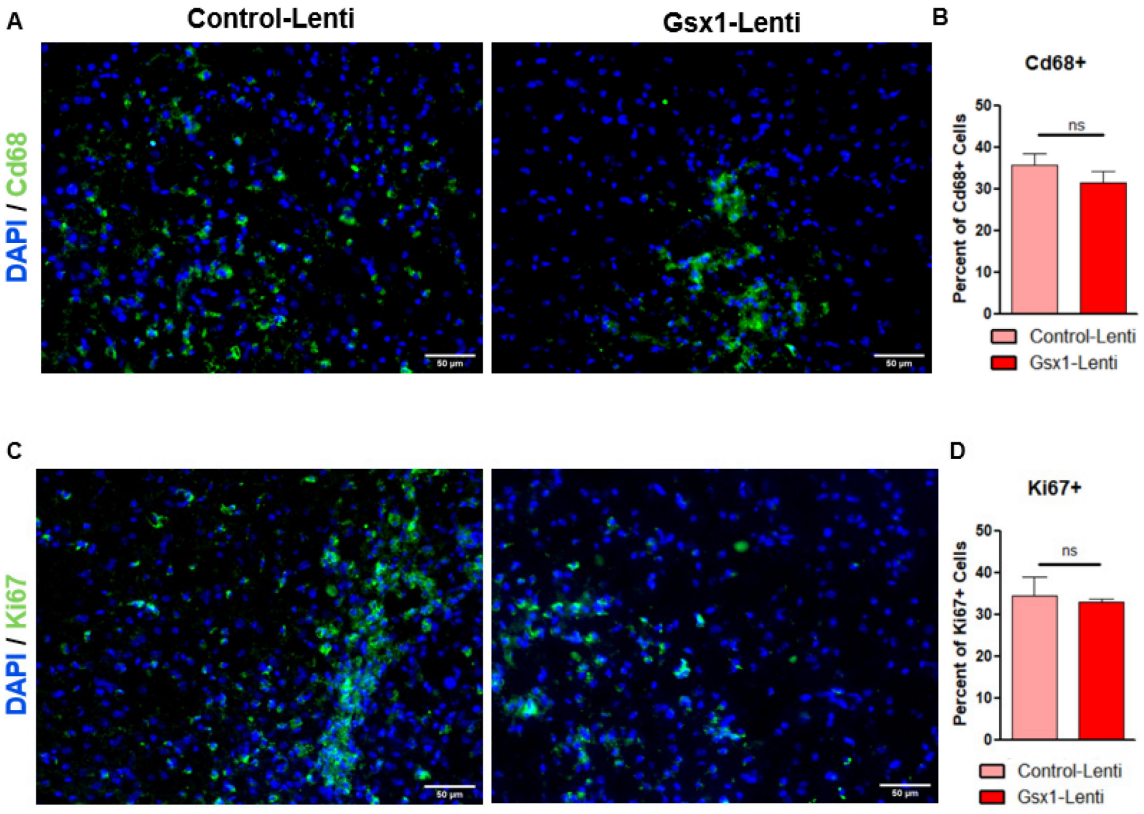


Figure 3: Lentiviral transduction did not affect inflammation or cell proliferation at the site of injury. (A) Representative immunofluorescence images of spinal cord sagittal section and (B) quantification of cells with inflammation marker Cd68 expression at 3 DPI. (C) Representative images and (D) quantification of cells with cell proliferation marker Ki67 expression at 3 DPI. Mean + SEM; n = 3. Students' t-test. ns = $P > 0.05$.

3.3 Delayed injection of lentivirus reduced cell death after SCI

After SCI, apoptosis has been observed for up to three weeks.[11] Caspase activation, used to measure cell death, occurs in both neurons and oligodendrocytes at the site of injury and spreads to areas that were not affected by the initial injury. Clinically, it is uncommon to have a SCI that severs every axon, like a transection, yet patients still experience complete paralysis. This widespread cell death may contribute to the permanent deficits seen by patients who do not have complete injuries.[12]

Figure 4A shows a significant reduction in Caspase3 activation in the experimental group compared to the control group at 14DPI. Quantification of the percentage of Caspase3+ cells shows that lentiviral transduction of the transcription factor was able to reduce cell death around the site of injury in the experimental group (12.57%±0.95; n=3) when compared to the control group (15.79%±0.60; n=3). (**Figure 4B**)

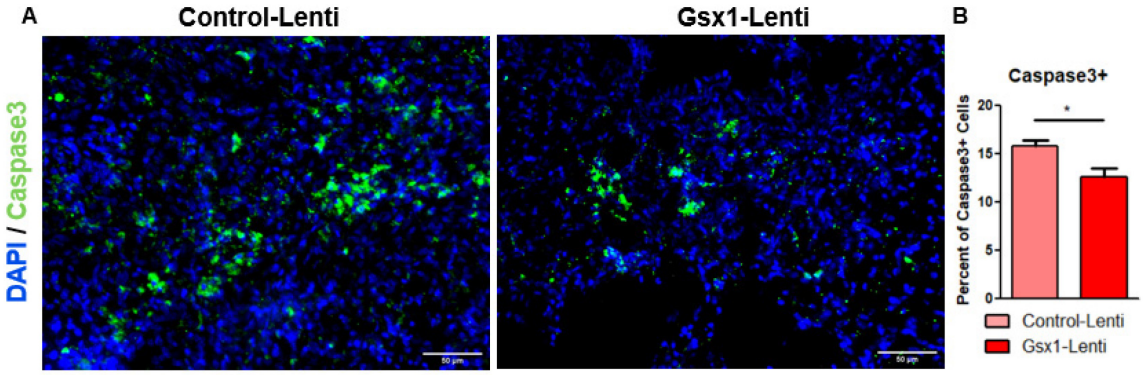


Figure 4: Delayed Gsx1 treatment reduced cell death at 14DPI. (A) Representative immunofluorescence images of spinal cord sagittal section and (B) quantification of cells with Caspase3+ expression at 14 DPI. Mean + SEM; n = 3. Students' t-test. * = $P < 0.05$.

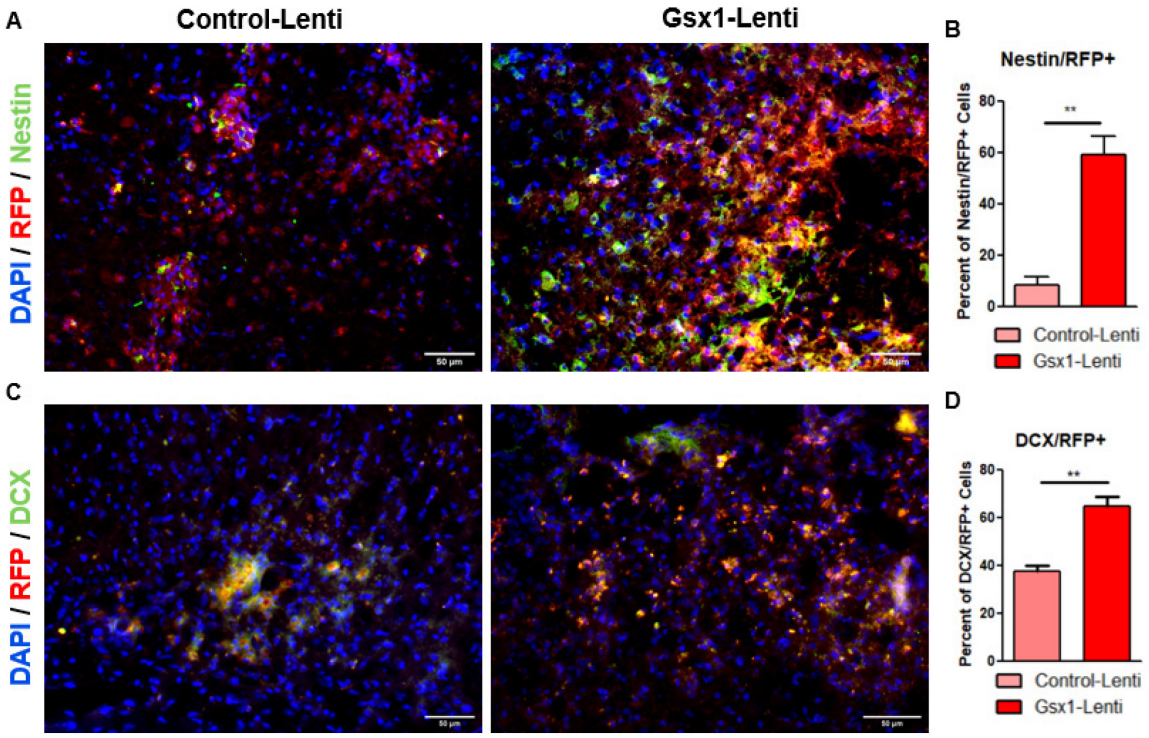
3.4 Delayed injection of lentivirus induces neurogenesis and changes the neuronal population at the site of injury

Neurogenesis is important in reestablishing severed connections after a SCI across the site of injury. We examined Nestin expression, a NSPC marker, to determine if the therapy injected one day after injury could activate NSPCs that are normally not activated in the spinal cord. **Figure 5A** shows a marked increase in Nestin+ cells between the two groups at 3DPI, meaning that more NSPCs were activated after the delayed experimental injection than the control injection. The quantification of the percentage of Nestin+ cells that were colabeled with RFP expression in **Figure 5B** confirms this difference in means (Control-Lenti: 8.438%±3.30; n=3; Gsx1-Lenti: 59.28%±7.13; n=3).

At 14DPI, we examined doublecortin (DCX) expression. DCX is a microtubule-associated protein found in pre-migratory neuroblasts.[13] These new cells migrate into the injured area to replace neurons and are therefore crucial in restoring the lost circuitry in SCI.[14] In **Figure 5C**, there is a marked increase in DCX+ cells colabeled with RFP in the experimental group. This is quantified in **Figure 5D**, where there is a statistically significant increase in the population of immature neurons in the experimental group (64.76%±4.086; n=3) compared to the control group (37.84%±2.253; n=3).

Early on, NSPCs are activated. Two weeks later, we see an increase in immature neurons. At 35DPI, we wanted to examine the differentiation of these activated neurons by studying the population of mature neurons present at the site of injury. We stained for glutamatergic interneurons, GABAergic interneurons, and cholinergic neuronal marker. (**Figure 5E**) After quantifying the percentage of vGlut2+, GABA+, and ChAT+ cells in both groups, we found that delayed treatment of the lentiviral gene therapy increases the

percentage of cholinergic neurons near the site of injury (72.31%±2.426; n=3 compared to 56.84%±3.789; n=3 in the control group) but does not change the percentage of glutamatergic (21.96%±0.858; n=3 compared to 18.81%±3.920; n=3 in the control group) and GABAergic neurons (7.140%±0.729; n=3 compared to 6.241%±0.301; n=3 in the control group). (**Figure 5F**) These results indicate that delayed injection of the lentiviral therapy activates NSPCs early on which differentiate into cholinergic neurons at 35DPI.



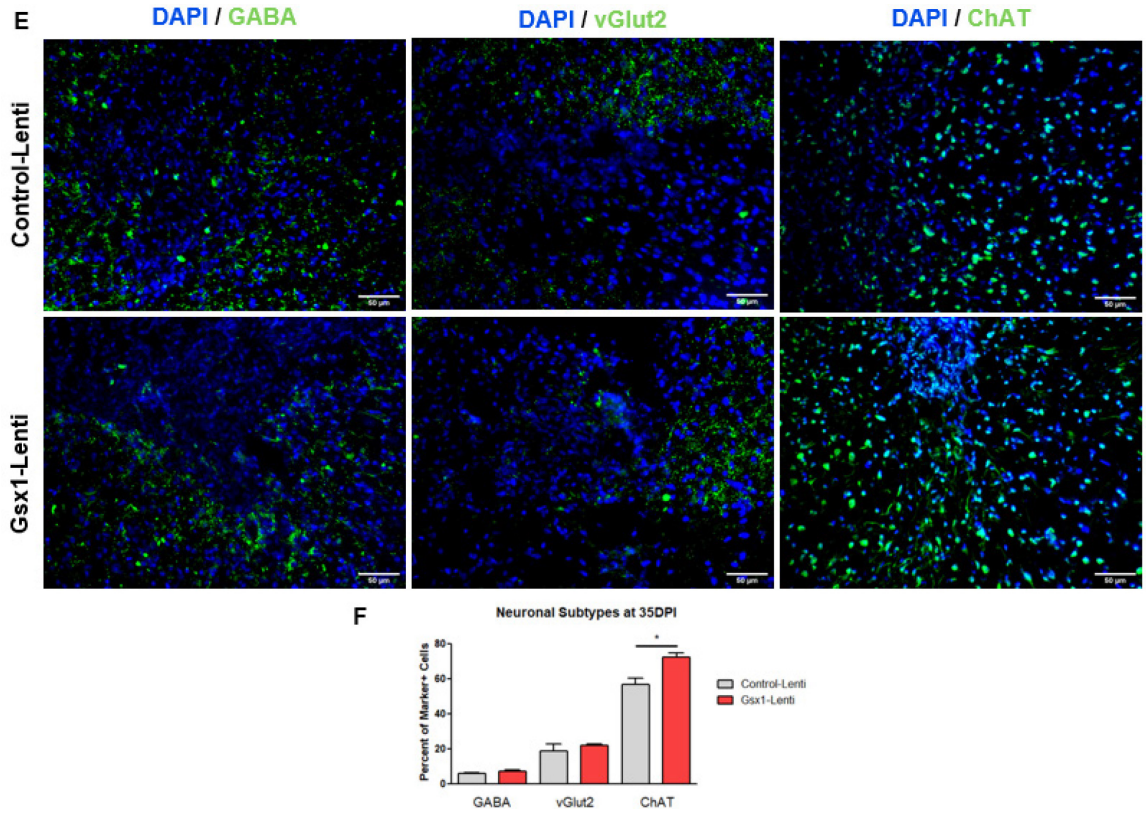


Figure 5: Delayed injection of gene therapy increases NSPC activation and generation of cholinergic neurons at the site of injury. (A) representative immunofluorescence images of spinal cord sagittal section and (B) quantification of cells with Nestin+/RFP+ colabeled cells at 3 DPI. (C) representative images and (D) quantification of cells with DCX+/RFP+ colabeled cells at 14 DPI. (E) representative images and (F) quantification of vGlut2+, GAGA+ and ChAT+ cells at 35 DPI. Mean + SEM; n = 3. Students' t-test. * = $P < 0.05$, ** = $P < 0.01$.

Chapter 4

Discussion

Timing of therapeutic intervention is as important as the therapy itself. Although the lentiviral gene therapy showed promising results during initial studies, it was important to adjust the animal model to account for the treatment delay that occurs in clinical spinal cord injury. Clinically, there is no standard for timing of surgical intervention for decompression.[15] There are many variables that must be evaluated on a case by case basis. First responders must transport the patient to a hospital and monitor the patient's vital signs to ensure that the patient is healthy enough to survive the surgical decompression. The surgeon typically orders X-ray or MRI imaging prior to surgery to determine what the damage is and once the patient is stable, surgical decompression can take place.

Meta-analyses of clinical data on decompression found neurological benefit when surgical intervention was performed within 24 hours of injury.[16, 17] Additionally, the Surgical Timing In Acute Spinal Cord Injury Study (STASCIS) enrolled over 300 patients with acute cervical SCI and found that patients that underwent surgery less than 24 hours after injury were 2.8 times more likely to have at least a 2 grade AIS improvement six months after injury.[18] With these findings in mind, the gene therapy injection that we evaluated was injected into the spinal cord in a mouse model 24 hours after the initial SCI. In that time, the site of injury continues to change from the immediate injury phase to the acute injury phase, as summarized by **Figure 6**.

We validated the delayed therapeutic injection by analyzing the RFP signal and immunohistostaining for the transcription factor that the lentivirus carried. The lentivirus

was effectively delivered to the cells at the site of injection and injury. The RFP+ cell population appears to reduce over time, which could be caused by cell death or differentiation over time leading to loss of RFP gene expression. Furthermore, we analyzed the expression of the transcription factor in the lentivirus and found successful overexpression in the experimental group. Overall, we confirmed that the treatment could still infect cells at the site of injury, even when injected a day after injury.

With delayed treatment, we found no changes in inflammation or proliferation in the days following treatment, a reduction in cell death two weeks after treatment, and an activation of NSPCs which resulted in an increase in cholinergic neurons.

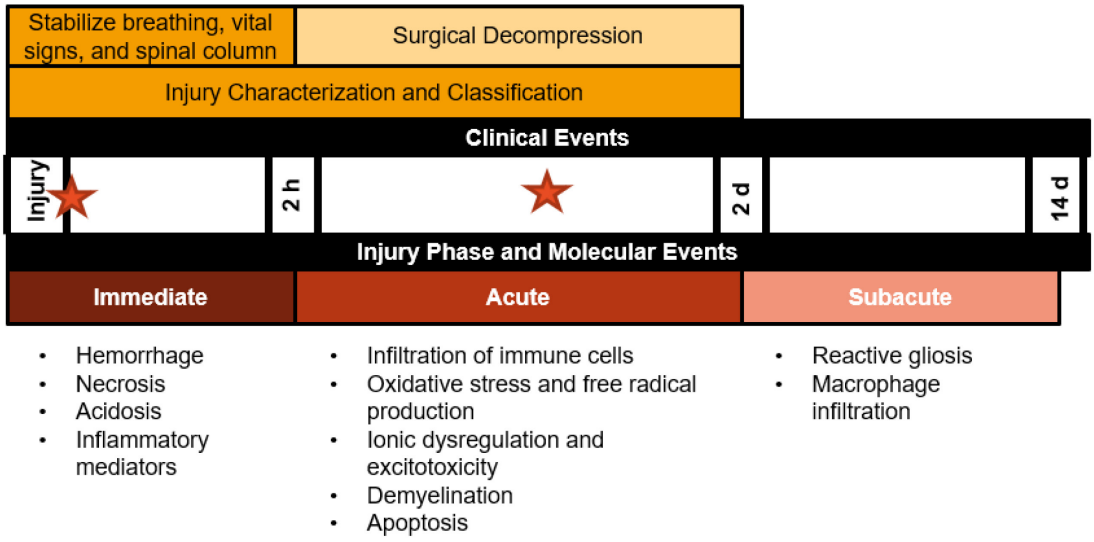


Figure 6: Timeline shows clinical and molecular events that occur in the first few days after SCI. Leftmost star shows the therapeutic intervention of the previous animal model for testing this gene therapy. The second star shows therapeutic intervention about one day after injury, which is more consistent with clinical surgical decompression but with many changes to the microenvironment of the injury. Figure adapted from Siddiqui, Khazael, & Fehlings, 2015. [19]

4.1 Proliferation and inflammation unchanged with delayed treatment

After initial injury, the body's first response is an attempt to reduce the extent of the injury. An increase in proliferation is seen, most of which become oligodendrocytes and reactive astrocytes, and is confined to a thin layer around the lesion.[20] These new cells organize around the edges of the lesion and begin to form the glial scar, a physical border that separates the damaged tissue from the adjacent healthy neuronal tissue.[21] Injection of the lentiviral therapy immediately after injury resulted in an increase in proliferation at the site of injury. However, the delayed injection of the same therapy did not produce an increase in proliferation. We speculate that the lack of effect on proliferation may be due to the negative microenvironment that develops between injury and treatment. Hemorrhaging, necrosis, oxidative stress, and ionic imbalances lead cells down apoptotic pathways. These environmental cues may affect the cell's decision to proliferate in response to the therapeutic injection.

Another important tool used by the body to mitigate the effects of injury is inflammation. Expression of proinflammatory cytokines have been detected as early as 30 minutes after SCI, followed by immune cell infiltration.[22] It is common to think of inflammation as detrimental, however, studies have shown that suppressing the immune response after traumatic brain injury could worsen outcomes. Inflammation in the early stages can prepare the site of injury for future repair.[23, 24] Our results show that inflammation three days after injection was not affected by delayed treatment, allowing the spinal cord to appropriately respond to injury.

4.2 Attenuation of cell death two weeks after therapeutic intervention

Cell death is a consequence of mechanical injury. Since cellular debris is toxic to cells, damaged cells are labeled for clearance.[25] The growth factors, cytokines, and chemokines released by cells recruit inflammatory cells to clear the debris.[21] Although localized inflammation is beneficial in these initial stages of injury, chronic inflammation can spread past the original injury location and cause an increase in size of the lesion. Delayed lentiviral gene therapy effectively reduced cell death two weeks after administration. A decrease in cell death leads to reduced debris and tissue damage by reducing the spread of destructive inflammation. Therefore, the treatment was able to help contain the spread of secondary injury.

4.3 Delayed treatment increases neurogenesis after injury

Although the glial scar acts to protect healthy tissue from dysfunctional tissue by creating a physical barrier[21], the imbalanced microenvironment limits new growth and tissue repair[26, 27]. After injury, the microenvironment influences NSPCs to differentiate into more astrocytes and oligodendrocytes, not neurons, which is the main reason for limited recovery after SCI.[27-29] Neurons are lost to the initial mechanical injury and their regeneration is limited by these environmental cues. The risks of exogenous cell transplants have prompted researchers to try to find methods of motivating endogenous stem cells to differentiate into neurons and repair the damaged cord. Gsx1 expression in the developmental stages affects the differentiation of NSPCs into necessary interneurons that enable communication between the motor and sensory neurons of the central nervous system. In a previous study conducted by our lab, we found that Gsx1 expression in the cells of the mature spinal cord that underwent injury could induce neurogenesis and promote functional recovery. Therefore, we analyzed the delayed treatment's effect on activating endogenous NSPCs and influencing them to differentiate into the mature neurons of the spinal cord.

Three days after injection, we saw an increase in NSPC activation through Nestin staining in the experimental group. Reactivation of Gsx1 expression at the site of injury However, NSPC activation without directed differentiation could create more astrocytes and insufficient neurons for restoring the local circuitry.[30] Two weeks after injection, immunohistochemical analysis illustrated an increase in immature neurons. Consequently, 35 days after injection, we saw in increase in cholinergic neurons in the experimental group. Our results show that the delayed injection of the lentiviral Gsx1

treatment was able to change the proportion of neuronal subtypes at the site of injury by increasing interneurons that support motoneurons. Motoneurons are responsible for integrating motor commands from the central nervous system and sending an output that affects muscular contraction.[31] These cholinergic interneurons can therefore replace neurons lost after injury and restore the severed connections.

Chapter 5

Conclusion and Future Work

Clinically, surgical intervention would not occur immediately after injury. We have shown that a one-day-delayed injection to the injured spinal cord of a lentiviral gene therapy can still remodel the site of injury in a hemisection SCI mouse model. Although inflammation and proliferation were not affected, studies show that these are important aspects of the body's response in the early stages of injury. Cell death was effectively reduced two weeks after injection, reducing the risk for chronic inflammation. Additionally, Gsx1 overexpression induced by the delayed treatment activated NSPCs and influenced them to become cholinergic neurons, important interneurons for modulating locomotion. These results indicate that this gene therapy could be effective at restoring locomotor function after SCI in realistic clinical settings.

For future studies, a long-term behavioral study tracking functional recovery would be important to determine if these cellular/molecular changes are enough to affect overall locomotor function. Additionally, since most clinical SCIs are not hemi-sections, performing a delayed injection on more clinically relevant injury models, such as contusion or compression, would further elucidate the clinical applicability of this gene therapy. The identification of a time window for effective gene therapies is necessary if we hope to translate them to therapies in humans. Our studies represent a first step in this effort.

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