NEURAL STEM CELL TRANSPLANTATION
TO TREAT LUMBOSACRAL SPINAL CORD INJURY

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Wise Young
And approved by

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ABSTRACT OF THE DISSERTATION

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By NING CHIANG

Dissertation Director:

Wise Young

Abstract

A third of human spinal cord injuries (SCIs) involve the lumbosacral spinal cord (LSC), but few studies are on LSC injury (LSCI) and no functional treatments are available to date. Unlike cervical or thoracic SCI that damages primarily the fiber tracts, LSCI disrupts both fiber tracts and neurons comprising the motor centers for walking, bladder, bowel and sexual functions. Thus, treatments for LSCI need not only axonal regeneration but also neuronal replacement. The ideal therapy is to transplant cells that have neuronal potential that can replace the damaged or lost neurons after LSCI. Initially, I cultured neural stem cells (NSCs) isolated from the subventricular zone (SVZ) of neonatal Fischer 344 rats and other cell types as the sources for transplantation. However, those cells either remained as NSCs or produced astrocytes instead of neurons after transplantation. Neither lithium treatment nor silencing of the phosphatase and tensin homolog (PTEN) gene increased the percentage of neurons in culture. Silencing PTEN reduced neurons and produced more oligodendrocytes. Therefore, I transplanted human induced-pluripotent stem cell-derived neural stem cells (iPSC-NSCs) into immune-
compromised Rowett nude (RNU) rats after LSCI. These human iPSC-NSCs survived, migrated and grew long processes 10 weeks after transplantation, showing the potential of human iPSC-NSCs as a future therapy for LSCI. However, the transplants did not improve functional recovery, and the reasons why will be discussed.
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<tbody>
<tr>
<td>BMSC</td>
<td>Bone marrow stromal cell</td>
</tr>
<tr>
<td>CD29/68/90/105</td>
<td>Cluster of Differentiation 29/68/90/105</td>
</tr>
<tr>
<td>C1-8</td>
<td>Cervical spinal cord segment 1-8</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPG</td>
<td>Central pattern generator</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CST</td>
<td>Corticospinal tract</td>
</tr>
<tr>
<td>DCX</td>
<td>Doublecortin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>E13/13.5/14</td>
<td>Embryonic day 13/13.5/14</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ENSC</td>
<td>Embryonic neural stem cell</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>F344 rat</td>
<td>Fischer 344 rat</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GA muscle</td>
<td>Gastrocnemius muscle</td>
</tr>
<tr>
<td>GAD65/67</td>
<td>Glutamic acid decarboxylase 65/67-kilodalton isoform</td>
</tr>
<tr>
<td>GalC</td>
<td>Galactosylceramidase</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GRP</td>
<td>Glial restricted progenitor</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>H&amp;E staining</td>
<td>Hematoxylin and eosin staining</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
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<tr>
<td>iPSC-NSC</td>
<td>Induced pluripotent stem cell-derived neural stem cells</td>
</tr>
<tr>
<td>ITSF</td>
<td>Intermediate toe spread factor</td>
</tr>
<tr>
<td>ITSS</td>
<td>Insulin-transferrin-sodium selenite media supplement</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>L1-5</td>
<td>Lumbar spinal cord segment 1-5</td>
</tr>
<tr>
<td>LSC</td>
<td>Lumbosacral spinal cord</td>
</tr>
<tr>
<td>LSCI</td>
<td>Lumbosacral spinal cord injury</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule-associated protein 2</td>
</tr>
<tr>
<td>MASCIS impactor</td>
<td>Multicenter animal spinal cord injury study impactor</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>Muse cell</td>
<td>Multi-lineage differentiating stress enduring cells</td>
</tr>
<tr>
<td>Nestin</td>
<td>Neuroectodermal stem cell marker</td>
</tr>
<tr>
<td>NF</td>
<td>Neurofilament</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>NRP</td>
<td>Neural restricted progenitor</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>Olig2</td>
<td>Oligodendrocyte transcription factor 2</td>
</tr>
<tr>
<td>P0/1/2</td>
<td>Postnatal day 0/1/2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase/Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKB/Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PS</td>
<td>Penicillin Streptomycin</td>
</tr>
<tr>
<td>PSA-NCAM</td>
<td>Polysialylated neuronal cell adhesion molecule</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RNU rat</td>
<td>Rowett nude rat</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>S1-5</td>
<td>Sacral spinal cord segment 1-5</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>SD rat</td>
<td>Sprague Dawley rat</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum-free medium</td>
</tr>
<tr>
<td>SITS</td>
<td>Static intermediate toe spread</td>
</tr>
<tr>
<td>SSEA-3</td>
<td>Stage-specific embryonic antigen 3</td>
</tr>
<tr>
<td>SSI</td>
<td>Static sciatic index</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STS</td>
<td>Static toe spread</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>SYP</td>
<td>Synaptophysin</td>
</tr>
<tr>
<td>T1-13</td>
<td>Thoracic spinal cord segment 1-13</td>
</tr>
<tr>
<td>TA muscle</td>
<td>Tibialis anterior muscle</td>
</tr>
<tr>
<td>TSF</td>
<td>Toe spread factor</td>
</tr>
<tr>
<td>Tuj1</td>
<td>Class III β-tubulin</td>
</tr>
<tr>
<td>TUNEL staining</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling staining</td>
</tr>
<tr>
<td>VGluT1/2</td>
<td>Vesicular glutamate transporter 1/2</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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</tbody>
</table>
Introduction

Spinal cord injury (SCI) is a serious problem and no effective treatment is available. Among all types of SCI, lumbosacral SCI (LSCI) has the most severe impact on function, causing flaccid paralysis of legs and pelvic organs, but is the least studied. The first two chapters of this dissertation will provide general background information and common methods. The subsequent four chapters will each cover a topic, beginning with literature review, rationale and specific methodology, followed by results and discussion. Chapter III will describe different cell sources for transplantation that I have tested, including neonatal and adult stem cells. Chapter IV will describe my experiments to promote neurogenesis of rat neural stem cells (NSCs) and human induced pluripotent stem cell-derived neural stem cells (iPSC-NSCs) in culture. Chapter V will show how the human iPSC-NSCs behave after transplantation, and Chapter VI will explain how these cells affect functional recovery.
Chapter I: Background

1. Spinal cord injury (SCI)

The spinal cord connects the brain to the body: it conveys the sensory signals from the body to the brain, conducts the motor signals from the brain to the body, and processes both somatic and autonomic inputs and outputs. Injury to the spinal cord disrupts these functions, causing the loss of sensory, motor and automatic functions below the injury level. Spinal cord injury (SCI) can be divided into traumatic and non-traumatic SCI. Traumatic SCI occurs when external physical force damages the spinal cord (such as fall, sports, violence or car accident), while non-traumatic SCI occurs when a disease damages the spinal cord (such as tumor, ischemia, infections, inflammation and toxins) (Young, 2014).

In traumatic SCI, “primary” (acute) injuries of the spinal cord is followed by “secondary” (chronic) injury resulting from the responses of the spinal cord to the primary injury, damaging white and gray matter. SCI can be distinguished temporally: acute (less than 48 hours), subacute (48 hours to 14 days), intermediate (14 days to 6 months) and chronic (more than 6 months). The primary injury causes the initial mechanical disruption and dislocation of the vertebral column, thus damages the spinal cord by contusion, compression or transection. The secondary injury includes cell permeabilization and pro-apoptotic signaling due to the destruction of the microvascular supply of the spinal cord. Inflammatory cells such as lymphocytes, neutrophils and macrophages infiltrate to the injury site, resulting in swelling and damaging more regions of the spinal cord. The cytotoxic by-products causes further necrotic and apoptotic cell death, creating a harsh environment. The
cascade results in the death of both neurons and glia, disrupts the structure of the spinal cord, and forms glial scar and cystic cavities. Cystic cavities are the result of accumulated extracellular fluid such as cerebrospinal fluid (CSF) (Ahuja et al., 2017).

Interestingly, traumatic SCI occurs more in male (80%) than in female (20%) (Chen et al., 2016). In North America, the most common causes of traumatic SCI between 2010 to 2014 are traffic accidents (40%), falls (30%) and sports (15%). In these cases, the spinal cords mostly contused instead of crushed or transected. Therefore, among all animal models, contusion model is the closest to real-life human experience.

The human spinal cord has four sections: cervical (C1-C8), thoracic (T1-T12), lumbar (L1-L5) and sacral (S1-S5). In the general population in the US, traumatic SCI is more frequent at the level of cervical (60%) spine than thoracic (30%) and lumbosacral (10%). However, lumbosacral SCI (LSCI) actually occurs much higher (close to a third), mostly because of mistakenly diagnosed as lower thoracic injury. Its importance is thus underestimated.

2. **Lumbosacral spinal cord injury (LSCI)**

Spinal cord segments do not match completely with vertebral segments. In cervical and thoracic parts, spinal and vertebral segments roughly match one-on-one, but there is a shift as it goes lower to the lumbar and sacral parts. Because of this mismatch between the segments of spinal cord and vertebra in the lower part of the spinal cord, ten lumbosacral spinal cord segments (L1-S5) are squeezed into three vertebral segments of T11, T12, and L1. Therefore, lumbosacral spinal cord injuries (LSCI) often damage multiple lumbar and sacral spinal cord segments. The LSC
contains motor centers for the legs and pelvic organs, including not only central pattern generator (CPG) for walking but also sacral centers for bladder, bowel, and sexual functions. LSCI causes flaccid paralysis with muscle atrophy, as well as severe bladder, anal and sexual dysfunction.

Few studies have focused on LSCI, perhaps due to underestimation of the number of LSCI patients. Up to date, the majority of SCI studies have been tested on either cervical or thoracic in rodent models. Because the LSC is located from T11 to L1, upper lumbar SCI are often misclassified as thoracic SCI, over a third of SCI are actually LSCI. Until recently, no standardized animal LSCI model was available. Consequently, few therapeutic treatments have been studied for LSCI. Thus, this dissertation will focus on treatments for LSCI in an animal model.

3. Strategies for treating LSCI

To repair the damage from SCI, axons and neurons need to regenerate. Injured central nervous system (CNS), including the brain and the spinal cord, have limited regenerative capacity with poor endogenous axonal regrowth and remyelination (Don et al., 2012). Therefore, spinal cord regeneration is a major objective of research (Young, 2014).

The distribution and proportion of fiber tracts (white matter) and neurons (gray matter) are different between higher and lower spinal cord. The gray matter is surrounded by the white matter. If the proportion of gray matter is larger, the gray matter will be easier to be damaged. From the cross-sections of spinal cord, lumbosacral part has much larger proportion of gray matter compared to cervical and thoracic. Cervical and thoracic SCIs damage long fiber tracts that connect the brain
and lower spinal cord, while LSCI damage not only fiber tracts but also neurons that the fibers connect to and control muscles. Therefore, regenerating axons may not be sufficient to restore functions in LSCI. Neuronal replacement is required in addition to axonal regeneration. Thus, cell transplantation is necessary to restore function after LSCI.

While many therapies regenerate axons in the spinal cord, few researchers have tried to replace neurons in injured spinal cords. Not only must regrown or transplanted neurons survive, they must themselves be innervated by descending and local axons and send axons to connect with other neurons and muscles. The new neurons must be incorporated in the circuitry of the spinal cord.

Neural stem cells (NSCs) can reproduce themselves and differentiate into three different cell types of the nervous system: neurons, astrocytes and oligodendrocytes. Because of its ability to self-renew (proliferate) and differentiate into neural cells (multipotency), NSCs are ideal candidates for transplantation therapy.

We investigated cells suitable for transplantation in three respects. First, whether the cells survive transplantation without immunosuppression. Second, whether the cells produce or differentiate into neurons after transplantation. Third, whether the cells incorporate into the neuronal circuitry by accepting and making synapses with other neurons.

4. Hypothesis

We would like to examine whether neural stem cells (NSCs) from several sources could survive, migrate and differentiate to neurons to help functional recovery of rats after LSCI. Our stepwise hypotheses include:
1) In the tissue with or without LSCI, NSCs make mostly astrocytes.

2) Pretreated NSCs will show neuronal potential in culture.

3) After transplantation, pretreated NSCs will differentiate into neurons.

4) After transplantation, differentiated neurons will migrate and form synapses.

5) After transplantation, differentiated neurons will help functional recovery.
Chapter II: Materials and Methods

1. Investigational plan

A. Explore stem cell sources for LSC transplantation (Chapter III)

   I transplanted stem cells from different sources into uninjured LSC to see whether they survive, migrate, and differentiate into neurons. The cells were green fluorescent protein (GFP)-labeled (either by transfection or isolated from GFP animals) so they can be visualized under microscope after transplantation. The cell sources mainly include rat neonatal NSCs and adult NSCs.

B. Characterize and manipulate NSCs in culture (Chapter IV)

   To encourage NSCs to produce more neurons to replace damaged and lost neurons, I applied lithium and PTEN U1 adaptor to the cultured cells to promote neurogenesis, and used immunofluorescence staining with different antibodies as markers for specific cell types.

C. Characterize NSCs transplanted into injured LSC (Chapter V)

   I applied the LSCI model (developed by Wen et al in SD rats) to injure RNU rats at L4-L5 with the MASCIS Impactor. Two weeks after the injury, human iPSC-NSCs transfected to express GFP were transplanted into the LSC. To characterize these transplanted cells, I did immunofluorescence staining with various antibodies to identify what cell types these NSCs had become.

D. Examine functional recovery after NSC transplantation into injured LSC (Chapter VI)
I obtained standing and walking footprints weekly to assess functional recovery before injury, after injury, and after transplantation until the animals were euthanized 12 weeks after injury (10 weeks after transplantation).

2. Rat neonatal NSC isolation and culture

Neonatal (P1-P2) Fischer 344 rats were anesthetized on ice and decapitated with a scalpel. The brain was removed and placed into ice-cold 1X PBS in a 60-mm petri dish. Tissues from the subventricular zone (SVZ) were dissected out, transferred to another petri dish containing ice-cold PBS, cut into small pieces with forceps, homogenized by pipetting, filtered with a 70-μm cell strainer (Falcon #352350), and transferred into petri dish coated with laminin (Invitrogen #23017-015) and poly-L-lysine (Sigma #P1274) and contained serum-free medium (SFM). The SFM contained DMEM/F12 (Gibco #11320), ITSS (Sigma #13146), Heparin (APP #63323-504031), Putrescine (100X), Progesterone (1000X), 30% glucose, 1M Hepses buffer, B27 Supplement (Thermo Fisher #17504044), EGF, FGF (Peprotech #100-18C), and Penicillin Streptomycin (PS) (100X). For passaging, cell suspension was centrifuged at 250g for 5 min, supernatant removed, TrypLE (Gibco #12604) added, and placed in 37°C water bath for 5 min to separate the cells. SFM was added to wash the cells, the solution was centrifuged at 250g for 5 min, the cell pellet was resuspended in SFM, and the cells were counted with a hemocytometer and transferred to new plates.

3. Human iPSC-NSC culture and transfection

We were fortunate to get human iPSC-NSCs donated from Dr. Ki-Bum Lee’s laboratory in Biomaterials Science at Rutgers. These cells were originally from Dr. Alysson Muotri’s lab at UCSD, and were induced from fibroblasts to iPSCs with
retroviral vector transduction and were already derived into NSCs with medium induction. The iPSC-NSC medium contained DMEM/F12 (Thermo Fisher #11320033), Neurobasal (Thermo Fisher #21103049), B-27 Supplement (Thermo Fisher #17504044), N-2 Supplement (Thermo Fisher #17502048) and FGF (Peprotech #100-18C). For cell expansion, StemPro Accutase (Thermo Fisher #A1110501) was used to dissociate the cells, which were then replated into petri dishes coated with Matrigel (Corning #CB-40234). To label the cells with GFP, plasmid pEF1alpha-AcGFP1-N1 Vector (Takara #631973) was amplified with Subcloning Efficiency DH5α Competent Cells (Thermo Fisher #18265017) in LB medium containing Kanamycin (Thermo Fisher #15160-054) with Plasmid Mega Kit (Qiagen #12181) and transfected via Lipofectamine 3000 Reagent (Thermo Fisher #L3000-008) and Opti-MEM (Thermo Fisher #31985062), and were selected with Geneticin (G418 sulfate, Thermo Fisher #10131035). After transfection, Geneticin was added to the NSC culture every time, first at final concentration of 500 μg/mL as selection, then decreased to 250 μg/mL as maintenance. All petri dishes were coated with coating solution containing 200 μL Matrigel (Corning #11320033).

4. Animals

The animals studied include wild-type (WT) and GFP Fischer 344 (F344) rats, WT and GFP Sprague-Dawley (SD) rats, and Rowett nude (RNU) rats. F344 and SD rats are in-house self-bred. We purchased RNU nude rats with 150-250 g body weight from Charles River. The Rutgers Institutional Animal Use and Care Committee approved all animal procedures and protocols. Sean was the main animal caretaker, who expressed the rat bladder daily, weighed the rats weekly, and gave medications
to the rats according to the protocols. When Sean was away, I took care of all the animals.

5. **Lumbosacral spinal cord contusion**

After anesthesia with isoflurane (Isoflurane VIP3000 Vaporizer MDS Matrix, 5% induction and 2% maintenance), the rat was shaved (Oster Golden A5) on the back (from behind the ears to the hip) and put onto the table with heating pad (Sunbeam #732-500) and drape. First, I made a midline incision to cut the skin, and blunt-dissected fat to separate it from the muscle. Then, I made two parallel incision to the muscle along the spinal column and identified the spinal processes of T13 and L1 with the back side of the scalpel (#15 blade with #3 holder). The rongeur (FST #16021-14) was used to remove the top of T13 and L1 vertebral segments (laminectomy) to create a rectangular window to expose the underneath spinal cord. Then the rat was transferred onto a sponge, the spinal column was clamped and suspended at T12 and L2, and the spinal cord was contused by dropping a 10-gram rod 12.5-mm onto T13 and L1 vertebral junction with the MASCIS Impactor (Model #3). After contusion, a piece of fat was cut to cover the exposed spinal cord, then the surrounding muscles and tendons were sutured (CP Medical #684S, 3/0, NFS-1 silk) with a needle holder (FST #12002) and the skin was closed with clips (Michel Clips, 14 mm) by using a clip applicator (Weck #522-110). Blood was removed by gauze (Henry Schein 2” x 2”), cotton pellets (Richmond) or cotton tipped applicators (Henry Schein, 6”).

6. **Cell transplantation**
The human iPSC-NSCs were injected into the LSC two weeks after LSCI, using a Hamilton syringe (Hamilton #80014) to insert a 33-gauge needle (Hamilton #7803-05, customized 0.69”, beveled at 30 degrees) 1 mm deep at a 60-degree angle with a micromanipulator (Narishige #MM-308040). In LSCI, the cells were injected into the left and right dorsal root entry zones at the rostral and caudal edges of the lesion (4 injection sites). In uninjured spinal cords, the cells were injected into 2 sites. Each site received 1 µL solution with $10^5$ cells injected slowly (over 3 minutes) and the needle held in place for 2 minutes to prevent backflow of the cells through the needle track (total of 5 minutes). For SD and RNU rats, the dura needed to be poked with a bent needle before injection.

7. Fixation and sectioning

The rats were first anesthetized with ketamine and xylazine or isoflurane, then cannulated through the aorta, injected with 1 ml of 1% lidocaine, perfused with 200 ml of saline at 35 ml/minute to wash out the blood, and fixed with 300 ml of 4% paraformaldehyde (PFA) at 25 ml/minute. Sean did the perfusions for me.

After perfusion, I removed the spinal cord from the spinal column and immersed it in 4% PFA overnight, then placed it in 30% sucrose solution until the tissue had sunk to the bottom of the container. The spinal cord was taken out and then frozen in O.C.T. Compound (Sakura Finetek #4583; VWR #25608-930) in a plastic mold (Sakura Finetek #4566; VWR #25608-924) and stored frozen at -80°C. On the day of sectioning, the block would be mounted in the chamber of the cryostat microtome (Bright #OTF5000) at least 30 min before sectioning. The block was cut into 30 µm-thick slices at -30°C (sample temperature: -25°C; chamber temperature: -
30°C) with the cryostat microtome. The sections were cut either coronally or horizontally. The sections were attached to charged slides (Globe Scientific #1358) and stored at -80°C.

8. Immunofluorescence staining and imaging

Tissue slices were washed 3 times with 1X PBS to remove OCT and then blocked with 5% normal goat serum (NGS) for 1 hour at RT. Primary antibodies with optimal dilution ratio were applied overnight at 4°C. After 4 washes, secondary antibodies were applied for 1 hour at room temperature. Then, DAPI or Hoechst was added for nuclei staining. After staining, I mounted the slides with antifade mounting medium (Sigma #F4680) and coverslip (Globe Scientific #1415-15).

Primary antibodies included markers for different lineages of NSCs, including nestin for NSCs, PSA-NCAM for neural-restricted precursors (NRPs), A2B5 for glial-restricted precursors (GRPs), GFAP for astrocytes, GalC for oligodendrocytes, Tuj1 for immature neurons, NeuN for mature neurons, NF for mature neurons and axons, MAP2 for mature neurons and dendrites, synaptophysin (SYP) for synaptic vesicles, VGluT1 for excitatory neurons. For human specific markers, STEM101 was for human nuclei and STEM121 was for human cytoplasm. GFP was used to amplify transfected GFP in human iPSC-NSCs. Detailed information including host, vendor, catalogue number and dilution ratio of primary antibodies was described in Table 1.

Stained sections were first examined under deconvolutional microscope (Zeiss Axiovert 200M), then transferred to confocal microscope (Zeiss LSM 510 and LSM 800) for higher resolution. Pictures would be further analyzed with Zeiss software and ImageJ.
9. **Graphs and statistics**

Graphs were made with GraphPad Prism 7. Results were showed as means ± standard error of the mean (SEM). Statistics were analyzed with StatView by using analysis of variance (ANOVA). Asterisk indicated $p < 0.05$. 
Chapter III: Exploring cell sources for transplantation in uninjured LSC

1. Literature review: Cell sources for LSCI transplantation

A. Embryonic NSCs

Embryonic NSCs (ENSC) can survive and help functional recovery after SCI. ENSCs from the cerebral cortex of E14 mice can reduce release of macrophages and inflammatory cytokines after SCI (Cheng et al., 2016). Moreover, ENSCs isolated from the spinal cord of E13.5 rats can improve functional recovery (Jin et al., 2016).

Kumamaru’s group transplanted NSCs derived from E14 F344 rats into female F344 rats and found conditioning lesions could induce robust sensory axon regeneration (Kumamaru et al., 2018). Human NSCs derived from fetal spinal cord can also incorporate into specific target area in primate hosts. Later, they found that E14 rat embryonic NSCs transplanted into rat with cervical (C4) SCI could regenerate corticospinal tract (CST) axons (Kumamaru et al., 2019). Furthermore, they found that corticospinal axons tend to regenerate into the area of motor interneurons instead of sensory interneurons.

Human ESC-derived NSC (H9 ESC line) has been used as the cell source for treating both cervical and thoracic SCI in both SD and F344 rats. Rodent fetal NSCs isolated from midbrain, hippocampus and subventricular zone (SVZ) were used on thoracic (T10) SCI (Zhao et al., 2016).

B. Neonatal and postnatal NSCs
Neonatal NSCs are early postnatal NSCs, and they reside in limited regions of the nervous system, including SVZ, hippocampal dentate gyrus and around the central canal in the spinal cord. However, neonatal NSCs are more active and migratory. Rat neonatal NSCs from the SVZ that migrated radially tend to produce astrocytes and oligodendrocytes, while the ones migrated bidirectionally (rostrally and caudally) tend to produce more neurons, especially interneurons (Suzuki and Goldman, 2003).

Recently, Du et al transplanted NSCs isolated from neonatal rat hippocampus into T10 contused male SD rats (Du et al., 2019). After four weeks, locomotor, sensory and function and neuropathic pain were improved. NSCs can also be derived from other types of stem cells including bone marrow stromal cells (BMSC). BMSC-NSCs were transplanted into thoracic (T10) contused SCI rat and helped functional recovery (Zhilai et al., 2011). NSCs grown from human cell lines can produce neurons that innervate endogenous neurons (Lu et al., 2012).

However, the cells may already be committed to certain types of neurons. Thus, NSCs isolated from the brain may not produce the right types of neurons as NSCs isolated from the spinal cord. Limitations of proliferation and differentiation may also prevent adult NSC participation in spinal cord circuitry.

C. **Mesenchymal stem cells and Muse cells**

Mesenchymal stem cells (MSCs) present in many tissues including adipose, dermis, blood, bone marrow and umbilical cord. The most commonly used MSCs are from dermal fibroblast and bone marrow (BMSC/BM-MSC). Because of their
trophic and anti-inflammatory properties, MSCs can repair tissue damage. Tissue stem cells are usually limited to generate the cells where they reside. In contrast, although MSCs belong to the mesodermal lineage, they can also differentiate to endodermal and ectodermal lineages. However, the ratio of this cross-lineage is very low, indicating that only a small subpopulation of MSCs is pluripotent-like. Therefore, MSCs are generally considered multipotent instead of pluripotent (Wakao et al., 2012).

In 2010, multi-lineage differentiating stress enduring cells (Muse cells) were discovered in adult mesenchymal tissues by Dezawa’s group (Kuroda et al., 2010). These cells can generate cells representative of all three germ layers from a single cell, which fulfill the triploblastic and self-renewal requirements for pluripotency compared to other MSCs. In addition, Muse cells comprise only around 1% of the total MSCs and express pluripotency markers such as Nanog, reduced expression 1 (Rex1), sex-determining region Y box 2 (Sox2), octamer binding transcription factor 3 and 4 (Oct3 and Oct4) unlike other MSCs. Muse cells can be isolated from MSCs by incubating in trypsin solution for over 16 hours, which shows their stress-enduring ability. Because Muse cells are a subset of MSCs and are pluripotent stem cells, they can be selected by CD-105 (endoglin), a marker for MSC, and stage-specific embryonic antigen 3 (SSEA-3), a surface marker for human ESC with fluorescence activated cell sorting (FACS). From MSC culture, SSEA-3 alone will be sufficient to identify Muse cells from other MSCs (Dezawa, 2016). Compared to other MSCs, Muse cells have higher homing rate to damaged tissue, higher survival after long time and higher rate of
differentiation. In addition, like other MSCs, Muse cells do not form tumors. Muse cells could repair tissues in stroke (Uchida et al., 2016), hepatitis and muscle degeneration (Kuroda et al., 2010). With pros of MSCs and without cons of other MSCs, Muse cells are a promising source for treating SCI.

D. Induced-pluripotent stem cells (iPSCs)-derived NSCs

Unlike embryonic stem cells, iPSCs can be obtained from adult somatic cells such as fibroblasts from the skin. By adding the four Yamanaka transcription factors (Klf4, Oct4, Sox2, and c-Myc), these cells can regain pluripotency. Therefore, fibroblasts can be isolated from the skin of the patient and modified into iPSCs, grown into NSCs, and then transplant back into the same patient. This autologous transplantation avoids ethical issues like embryonic cells and does not need any immunosuppressants to prevent rejection of foreign cells, making it highly clinical applicable. However, iPSCs could form tumors, thus they need to be induced to NSCs before transplantation. Culture system has been established to derive iPSCs into NSCs, and specific reagents and conditions can make the transplanted iPSC-derived NSCs survive, migrate and differentiate into neurons, astrocytes and oligodendrocytes (Yuan et al., 2013).

Transplanted iPSC-NSCs can survive and differentiate into neurons and glia after SCI. Studies have shown massive regeneration of neurons after human iPSC-NSCs were transplanted into nude rats (Lu et al., 2014). These exogenous neurons not only survived and extended long axons but also formed synapses to the endogenous neurons. However, functional recovery was not obvious.

2. The study
A. Rat MSCs from bone marrow

To see whether muse cells were suitable for transplantation in LSC, I transplanted MSCs (muse cells) isolated from the bone marrow of GFP F344 rat (by Dr. Fumitaka Ogura) into uninjured LSC of WT F344 rat with two injection sites (laterally left and right). Then, the spinal cord was taken out, sectioned, and IHC was performed to see if these muse cells survived, migrated and differentiate.

B. Rat neonatal MSCs from blood (NO1.1 cells)

NO1.1 cells were isolated from the whole blood of neonatal (P0-P1) SD rat and transfected with GFP (using Lipofectamine). To see whether this type of cell was good for transplantation, I transplanted NO1.1 cells into uninjured LSC (two injection sites) and used IHC to examine the characteristics of these cells.

C. Rat neonatal NSCs from subventricular zone

To see whether neonatal NSC was a good candidate for transplantation in LSC, I transplanted NSCs isolated from the SVZ of neonatal GFP F344 rats with two injection sites. After that, the LSC was taken out, sectioned, and IHC was performed to examine whether these neonatal NSCs survived, migrated and differentiated to neurons.

3. Additional methodology

A. Adult rat MSC (muse cell) isolation

Adult MSC (muse cells) were isolated from the bone marrow of GFP Fischer 344 rat (by Dr. Fumitaka Ogura from Dr. Mari Dezawa’s lab). Then the cells were transplanted into uninjured lumbosacral spinal cord of WT Fischer rat.
The cell number for transplantation was $2 \times 10^5$ cells ($10^5$ cells/site). The animal was perfused after 12 days and frozen sectioned coronally.

4. Results

A. NO1.1 cells transplanted into uninjured LSC

From the results of IHC, NO1.1 cells transplanted into uninjured LSC survived after 7 days. Under the GFP channel of epifluorescence microscope, there were two bright spots on the whole mount LSC from both dorsal and ventral view, showing the two injection sites of GFP transfected NO1.1 cells. The cells stayed in a spindle shape and did not migrate much. After sectioned coronally and stained, the transplanted cells had a round cluster under Deconvolutional microscope. The cells showed no colocalization with GFAP or NeuN, but a little with NF, suggesting that these cells differentiated into immature neurons with axonal potential (NF) but not mature neurons yet (NeuN). These cells did not differentiate into astrocytes (GFAP) either after 7 days.

B. Rat adult MSCs (muse cells) transplantation into uninjured LSC

From the results of IHC, the MSCs (muse cells) from the bone marrow of adult F344 GFP rat survived 12 days after transplantation. Looking at the whole mount spinal cord, the GFP cells could be seen very vaguely (bright in another animal) around the two injection sites from both dorsal and ventral view. After frozen sectioned coronally, the cells follow the needle track and formed a spindle shape at the injection site. Interestingly, these cells seemed to stay in the globular shape and did not migrate much out of the injection site. The GFP cells did not
colocalize with GFAP, but they overlapped a little with CD90 (MSC and neuronal marker) and ED1 (also called CD68, monocyte and macrophage marker), indicating that these cells had properties of MSCs, monocytes and macrophages, but did not differentiate into astrocytes.

C. Rat neonatal NSCs transplanted into uninjured LSC

I have transplanted neural stem cells isolated from subventricular zone (SVZ) of GFP neonatal (P1) Fischer rats into uninjured LSC of WT Fischer rats. The rats were kept for 1 week, 4 weeks and 7 weeks. The results showed that these NSCs survived after 7 weeks (Fig. 1). Under epifluorescence microscope, the whole mount spinal cord showed faintly two bright GFP spots at the two injection sites from both ventral and dorsal view. After sectioned horizontally, the cells migrated along the longitudinal line of the spinal cord, forming a line of about 600 μm after 1 week (Fig. 1A) and around 1 mm after 4 weeks (Fig. 1B) and 7 weeks (Fig. 1C). Interestingly, although the length of migration was the shortest at 1 week, the length of migration at 7 weeks did not look longer than that of 4 weeks, suggesting some limitation of migration.

From confocal images, the GFP cells colocalized partially with Nestin (Fig. 2A). This colocalization already appeared at 1 week after transplantation (Fig. 2A1), and it could still be seen after 7 weeks (Fig. 2A2-A4). The GFP cells grew long processes. Since these were horizontal sections, most processes formed bundles along the axis of the spinal cord (Fig. 2A1 & A4), but some processes grew laterally (Fig. 2A2 & A3). Interestingly, it seemed that these lateral
processes expressed more Nestin than that of the horizontal ones. This indicated that some transplanted cells remained as NSCs and migrated laterally.

I would like to further examine whether the transplanted NSCs had differentiated into specific cell types. For NRPs, there was no PSA-NCAM expression at 4 weeks after transplantation (Fig. 2B), indicating that the NSCs did not show neuronal potential. To make sure whether the cells could become neurons, I also examined Tuj1 expression (Fig. 2C), and found no GFP & Tuj1 colocalization either at 1 week (Fig. 2C1) or 7 weeks (Fig. 2C2-C3). In addition, the cells did not express neurofilament (NF) after 1 week, either (Fig. 2D). Both Tuj1 and NF results indicate no neurons were produced after transplantation.

At last, I used GFAP staining to examine whether the NSCs had become astrocytes (Fig. 2E). At 1 week after transplantation, only a few cells showed GFAP (Fig. 2E1). At 4 weeks, GFAP signals were not obvious (Fig. 2E2). At 7 weeks after transplantation, some GFAP colocalized with GFP processes (Fig. 2E3). In summary, neonatal NSCs isolated from F344 GFP rats transplanted into uninjured F344 WT rats at T13-L1 survived, migrated both horizontally and laterally, grew long processes and mostly retained NSC marker. Some NSCs differentiated into astrocytes but did not become neurons 7 weeks after transplantation.

5. Discussion

A. Transplantation into uninjured LSC might not be representative

From the results of NO1.1 and adult MSCs (muse cells), it seemed that although these two types of cells could survive, they did not migrate in the LSC.
Compared to them, neonatal NSCs had a better migration ability, both longitudinally and laterally. Since neonatal NSCs produced astrocytes instead of neurons, other cell sources with neuronal potential should also be considered. However, because the NO1.1 and muse cells were transplanted into uninjured LSC mainly to see survival, they might behave very differently in injured LSC.

From studies on muse cells, muse cells would be activated and attracted by damaged cells, and they could even engulf those damaged cells, acting like macrophages and monocytes. Muse cells have the ability to detect signals from damaged tissues, home to the damaged tissue, and differentiate into cells compatible with the homing tissue after integration (Dezawa, 2016). In fulminant hepatitis and muscle degeneration models, Muse cells were found to home into liver and muscle, spontaneously differentiated into hepatocytes and skeletal muscle, filled up and repaired the damaged tissue (Kuroda et al., 2010). Therefore, it is very likely that these muse cells could have migrated if transplanted into LSCI rats. Studies have shown that muse cells transplanted into stroke and SCI could repair lost cells. Therefore, although muse cells were not further investigated in this dissertation, it would be a great candidate for transplantation therapy.

It is believed that injury will activate regeneration because cells are not supposed to be in growing mode in a normal intact tissue. Unwanted cell growth is dangerous, which might form tumors. Needle injection might be a type of minor injury, but the severity should not be comparable to that of real spinal cord injury. The inflammatory response after injury would provide a very different
environment from normal tissue, thus affect the behavior of transplanted NSCs. Those extrinsic factors would play an important role in differentiation and maturation.

However, even in uninjured spinal cord, there was study showing NSCs were restricted to glial lineage (Cao et al., 2001). In 2001, Cao’s group used NSCs isolated from E14 rat cerebral cortex and adult rat SVZ. These NSCs could produce neurons, astrocytes and oligodendrocytes in culture. However, after transplantation into T8 spinal cord of F344 rats, most NSCs differentiated into astrocytes and some oligodendrocytes, while some remained undifferentiated NSCs and no neurons were detected in the uninjured animal. In T8-contused injured animals, NSCs were either undifferentiated or became astrocytes, and no neurons or oligodendrocytes were seen. Although the NSCs I transplanted into uninjured animals were neonatal (P1) NSCs instead of embryonic or adult NSCs, like in Cao’s study, I found undifferentiated NSCs, some astrocytes and no neurons. By looking at Cao’s work, it seems that the key difference between transplantation into normal (uninjured) and injured spinal cord is the production of oligodendrocytes: some in normal tissue and none in injured tissue. In both normal and injured spinal cord, NSCs either remained as NSCs, differentiated into astrocytes but not neurons. My results confirmed this astrogliosis instead of neurogenesis from NSCs in uninjured spinal cord. Further experiments could also be done in injured animals to see if neonatal NSCs behave differently.

B. Cell type and migration
According to the glial scar theory, astrocytes are not desired to be seen in injured spinal cord. Du et al found that the astrocytes had different morphology between SCI/vehicle and sham/NSC treated animals after NSCs isolated from neonatal rat hippocampus were transplanted. NSC-treated group also showed decreased GFAP and increased NF expression (Du et al., 2019). I did not quantify the total GFAP and NF expression or morphology, but I estimated there were 20% astrocytes (GFAP) and 0% neurons (NF), so the morphology of astrocytes could be a very interesting aspect to investigate. I did not look at control animals, thus I was not sure whether there was change in the number or morphology of the cells I transplanted, and how the transplanted cells affected the endogenous host cells. For oligodendrocytes, it would be especially interesting to see if transplanted cells would myelinate host neurons or if host oligodendrocytes would myelinate transplanted neurons.

Lien et al transplanted human embryonic NSCs (H9 human ESC-derived NSCs) into C5 hemisectioned RNU rats and found the NSCs that differentiated into neurons did not migrate, but the NSCs that differentiated into astrocytes migrated into long distance (9 segments from cervical to mid-thoracic). The study observed for 18 months, and those astrocytes migrated about 2-3 mm per month. Furthermore, those astrocytes integrated into the host blood-spinal cord barrier and showed colocalization with glutamate transporter (VGluT1), indicating they were functional. Maybe it was because the cell source was different (mine was neonatal rat NSC, not human embryonic NSC), I observed almost no neurons, but I did see long-distance migration. However, since I observed only some astrocyte
and no neurons, I would not be able to see how far the neurons have migrated. Interesting, the cells I found migrated laterally only expressed nestin, not GFAP, indicating that these migrating cells were still undifferentiated NSCs instead of astrocytes. In Suzuki’s study in 2003, the NSCs that migrated rostrally and caudally (bidirectionally) were neurons and that migrated laterally (radially) were astrocytes or oligodendrocytes (Suzuki and Goldman, 2003). In contrast, in my results, the NSCs that migrated rostrally and caudally were mostly still NSCs with almost no neurons and that migrated laterally were NSCs and astrocytes. It would be interesting if I further quantify both the migration direction and distance of each type of cells including NSCs, neurons, astrocytes and oligodendrocytes to see if each of them has a preference or tendency.

C. Pre-transplantation treatment is needed in culture for neonatal NSCs

From the results of neonatal NSCs, we learned that although these cells could survive, migrate and grow long processes, they tended to either stay as NSCs or differentiate into astrocytes instead of neurons. Although I have not quantified the number of each type or examined the expression of markers for oligodendrocytes, to my observation, I estimated that around 40% of NSCs remained as NSCs (Nestin) and around 20% of NSCs became astrocytes (GFAP) and 0% neurons (Tuj1). I wonder what the remaining 40% cells were. It is possible that those cells were oligodendrocytes. Further staining and quantification could reveal more exact ratio of different cell types.

Our goal was to replace damaged or lost neurons to treat LSCI, and my results showed that these neonatal NSCs did not differentiate into neurons.
Therefore, we would need to treat these cells and promote them into the neuronal lineage in culture to make them ready for transplantation. In the next chapter, I would describe further experiments about trying to produce more neurons in culture.
Chapter IV: Characterizing and manipulation of NSC culture

1. Literature review: Strategies to help cell survival and promote neurogenesis

A. Trophic factors to protect transplanted cells and promote neurogenesis

Trophic factors are essential for survival of all cell types. Epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) are two of the key trophic factors essential for NSCs. Research has shown the conditioned medium contacting these trophic factors can help NSCs survive and grow (Cheng et al., 2017). Lu’s group initially found 9 growth factors that could significantly support cell survival and growth when embedded in fibrin matrix, and later found that a minimum of 4 factors to form a “cocktail” with the NSCs, the gradual release of these factors can help the transplanted cells survive, migrate and differentiate. These 4 factors are brain-derived neurotrophic factor (BDNF), basic-fibroblastic growth factor (bFGF), vascular endothelial growth factor (VEGF), and MDL28170, a cell death inhibitor (Robinson and Lu, 2017).

In general, after transplanted into injured spinal cord, NSCs tend to differentiate into astrocytes (Cao et al., 2001). The reason of this astrogliosis include cytokines such as interleukin-6 (IL-6) released by activated macrophages and microglia. Therefore, most studies tried to modify expression of specific genes to promote neurogenesis of NSCs.

B. Gene manipulation toward neuronal lineage

Gene manipulation can be done before transplantation to direct NSCs to differentiate into desired lineages. It is known that NSCs tend to differentiate into
astrocytes after SCI because of the inflammatory response caused by secondary injury. Therefore, more neurons instead of astrocytes are needed for recovery. There are certain genes that can convert astrocytes into neurons (Noristani et al., 2016), which will be highly beneficial for treating SCI. Many methods have been used to deliver transcription factors (which regulate gene expression) into the cells, including plasmid transfection and lentiviral transduction. However, delivery methods associated with virus raise concerns when applying clinically. Recent study has shown that the dose of transplantation may also affect the fate of differentiation (Piltti et al., 2017). Together, safer ways to direct differentiation of transplanted NSCs need to be further investigated.

There are two genes that have been identified to control neurogenesis and cell survival: Nogo and mTOR. Neurotrpohin is another target. Neurotrophin-3 (NT-3). Erythropoietin (EPO) has also been used to promote survival, regeneration and functional recovery (Wu et al., 2015) (Zhao et al., 2016). Other target genes include Negr1, ERK and IGF. Many of the targets are transcriptional factors that affect axon growth (Carmel et al., 2015). For spinal cord regeneration, there have been three theories explaining the limitation of regeneration: axonal growth inhibitor (especially Nogo), glial scar, and the role of chondroitin sulfate proteoglycan (CSPG) (Young, 2014). In addition to cell survival, since our goal is to replace lost or damaged neurons, we would like to direct the NSCs toward the neuronal lineage and produce more neurons.
The glial scar theory is somewhat misleading. Studies claim that astrocytes accumulate at the injury site and form a cluster that becomes an obstacle, therefore preventing axons from growing across. However, many studies have shown that the so-called glial scar will not prevent regeneration. CSPG cannot stop regeneration, either.

Together, spinal cord has regenerative ability that are normally suppressed by both extrinsic and intrinsic factors, but it can be activated by injury, mediated by the PTEN/AKT/mTOR, cAMP, and GSK-3β pathways, to stimulate neural growth and proliferation.

C. Lithium

Lithium is a kind of alkali metal that has a monovalent cation. Lithium has already been used as a drug for bipolar disorder and hematopoietic disorders, thus relatively safe. As a drug, lithium has many effects on the brain and blood (Young, 2009).

Rat NSC culture isolated from olfactory bulbs and SVZ showed that lithium could suppress GSK-3β and activate NF-AT, and could promote neural precursor cell proliferation, but cannot promote survival of neural precursor cells (Qu et al., 2011). The study showed that instead of inositol depletion, lithium increases inhibitory phosphorylation of GSK-3β, which suppress GSK-3β and activates NF-AT. Moreover, downstream of GSK-3β, it was NF-AT instead of β-catenin that resulted in lithium-induced proliferation of NSCs. This GSK-3β-NF-AT signaling pathway is not dependent on neurotrophic factors.
Animal studies showed beneficial effect of lithium in the central nervous system. Lithium could stimulate NSC isolated from hippocampus and SVZ to produce more neurons (Chen et al., 2000) (Vazey and Connor, 2009) and decrease the amount of astrocytes (Kim et al., 2004). Two main pathways can explain the effects: one involves inositol depletion, and the other involves glycogen synthase kinase 3β (GSK3β). Lithium can inhibit GSK3β and STAT3, it can increase β-catenin.

However, lithium can also participate in a non-GSK3β but STAT3-dependent way. Zhu et al found that lithium could suppress NSC astrogliogenesis and promote NSC proliferation and neurogenesis (Zhu et al., 2011), and lithium inhibit astrogliosis via a non-GSK3β-mediated inhibition of STAT3.

D. PTEN U1 adaptor

PTEN is known to be a tumor suppressor gene found in patients with cancer. Therefore, inhibition of PTEN could promote proliferation and possibly neurogenesis. PTEN is involved in the mammalian target of rapamycin (mTOR) pathway, which is a negative regulator of mTOR. mTOR is a target for treating traumatic central nervous system (CNS) injuries including traumatic brain injury and spinal cord injury (Don et al., 2012). Like mentioned earlier in Chapter I, traumatic CNS injuries involve two phases: acute (primary) phase and chronic (secondary) phase. There are two pathways that can be the target for treatment: phosphatidylinositol 3-kinase (PI3K)-Akt-mTOR pathway, and Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway. mTOR could promote neuronal cell growth, axon regeneration and neuronal
survival. Therefore, suppressing mTOR inhibitor such as PTEN will be a good candidate for treatment to promote neurogenesis.

In 2010, it was found that PTEN deletion could enhance corticospinal neuronal regeneration (Liu et al., 2010). PTEN deletion increases corticospinal tract (CST) sprouting in mice. Genetic activation of mTOR promotes optic axon regeneration. Liu’s study found that downregulation of mTOR negatively affect the sprouting ability of cortical neurons. After T8 dorsal hemisection, PTEN deletion promoted CST regeneration and form synapses. In 2015, it is further found that PTEN deletion enhances CST neuronal growth and motor functional recovery after spinal cord injury in mice (Danilov and Steward, 2015). PTEN knockout mice received contusion injury at C5 recovered better in CST regeneration and forelimb gripping performance. These studies all show that axons can grow through glial scars and regenerate, and deletion of PTEN can result in axonal regrowth and CNS regeneration, indicating that this PTEN/Akt/mTOR pathway plays an important role in CNS traumatic injuries including spinal cord injury (Young, 2014).

U1 adaptor is a tool to silence gene expression. Created by Dr. Sam Gunderson’s group, U1 adaptor are oligonucleotides that composed of a target domain and an U1 domain (Goraczniak et al., 2009). The target domain is complementary to the target gene’s terminal exon. The U1 domain binds to the U1 small nuclear RNA component of the U1 small nuclear ribonucleoprotein (U1 snRNP) splicing factor. This U1 snRNP targeting pre-mRNA will block poly(A)-tail addition, resulting in degradation of the pre-mRNA in the nucleus, causing
gene silencing. U1 Adaptors can inhibit both endogenous and reporter genes in a sequence-specific manner. Compared to small interfering RNA (siRNA), U1 adaptors have less off-target effects and multiple adaptors could further enhance gene silencing. Therefore, U1 adaptor designed to target PTEN could be a good method to inhibit PTEN expression.

E. Induced-pluripotent stem cells (iPSCs) and derived neurogenesis

To replace neurons in the injured lumbosacral cord, we need a suitable and reliable cell source for transplantation. One source is neonatal neural stem cells. However, my results from Chapter III showed that rat neonatal NSCs did not produce neurons.

Induced-pluripotent stem cell (iPSC) is another source for transplantation. Derived from adult fibroblasts or other differentiated cells, iPSCs can produce cell types from epithelial, mesothelial, and endothelial layers. They do not have to be isolated from embryos or fetuses, therefore avoid ethical issues and could be transplanted autonomously. However, iPSC must be differentiated before transplantation or else they may form tumors.

Most investigators create iPSCs by inducing primary dermal fibroblasts with retroviral vectors that express the four Yamanaka genes: OCT4, SOX2, KLF4 and c-MYC. Several methods stimulate iPSCs to produce NSCs, including spontaneous differentiation, chemical induction with retinoic acid in serum free medium, or growing iPSCs on mouse stromal feeder cells. NSCs can differentiate into neurons, astrocytes and oligodendrocytes. Since LSCI needs neuronal
replacement, we prefer having iPSC-NSCs toward the neuronal lineage, which are ready to be transplanted and become neurons.

2. The study

A. Lithium treatment in culture

Since we want to use NSC to replace lost neurons, lithium might stimulate the NSCs and make them ready for transplantation.

I have isolated NSCs from the SVZ of WT neonatal (P1) Fischer rats and gave different treatments to promote NSCs to differentiate into neuronal lineage. The newly isolated cells were cultured for two weeks to form secondary neurospheres, then were treated with different concentrations of fetal bovine serum (FBS), lithium, and the combination of the two for a week. Then I stained markers for different types of cells of the NSC lineage to see the effects of FBS and lithium on differentiation.

B. PTEN U1 adaptor treatment in culture

In order to make the NSCs ready for transplantation, I have also treated the NSCs with different concentrations of PTEN U1 adaptor (provided by Dr. Samuel Gunderson) to silence *pten* gene. After treating for a week, I stained the same markers for different types of cells of the NSC lineage to see the effects of PTEN silencing.

C. Induced pluripotent stem cell (iPSC)-derived NSCs

Since in previous studies, human iPSC-NSCs have been transplanted into RNU rat with cervical SCI and had good survival and massive axonal growth, I
would like to see if these cells could perform well in LSCI. Before transplantation, I would like to grow them in culture and examine their characteristics by using antibodies as markers for different cell types.

3. Additional methodology

A. Automatic counting and decision tree analysis

Staining pictures were taken by IN Cell Analyzer 1000 (GE Healthcare) and analyzed with decision tree analysis tool in the multi-target analysis module. First, nuclei staining (Hoechst) signal was used as reference to identify the cell, then one fluorescence channel was used to detect the signal of that specific marker. A radius and threshold were set to detect the intensity of signals surround the nucleus. The program then could be directed to do automatic counting on total cell number, positive cell number and percentage of positive cell out of total cell. With decision tree analysis with two markers, the program could first divide all cells into positive and negative group based on the first marker, then further divide each group into two groups with positive or negative of a second marker.

4. Results

A. Lithium treatment in culture

The rat neonatal NSCs were treated with lithium at different dosages (1, 2, and 4 mM) to see whether it could produce more neurons and if there was dose effect (Fig. 3). The data were collected from 5 batches of cells. Among these 5 batches, I tested 3 different seeding densities (3000, 4000 and 600 cells/well in 96-well plate) and found no significant difference between different batches and
densities. Without FBS, the cell number was very low compared to the seeding number in both the control group without lithium (around 30 cells/field) and lithium-treated group (Fig. 3A, left panel). Moreover, high concentration of lithium (4 mM) actually decreased total cell count (around 25 cells/field) and there was also a decreasing trend from 1 mM, 2 mM to 4 mM, indicating some toxic effect (Fig. 3A, left panel). Therefore, we added 5% FBS to increase the base number of cells. FBS increased the number of total cells to almost 10-fold (from around 30 to 300 cells/field), while lithium decreased the total cell count in the presence of FBS, around 25% (from 300 to 200 cells/field) (Fig. 3A, right panel). 5% FBS did not affect the expression of Nestin (Fig. 3B), Tuj1 (Fig. 3D), A2B5 (Fig. 3E) or GFAP (Fig. 3F), but decreased the percentage of PSA-NCAM (from around 55% to 20%) (Fig. 3C) and GalC (from 40% to 10%) (Fig. 3G) positive cells, suggesting that FBS decreased the production of NRPs and oligodendrocytes. On the other hand, lithium did not significantly alter any of the six markers (nestin, PSA-NCAM, Tuj1, A2B5, GFAP or GalC) (Fig. 3B-3G). From the staining results, lithium did not promote neurogenesis of rat neonatal NSCs.

B. PTEN U1 adaptor treatment in culture

In the initial experiments, we added the oligonucleotides (PTEN-2, which was Cy3-conjugated) together with signaling peptides (TP-Trf) provided by Dr. Gunderson. However, in the group that did not have peptides, we could still see the Cy3 signal within the cell region after multiple washes during the staining
procedure. Therefore, we decided to add oligonucleotides without peptides in later experiments.

The rat neonatal NSCs were treated with PTEN U1 adaptor at different dosages (1, 5, 10, 20, 50 and 100 nM) to see whether it could promote neurogenesis and whether it was dose-dependent (Fig. 4). The data were collected from 5 batches of cells. Among these 5 batches, I had tried 3 different seeding densities, and did not find any significant difference between batches or density. First, I would like to see whether the cells could successfully take up the PTEN U1 adaptor or how well the efficiency was. Only 5% (at 1 nM) to 40% (at 100 nM) of the treated cells showed positive PTEN U1 adaptor signal, indicating that some cells did not take up the PTEN U1 adaptor, and this effect was dose-dependent (Fig. 4A).

Since only 5-40% of treated cells show positive PTEN U1 adaptor signal, the cells that did not take up PTEN U1 adaptor (PTEN U1 adaptor negative cells) might mask the real effects. Therefore, I used the decision tree analysis tool in multi-target analysis module in the IN CELL software to unmask the potential effects (Fig. 4H). My design was first divided all cells (10 cells, an example of 10 cells in the field, Fig. 4H2) into 2 groups: PTEN U1 adaptor positive (3 cells) and negative (7 cells), then in each group, those cells were further divided into two groups: cell type marker positive or negative (Fig. 4H1). My target is the percentage of cells taken up U1 adaptor and showing specific cell type marker, U1 (+) marker (+), out of the total cells taken up U1 adaptor (1 cell out of 3). On the other hand, the percentage of cells not taken up PTEN U1 adaptor but
showing a specific cell type marker, U1 (-) marker (+), out of the total cells not taken up U1 adaptor (2 cells out of 7), was served as internal control. By comparing marker positive cells between adaptor positive (33.3%) and negative group (28.6%), I could unmask the effect of U1 adaptor.

After the decision tree analysis, PTEN U1 adaptor decreased the percentage of Tuj1 positive cells (from around 30% to less than 20%) (Fig. 4D) and increased the percentage of GalC positive cells (from around less than 20% to 35%) (Fig. 4G). The percentage of Nestin (50% to 40%) (Fig. 4B) and PSA-NCAM (25% to 20%) (Fig. 4C) were slightly lower, while A2B5 (30%) (Fig. 4E) and GFAP (35%) (Fig. 4F) positive cells remained roughly the same. Notably, when the concentration was low (0 nM or 1 nM), there was almost no U1(+) cells. Therefore, I only focused on the difference between U1(-) internal control and U1(+) above 5 nM. The results suggested that PTEN U1 adaptor produced more neurons and fewer oligodendrocytes. Interestingly, this response was not dose-dependent.

C. Induced pluripotent stem cell (iPSC)-derived NSCs

The human iPSC-NSCs were cultured and transfected with GFP, and several markers were used to identify their fate (Fig. 5). From earlier passage, GFP positive cells were only around 50%, but in the later passage, the GFP percentage increased to almost 100%, indicating the transfection and the continuing neomycin (G418) selection for 2 months was successful (Fig. 5A). Ki67 positive cells were around 50%, suggesting half of the cells were still proliferating. Nestin positive cells were almost 100%, suggesting that these cells
still had properties of NSCs. These NSCs showed 25% PSA-NCAM, 90% Tuj1, 40% A2B5, 40% GalC and almost no GFAP (Fig. 5A). The results showed that human iPSC-NSCs had neuronal potential in culture, with some oligodendroglial but very little astroglial potential (Fig. 5B).

5. Discussion

A. Stages of neurogenesis

It is likely that the secondary neurospheres I cultured already passed the stage of neurogenesis in rats. In development, neural stem cells were first differentiated into neurons, followed by astrocytes, then finally oligodendrocytes. Since the tissues I initially isolated from the P0-P1 rats were not pure NSCs, I passaged two times and purified them by collecting suspended neurospheres and discarding non-wanted attached cells. The time lapse of passaging is around 10 days, and the treatment of lithium or PTEN U1 adaptor lasted for a week. Therefore, when the cells were fixed for staining, they were already around 17 days after isolation. It is possible that after this time, the cells already passed the stage of neurogenesis and had already entered the stage of astrogliosis. However, when I looked up studies using NSCs isolated from neonatal rat SVZ, time frame of two weeks seems very common. Further experiments could be done by fixing NSCs at different time points to examine the percentage of each cell types.

From the study of Lu et al., human embryonic stem cells (H9 ESC line) that were transplanted into immune-compromised RNU rats showed matured neurons at 3 months, then matured astrocytes at 6 months and matured oligodendrocytes at 1 year (Lu et al., 2017). Their results indicate that transplanted cells display
intrinsic human rate of maturation despite of the environment of rat. Although it was done in vivo, it may also be true in vitro.

Intrinsic factors may also contribute to the “imprint” of certain cell types at different stages. Therefore, the cell sources for transplantation could play a key role. Since the NSCs I used were isolated from the brain (SVZ), they may not behave the same as the cells in the spinal cord. Some studies used NSCs derived from embryonic spinal cord (either mouse, rat or human): rat E14 spinal cord (Hwang et al., 2014) (Kadoya et al., 2016), human embryonic/fetal spinal cord derived NSCs such as SPC-01 (Amemori et al., 2013) and NSI-566RSC line (van Gorp et al., 2013) (Kumamaru et al., 2019). The interaction between intrinsic and extrinsic factors would be an extremely important topic to explore.

B. Lithium effect

In Qu’s paper, lithium could increase the number of NRPs and has dose-dependent effect peaked at 3 mM. However, my results did not show increased cell number. On the contrary, when lithium was added, the cell number was decreased by a third without FBS and a quarter with FBS. Qu’s paper found that lithium promoted proliferation but not survival. If this was true, my results of decreased cell number suggested that the rate of proliferation did not surpass the rate of cell death. To my cells, FBS could successfully increase survival.

Zhu’s paper showed that lithium could promote neurogenesis and suppress astrogliosis. In Zhu’s paper, NSCs expressed almost all nestin (97%), some A2B5 (39%) and PSA-NCAM (16%), and very few GFAP (1%), Tuj1 (1%) and GalC (0.5%). This showed the cells were still mostly NSCs or precursor cells (GRPs
and NRPs), and very few differentiated astrocytes, neurons and oligodendrocytes. After transferred to neurobasal medium, the cells expressed more GFAP (42%) and less Tuj1 (32%), indicating more astrocytes than neurons. After treatment, lithium significantly decreased the percentage of A2B5(+) cells and increased PSA-NCAM(+) cells.

In contrast, after transferred to neurobasal, my results showed 55% PSA-NCAM(+) and 60% A2B5(+) cells, and lithium did not alter the percentage much. After 5% FBS was added, although PSA-NCAM(+) and A2B5(+) cells showed a slight increasing trend, neither was significant.

Zhu’s study mentioned that the effect of astrogliosis inhibition was prominent especially when the concentration is higher than 3 mM. The paper noted that there might be a toxic effect when the concentration is higher than 4 mM. However, my results did not show this toxic effect between 1, 2 and 4 mM of lithium.

C. The efficiency and effects of PTEN U1 adaptor

The efficiency of PTEN U1 adaptor could be greatly reduced in the absence of signaling peptides. The original PTEN U1 adaptor designed by Dr. Gunderson was to combine PTEN U1 adaptor oligonucleotides with signaling peptides. The peptides can make the cells internalize the oligonucleotides. When I first tested the PTEN U1 adaptor, I added both the oligonucleotides and peptides. However, in later experiments without adding peptides (after the peptides ran out), I could still see the signals of the oligonucleotides within the cells. Therefore, for the later experiments with multiple concentrations, I did not add any peptides, which could
decrease the efficiency of cells taking up the U1 adaptor. The final result showed that the efficiency could only reach 40% at 100 nM. In order to get higher efficiency, it would be better to either add peptides, or to use traditional transfection techniques such as electroporation or Lipofectamine.

PTEN suppression could promote neuronal cell growth instead of neurogenesis. Our initial design was to promote neurogenesis by suppressing PTEN with PTEN U1 adaptor. However, some studies have shown that inhibiting PTEN promotes cell growth instead of proliferation. Therefore, I could measure the area of cells in addition to counting cell number. The IN Cell Analyzer has the function to automatically measure total area of cells, which would be useful for further investigation.

Interestingly, although I saw the effects of decreased Tuj1 (neurons) and increased GalC (oligodendrocytes), I did not see a dose response. Theoretically, higher concentration would increase intake of the PTEN U1 adaptor, resulting in more PTEN silencing. It might already reach a plateau. To examine the expression levels of the pten gene, further experiments such as RT-qPCR and Western blot could be used to test the amount of RNA from the cell lysate. The results could be compared with that from fluorescence imaging to see the efficiency of silencing.

D. iPSC-NSCs could be a good source for transplantation

My result of about 90% Tuj1(+) neurons were similar to that of Israel’s study in 2012 (Israel et al., 2012). In Israel’s paper, they used FACS to do neuronal differentiation and purification, and got more than 90% neurons
expressing Tuj1 and MAP2. They also used electrophysiological methods to examine the properties of those neurons and found around 40% neurons had spontaneous excitatory or inhibitory synaptic currents. From immunostaining, around 15% of those neurons expressed VGluT1 and 8% expressed GABA. I only did PSA-NCAM and Tuj1 staining to detect NRPs and neurons, so it would be interesting to further investigate whether those Tuj1(+) cells could form functional synapses, either excitatory or inhibitory. My results showed around 0% astrocytes (GFAP), 40% oligodendrocytes (GalC) and 90% neurons. It was intriguing that the combination of astrocytes and oligodendrocytes exceeded 100%. Since these two were supposedly more determined cell types, it was unlikely that there were cells expressing both. Further imaging and analysis need to be down to confirm whether there were indeed that many oligodendrocytes.

The cell source could play a key role affecting treatment. For example, CST regeneration requires caudalized and homotypic neuronal grafts (Kadoya et al., 2016). Kadoya et al found that NSCs isolated from E14 rats had different ability to regenerate CST after cervical (C4) lesion: the cells from the spinal cord performed the best, while cells from the hindbrain were not as good, and cells from the telencephalon failed to help CST regeneration. Although I used neonatal NSCs instead of ENSCs, since my cells were isolated from SVZ, they may not be caudalized enough to perform well in regeneration.

Compared to 30-50% PSA-NCAM and 30-40% Tuj1 positive cells in rat neonatal NSCs despite lithium or PTEN U1 adaptor treatments, Human iPSC-NSCs showed 25% PSA-NCAM and 90% Tuj1 positive cells. It appears that
human iPSCs are better sources for neurons than NSCs isolated from the SVZ of neonatal rats.
Chapter V: Characterization of NSCs in injured LSC

1. Literature review

Studies on transplanting NSCs after SCI were overwhelmingly done in thoracic spinal cord (roughly 70%), with cervical in a lesser degree (roughly 30%), and very little lumbosacral. Therefore, we would like to find a good source of cells to treat LSCI. As mentioned in chapter IV, after rodent NSCs were transplanted into the injured spinal cord, they tended to differentiate into glia (mostly astrocytes) instead of neurons. Therefore, to produce more neurons ready for transplantation, especially to replace lost neurons after LSCI, NSCs would need treatment or induction. However, neither lithium nor pten silencing promoted neurogenesis of rat neonatal NSCs. I found that human iPSCs already showed neuronal potential in culture. Thus, unlike rodent NSCs, human NSCs seemed to behave differently, making them an alternative source for transplantation.

Lu, et al. (Lu et al., 2014) transplanted iPSCs into cervical hemisected spinal cords of Rowett nude (RNU) rats and showed that these cells not only survived but also grew out massive amounts of axons that extended long distances across the lesion site to distal cord and brainstem. The transplants did not produce teratomas.

Our lab has transplanted human umbilical cord blood mononuclear cells (UCB-MNC) into 77 RNU rat after a thoracic spinal cord (T9-T10 vertebral) contusion. 3 rats died after the injury, but none died after transplantation. The RNU rats tolerated spinal cord injury and transplantation well, supporting the feasibility of the proposed experiments to transplant human iPSC-NSCs into these rats. Later in 2016, Kadoya’s
group transplanted human iPSC-NSCs derived from fibroblasts into cervical (C4) SCI RNU rats, and they helped corticospinal regeneration.

Since rats would typically reject human cells, when human cells were transplanted into rats, immune-suppressed drug such as cyclosporin or tacrolimus was traditionally applied. Rowett nude (RNU) rats lack T-lymphocytes, thus are immune-compromised and do not reject transplanted cells from other individuals or species. Therefore, no immunosuppression is required (Wheeler et al., 2014). Although RNU rats do not have T cells, they still have B cells. Thus, they still have B-cell dependent inflammatory response.

2. The study

From the previous chapter, we knew that the human iPSC-NSCs we cultured had neuronal potential, which could possibly replace lost neurons after LSCI. Therefore, we would like to transplant these human iPSC-NSCs into RNU rats after LSCI to see if these cells could survive. If they could, we would examine what kind of cells they become, hopefully differentiate into neurons. We would use different markers for various cell types in the NSC lineage, including NSC, neuron, astrocyte, oligodendrocyte and their precursors.

3. Additional methodology

A. Animals

There were 3 batches of RNU rats being tested. The first batch had 6 animals (3 females and 3 males) and was kept for 8 weeks. 2 weeks after injury, all 6 animals were transplanted with human iPSC-NSCs and perfused 6 weeks
after transplantation. The second batch had 11 animals (original 10 plus 1 replacement), which were all females and kept for 12 weeks. 2 weeks after injury, the rats either received medium (medium injection control) or human iPSC-NSCs (cell transplantation). After that, the animals were kept for another 10 weeks. The third batch had 22 animals (original 20 plus 2 replacements), which were also all females and kept for 12 weeks. 2 weeks after injury, the animals either received no treatment (injury only control), medium (medium injection control) or human iPSC-NSCs (cell transplantation). After that, the animals were kept for another 10 weeks, and then perfused, sectioned and stained.

4. Results

A. Cell transplantation into injured lumbosacral spinal cord

I had transplanted human umbilical cord lining cells (isolated and frozen by Dr. Shao-Yun Hsu and Tzu-Hao Huang) treated with or without cytophosphamine (cultured by Zikuan Leng) into LSCI rats. Although I did not see any positive cells with human nuclei (Millipore #MAB1281) staining in the sagittal sectioned slices, I was able to do cell transplantation into LSCI animals and do frozen sectioning and immunofluorescence staining on the injured spinal cord. No detection of human cells might be due to rejection of the rat.

B. Human iPSC-NSCs survival, migration and differentiation after transplantation

With the human-specific maker STEM 101 and STEM121, the human iPSC-NSCs survived after 6 weeks after transplantation (from the first batch) and 10 weeks after transplantation (from the second and third batch) (Fig. 6). In the
mosaic picture of horizontal section, the middle oval shape was the injury site, and there were several cysts appeared rostrally to the injury site, showing that CSF had accumulated due to the blockage caused by the injury (Fig. 6A1-A2). Surrounding the injury site and along the axis of the spinal cord, there were massive amount of soccer ball-shaped macrophages that had autofluorescence, expressing signals on every channel (yellow part of Fig. 6A1). However, I could still identify the transplanted human cells by their stronger signal, slight different color and smoother shape (pink part of Fig. 6A2), which were very different from the morphology of macrophages (yellow part of Fig. 6A1).

Initially, I would like to visualize the human cells by the GFP we transfected. However, when I co-stained with human-specific antibodies, some human cells did not show GFP signal, suggesting that some transplanted cells had lost their GFP. Therefore, I used two human-specific antibodies, STEM101 and STEM121, to identify the human iPSC-NSCs I transplanted. Human nuclei marker (STEM101) positive cells could be found near the injury site, away the 4 injection points, indicating that these cells have migrated toward the injury site (white arrow in Fig. 6A1-A2). Notably, in one animal, some human nuclei were found in the dorsal roots (white arrowheads in Fig. 6A1-A2 & C1), showing these cells even migrated to the dorsal roots. Moreover, from the human cytoplasm marker (STEM121) positive cells, I could see the processes appear away from the injection sites toward both caudal and rostral ends of the sections (Fig. 6C3-C4). Since the sections were 15-mm long, it suggested that the transplanted cells could grew and extended their processes almost 7.5-mm both caudally and rostrally.
C. Transplanted human iPSC-NSCs became neurons instead of astrocytes or oligodendrocytes.

With markers for different NSC lineage, we could characterize what the transplanted cells had become. I saw some cell clusters near the injection sites (Fig. 6B & 6D), but I could only find one single cell showed proliferation marker (Ki67), suggesting that after 6 weeks (first batch) or 10 weeks (second and third batch) after transplantation, these cells had very little proliferation. For NSC marker (Nestin), the cells near the injection site and injury site showed mostly colocalization with human cytoplasm (STEM121) (Fig. 6C2), but the processes that were farther away from the injection sites and injury site, and did not show nestin (Fig. 6C3-4), suggesting that the cells grew processes toward the rostral and caudal end, and they gradually lost their NSC marker and had more differentiated and mature state. Notably, the cells found in the dorsal roots also showed NSC marker (Fig. 6C1), which were also in vicinity of the injection sites. The transplanted human cell nuclei interestingly lined up along the border of dorsal root, while the cell processes wrapped up like bundles in the dorsal roots.

To examine whether the transplanted cells had differentiated toward neurons, I used several neuronal markers, including Tuj1, NeuN, MAP2 and neurofilament (NF). Human nuclei marker (STEM101) colocalized largely with mature neuronal nuclei marker (NeuN) (Fig. 6D), while human cytoplasm marker (STEM121) colocalized largely with immature neuronal marker (Tuj1) (Fig. 6E1-E2) and mature dendritic neuronal marker (MAP2) (Fig. 6F), but very little with mature axonal neuronal marker (NF) (Fig. 6G). Interestingly, the processes
formed clusters or seemed more disoriented near the injection sites and injury site (Fig. 6C2). However, while the cells were found further away toward the ends, they looked more parallel to each other (Fig. 6C3 and 6E1), as if were following some track. The human cytoplasm marker (STEM121) colocalize very little with astrocyte (GFAP) (Fig. 6H), while the human nuclei marker (STEM101) did not colocalize with oligodendrocyte marker (Olig2) (Fig. 6I). Together, we learn that the transplanted human iPSC-NSCs had differentiated into neurons ranging from immature to mature, with more dendritic than axonal potential, very few differentiated into astrocytes, and did not differentiate into oligodendrocytes.

Since these transplanted human iPSC-NSCs appeared to be mostly neurons, we further examined whether they had formed synapses. The staining results showed that the human cytoplasm marker (STEM121) colocalized partially with the puncta of presynaptic vesicles (synaptophysin) (Fig. 6J1), suggesting that the transplanted cells had formed some synapses. With the feature of Airyscan from Zeiss LSM800 Microscope (Huff, 2015), I could see the synaptophysin puncta in higher resolution. Although there was no exact yellow colocalization between the red puncta (synaptophysin) and green cells (STEM121), the distribution of red was highly correlated with green: the red puncta were most dense where there were green cells (Fig. 6J2-J3). Blue Hoechst(+) cells with green STEM121(+) processes were transplanted human cells, while blue Hoechst cells without green process were RNU rat endogenous host cells. Around the non-green host cells, there were very few red synaptophysin puncta. In contrast, around the green transplanted human cells, there were very dense red synaptophysin puncta. This
indicated that the transplanted cells did form synapses. To further examine whether these synapses are excitatory, I used vesicular glutamate transporter 1 (VGlut1) as the marker. However, human cytoplasm (STEM121) did not colocalize with excitatory neuronal marker (VGlut1), indicating that these neurons did not form functional excitatory synapses (Fig. 6K).

5. Discussion

A. The guidance for cell and process growth

From the results, the transplanted human iPSC-NSCs survived, migrated, differentiated into neurons and grew long processes toward both rostrally and caudally. It was interesting that further away the cells from the injection site, more parallel these cells were to each other, as if they were migrating or growing along a track. It was possible that they were trying to innervate to the host cells, thus growing the same direction as the nerve fiber tracts. The injury site might be abundant with inflammatory factors that repel the transplanted cells, or there might be some gradient of certain growth factors that guided the cells toward specific orientations.

Both time and location of transplantation are important factors to treat LSCI. Li’s group studied NSCs transplantation in thoracic (T9) transected SCI, and found cells transplanted rostral to the injury site performed better than the cells transplanted caudal to the injury (Li et al., 2011). In addition, NSCs transplanted at subacute stage (7 days after injury) performed better than the one transplanted at acute stage (right after injury). They also found that there were more macrophages (CD68) in caudal region at acute stage. Looking at LSCI
studies, most researchers transplanted NSCs 1 week to 2 weeks, confirming that subacute stage is the better time for treatment. For time, I transplanted the cells 2 weeks after injury, which should fall into the subacute stage. Thus, my time point should be ideal. For location, I transplanted the cells at both rostral and caudal surrounding the injury site (2 points rostral and 2 points caudal). Ideally, I hope the cells could grow both upward toward the cerebral cortex and downward to the muscles. I could further examine and compare the number of cell bodies and processes between rostral and caudal direction, and see if the location of injection really matters.

B. **Cell types and synapse formation**

My results showed the iPSC-NSCs mostly differentiate into neurons with very few astrocytes and almost no oligodendrocytes. Compared to Lu’s paper (Lu et al., 2014), which they transplanted human iPSC-NSCs into cervical (C5) injured RNU rats, my results were mostly similar to that of Lu’s: both showed the cells expressed mostly Tuj1 and MAP2, but very little NF, indicating these neurons are more dendritic than axonal. However, since Lu’s paper showed massive axonal regeneration, does this mean that we should see more NF than MAP2 expression? Interestingly, my cells showed very little GFAP expression, while in Lu’s paper there was a lot more.

The transplanted human iPSC-NSCs appeared to differentiate into neurons, and these neurons also seemed to form synapses. I have examined synapses by staining synaptophysin, and have seen partial colocalization, indicating these neurons have formed synapses. However, although I have used the Airy Scan
function in the LSM800 microscope, it is still difficult to identify whether
synaptophysin puncta are really colocalizing with STEM121 (human cytoplasm).
We probably need even higher magnification. Since synaptophysin presents in
synaptic vesicles on the presynaptic side, we could also examine more specific
presynaptic and postsynaptic markers such as Homer and PSD95. Also, it would
be important to see the interaction between host and transplanted cells. For
example, it would be interesting to see whether host rat cells made synapses onto
transplanted human cells, or transplanted human cells made synapses onto host rat
cells, or even between transplanted cells themselves. Maybe the transplanted cells
would tend to be either presynaptic or postsynaptic. To further examine whether
these neurons and synapses were excitatory or inhibitory, I had already tried
VGlut1 as marker for excitatory neurons and found no colocalization. I could
also try other type of excitatory neuronal markers such as VGlut2. In addition to
that, I should also examine inhibitory neurons by using either GAD65 or GD67,
or even other specific neuronal types such as cholinergic or dopaminergic
neurons. However, since these transplanted cells still showed NSC marker near
the injection sites and still showed premature neuronal marker both rostrally and
caudally, they might not be mature enough to express specific functional neuronal
markers.

C. **Cell survival, trophic factors and scaffold**

In this study, I have not quantified cell survival. However, compared to the
pictures from other studies, it seemed that the cell survival and neurite growth was
not as robust. One reason might be because I did not add any additional growth
factors and neurotrophic factors with the cells as the “cocktail” of Lu’s group. Even so, since my cells were resuspended in NSC medium containing EGF, B27 and N2, there were still some protective effects to support transplanted NSCs. The number of types or amount might not be as many or as much as the 9-factor cocktail (or the later 4-factor cocktail), thus my cells did not survive or grow as much as that of Lu’s.

Another reason might be that I did not use any scaffold that some other studies used. There are many different types of scaffolds with materials that could support cell survival and growth. Lu’s group used fibrin matrix combined with growth factors cocktail. Hosseini’s and Dagci’s group used alginate/arginine scaffold (Hosseini et al., 2016). Yuan’s group used double layer collagen membrane (Yuan et al., 2014). More recently, there were some biomimetic 3D-printed scaffolds to treat spinal cord injury (Koffler et al., 2019). Together with NSCs, these scaffolds could support the transplanted NSCs (or neural progenitor cells, NPCs) and regenerate to form new connections (or “relay”) across the spinal cord in rodents. These scaffolds have the shape of the spinal cord lesion cavity (either contused or cut) that can be “personalized” and have an elastic texture similar to that of spinal cord. This method could possibly enhance the survival of transplanted cells.
Chapter VI: Functional recovery

1. Literature review

Human iPSC-NSCs have been transplanted into cervical SCI in RNU rats, and minimum functional recovery was found (Lu et al., 2014).

BBB score is the most popular method to study functional recovery after spinal cord injury in rats. It composed of a 21-point score that examine the movements of hip, knee and ankle, weight support and stepping coordination. It is the most standardized behavioral test for thoracic SCI, and most cervical SCI also used it to examine functional recovery. Other methods have been used: forepaw placement test for cervical SCI (C5) (Lu et al., 2017), plantar test and beam-walking test (Amemori et al., 2013).

However, because the injury level of what we do is L4-L5, which the rats can still walk, the popular BBB score method to measure functional recovery in cervical and thoracic SCI cannot reflect the recovery in LSCI. Wen et al. has established footprint analyses as the method for assessing functional recovery in LSCI (Wen et al., 2015). Wen’s study showed the consistence between footprint analysis, spinal cord sparing (neuronal and fiber loss) and muscle atrophy. This LSCI model would decrease the number of axons and diameter of myelin in peroneal and tibial motoneurons, and atrophy of gastrocnemius (GA) and tibialis anterior (TA) muscles. Therefore, footprint analysis could reflect or predict functional recovery after LSCI.

2. The study

Wen et al. has established the LSCI model and footprint analyses as the functional recovery method in SD rats. However, this model has never been used in
RNU rats. Since I would like to apply this method to examine the effects of human iPSC-NSCs transplanted into RNU rats after LSCI, my first goal was to establish this model and functional recovery method in RNU rats, and my second goal was to assess whether the human IPSC-NSCs transplanted into the LSCI RNU rats can help functional recovery after LSCI.

3. Additional methodology

A. Animals

There were 3 batches of RNU rats being tested. The first batch had 6 animals (3 females and 3 males) and was kept for 8 weeks. 2 weeks after injury, animals were transplanted with human iPSC-NSCs and perfused 6 weeks after transplantation. The second batch had 11 animals (original 10 plus 1 replacement), which were all females and kept for 12 weeks. 2 weeks after injury, the rats either received medium (medium injection control) or human iPSC-NSCs (cell transplantation). After that, the animals were kept for another 10 weeks. The third batch had 22 animals (original 20 plus 2 replacements), which were also all females and kept for 12 weeks. 2 weeks after injury, the animals either received no treatment (injury control), medium (medium injection control) or human iPSC-NSCs (cell transplantation). After that, the animals were kept for another 10 weeks.

B. Static footprint and foot placement

For standing footprint analysis, the rats were put into a transparent container (9.5” x 5.8” x 4.8”L) on top of an acrylic riser and pictures were be taken from
below with additional light source and a ruler. Each rat would have 3 pictures taken each time, every week for all 13 weeks (Week 0 to Week 12). With the pictures, static toe spreading (STS) and static intermediate toe spreading (SITS) were measured with ImageJ. STS is the distance between the first and the fifth toe, while SITS is the distance between the second and the fourth toe. Toe spread factor (TSF) and intermediate toe spread factor (ITSF) were then calculated by normalizing TS and ITS measurements to that at week 0 or week 2: \( \text{TSF} = \frac{\text{ETS} - \text{NTS}}{\text{NTS}} \); \( \text{ITSF} = \frac{\text{EITS} - \text{NITS}}{\text{NITS}} \). Static sciatic index (SSI) was then calculated with the formula: \( \text{SSI} = 108.44 \times \text{TSF} + 31.85 \times \text{ITSF} - 5.49 \). Therefore, the overall equation for SSI was: \( \text{SSI} = 108.44 \times \left[ \frac{\text{ETS} - \text{NTS}}{\text{NTS}} \right] + 31.85 \times \left[ \frac{\text{EITS} - \text{NITS}}{\text{NITS}} \right] - 5.49 \). SSI would be used to evaluate functional recovery.

The same 3 pictures were also used for foot placement. Each foot placement was categorized into either plantar, side or dorsal. Plantar: the paw was facing down and all 5 toes could be seen; side: not all 5 toes could be seen; dorsal: the paw was facing up and all 5 toes could be seen.

C. **Walking footprint**

For walking footprint analyses, a piece of white paper was placed on the floor of a polypropylene (PP) corridor (inner: 10 cm x 12 cm x 122 cm; outer: 12 cm x 13 cm x 122 cm; PP thickness: 1 cm). The hind limbs of the rat were painted with ink (right foot: black; left foot: red) and the rats were put on one end of the corridor, and encouraged to walk to the other end with a towel covering the corridor to create a shade that the rat would prefer to walk toward. The footprints
on the paper were then measured footprint length (FL), toe spreading (TS), intermediate toe spreading (ITS) and calculated sciatic function index (SFI).

4. Results

A. Batches and animal conditions

There were three batches of animals tested (Fig. 7). The first batch had 6 animals (3 females and 3 males) and was kept for 8 weeks, but only 5 left because one male was perfused early (or died) at day 6. They were all injured animals transplanted with human iPSC-NSCs. The male rats became ill and developed blocked bladder. After dissection, we found sperm plugs at the sphincter of bladder. Thus, we were able to collect 5 samples of animals with cell transplantation.

The second batch had 11 animals (original 10 plus 1 replacement), which were all females and kept for 12 weeks, but 3 were perfused early or died at week 3 (cell transplanted), 5 (medium injected) and 9 (medium injected). Therefore, the final number of samples collected was 3 in the medium group and 5 in the cell group, which made a total of 8.

The third batch had 22 animals (original 20 plus 2 replacements), which were all females and kept for 12 weeks, but 6 were perfused early or died at 2 (cell transplanted), 4 (medium injected), 6 (injury only), 7 (cell transplanted), 8 (injury only) and 11 (cell transplanted). As a result, the final number of samples collected was 3 in the injury only group, 8 in the medium group and 5 in the cell group, which made a total of 16. If we combine the second the third batch, which
had the same length of observation, then the total number will be 3 in the injury only group, 11 in the medium group and 10 in the cell group.

In the first and second batches, many animals had autotomy (which they bit their toes) (Fig. 7A, 7C1 & 7C2). When this happened, we treated them with Tylenol daily; however, if it did not help and the animals were not responsive, we had to perfuse the animals earlier than planned. Coincidently, these animals could not walk with proper stepping and all had dorsal dragging (Fig. 7C1). I noticed that this dragging was mostly asymmetric (Fig. 7A, 7C1, 7C3 & 7C4), and a “cross-leg” posture could be seen (Fig. 7C2).

In the second and third batches, many animals could walk and developed callus on their heels (Fig. 7B1). Many of the calluses grew worse and formed ulcer-like tissues and started swelling and bleeding (Fig. 7B2). We gave them Tylenol daily and applied topic antibiotics ointment. It is unknown whether callus would affect the animal’s walking. I noticed that while the callus was getting more serious possibly due to the heel keeping rubbing the ground, the toes were elevated from the ground. This might affect the accuracy of the toe spread we got. Most of the animals in this batch could walk (7D1-3).

Although I planned to do T13-L1 injury, when I dissected the samples after perfusion and checked the injection location with laminectomy, I found that I did some L1-L2 injury mainly in the third batch. It appeared that T13-L1 animals were non-walkers with autotomy, while L1-L2 animals were walkers with calluses. In the T13-L1 group, the autotomy started around 2 weeks after
injury, and increased gradually throughout time, reaching 30% at 12 weeks (Fig. 7E). In contrast, the L1-L2 group had zero autotomy.

B. Foot placement

Since not all animals had plantar foot placement, I characterized foot placement into three categories: plantar, side and dorsal. When mapping with the injury location of laminectomy (T13-L1 versus L1-L2), the patterns of foot placement were very different between T13-L1 and L1-L2 LSCI rats: T13-L1 animals changed to either side or dorsal after injury (Fig. 7F), while L1-L2 animals remained plantar placement after injury (Fig. 7G). Some L1-L2 animals changed to side placement 1 week after injury, but they all returned to plantar later. Therefore, for my functional recovery data, I would analyze these two distinct populations separately.

C. Static footprint

For static footprint analyses, toe spread (TS) and intermediate toe spread (ITS) were measured and sciatic index (SSI) was calculated (Fig. 8). SSI normalized to week 0 was compared between different treatment groups (Fig. 8A). Although there is no significant difference, there was a surprisingly trend that the injury only group had higher SSI than the medium injection group, and event higher than the cell transplantation group. However, since the laminectomy check revealed two types of injury location, I would like to examine T13-L1 and L1-L2 LSCI separately. The problem was that some former perfused animals were not checked thoroughly, resulting in not enough animals in each group. Therefore, based on the foot placement pattern I found in determined animals
(Fig. 7F-7G), I assigned the undetermined animals with either T13-L1 or L1-L2 injury retrogradely (post-hoc). Then I separately the SSI data into T13-L1 (Fig. 8B) and L1-L2 (Fig. 8C). In the T13-L1 group, injury only and medium did not show difference, but medium group had significantly higher SSI than the cell group after week 6 (with an exception on week 8). In the L1-L2 group, there was no significant difference between any groups. The medium and cell group were very close to each other, while the injury only group appeared a little bit higher SSI.

I noticed that in the T13-L1 group (Fig. 8B), the injury only group showed higher SSI even at week 2 before transplantation. Therefore, I wanted to calculate a new SSI that normalized to week 2 to see the effect of treatment instead of the original SSI that normalized to week 0 (that reflect the effect of the injury itself). Unlike the SSI normalized to week 0, the SSI normalized to week 2 showed that the medium group had the highest SSI (Fig. 8D). However, since there were two locations of injury, the data were separated by the retrogradely (post-hoc) assigned T13-L1 (Fig. 8E) and L1-L2 (Fig. 8F) groups. In the T13-L1 group, there was no difference between the medium and cell groups, but they both had higher SSI than the injury only group at week 5. In the L1-L2 group, the three groups were generally not significantly different to each other, but injury only group had higher SSI than the cell group at week 4 and 12.

The results suggested that medium and cells increased SSI in T13-L1 LSCI, but not in L1-L2 LSCI. The cells might even lower the SSI in L1-L2 LSCI.

5. Discussion
A. Bladder problem in male RNU rats after LSCI

I have done LSCI on male SD rats to confirm the problem Wen faced when establishing the LSCI model: male rats tend to have clotted bladder and could not survive after one week. 3 out of these 7 rats died after a week, possibly due to clotted bladders. From the dissection of these rats, we found sperm plugs in the sphincters of bladder. Therefore, later on, we only used female SD rats.

Similar to what was found in the male SD rats, male RNU rats also had blocked bladder after LSCI. In a separate project done in our lab by Hadi and Sonia, it was suggested that LSCI made the seminal vesicles secrete excessive semen, forming the semen plug that blocked the sphincter of the bladder. However, removing seminal vesicles means extra work of surgery besides LSCI. Therefore, to simplify the procedure, it is easier to use female rats. Notably, in our 38 female RNU rats, there was one female also had bladder problem: we needed to perfuse the rat at week 6 because the bladder could not be manually expressed (and the bladder accumulated about 12 mL of urine). Since female do not have seminal vesicles, I do not know what the cause of this blockage was.

B. T13-L1 versus L1-L2 LSCI

According to the laminectomy and foot placement results, the animals could be divided into either T13-L1 or L1-L2 groups. T13-L1 animals had mixtures of different types of stance and none of them could not walk with proper stepping, while L1-L2 animals had consistently plantar stance (with a few exceptions with temporarily side stance) and all could walk. This result is very different from the LSCI model paper. The model paper said the injury was T13-L1 and most of the
animals could walk. Further experiment such as retrograde tracing is needed to confirm where L4-L5 spinal cord actually locates in RNU rats. Even so, we could still learn from the results that one segment shift will cause a huge difference in behavior. As mentioned in the introduction section (Chapter I), 10 lumbosacral spinal cord segments (L1-S5) are squeezed into 3 vertebral segments of T11, T12, and L1, it is not surprising that 1 segment shift can make a huge difference.

Autotomy rates were distinctively different between T13-L1 and L1-L2 animals. T13-L1 autotomy started around 2 weeks and reached to 30% autotomy rate, while L1-L2 animals had none. 30% of the T13-L1 rats needed to be sacrificed earlier than planned, greatly decreasing data points. Thus, T13-L1 may not be a good model for LSCI before future researchers solve this problem. Since autotomy in rat is similar to neuropathic pain in human, researchers need to find treatments other than just Tylenol to control the symptoms to preserve the sample size of LSCI animals. Therefore, L1-L2 is a better model for LSCI in rats for now.

C. The problem of toe spread

The toe spread we measured might not reflect the real toe spread of the animal. While handling the first batch, we used a 4L beaker as the chamber for static footprint. Since the bottom of the beaker was not big enough to let the rat to freely put their feet touching the ground simultaneous, and the walls were too high and the rats liked to place their forelimbs to the wall in a standing pose, the measurement we got might not reflect the real toe spread when the four limbs were all touching the ground. Also, since there was a curve on the edge close to the bottom of the beaker, some part of the animal’s feet was not placing the flat
surface of the bottom of the beaker, the measurements we got might not be correct, either. Later, we substituted the beaker with acrylic chamber, which was a much better tool.

In the second and the third batch, we obtained a bigger chamber, but the toe spread might not be measured correctly still. For non-walkers, some of them had their feet facing the sides or dorsal dragging, making the toe spread either invisible or not reflecting the real length. For walkers, like mentioned before, had calluses and had elevated toes away from the ground, which would also make toe spread measurement unreliable.

An alternative way to depict the behavior without measuring the toe spread is the count of different types of foot placement. I categorized the position of the hindlimb of the animal into plantar, side or dorsal. For T13-L1 animals (non-walkers), The error bars of the percentage for each category was huge. It was probably because I only took 3 pictures from each rat (and the percentage was either 0%, 33% 66% or 100%). If I increase to 5 pictures (or even 10 pictures), I could get more different values of percentage (0%, 20%, 40% 60%, 80% or 100%), then I may decrease the error bars. For non-walkers, maybe some adjustments of the traditional BBB score for thoracic SCI could also work. The BBB score looks at movements of all three joints (hip, knee and ankle). We could modify the BBB score by only focusing on knee movements, then we would be able to tell the recovery of rats with T13-L1 injury. For walkers, further experiments could be done with video recording and analysis (such as DigiGait
and Motion Sequencing) to examine animal behavior in addition to toe spread, like the length of gait or frequency.

Nerves and muscles will be other places to be examined indirectly to behavior. Priya and I have collected gastrocnemius (GA) and tibialis anterior (TA) muscles of the third batch, and we plan to do paraffin sectioning and hematoxylin and eosin (H&E) staining to see how much atrophy was caused by the injury. We have also collected some of the sciatic nerves. Currently I could only find the mapping of human spinal cord to nerve and muscle, but not the mapping for rats. By comparing the location of laminectomy and muscle atrophy, we could better match spinal cord and the corresponding nerve and muscle in RNU rats.

D. Other types of functional tests

There are very few LSCI studies, and van Gorp’s group is one of them. They did LSCI on T13 vertebral segment (L3 spinal cord) in SD rats transplanted with human fetal spinal cord-deprived NSC, and used open field, gait (CatWalk), inclined ladder, and single frame tests for functional recovery (van Gorp et al., 2013). From my results, T13-L1 LSCI rats could not walk, while L1-L2 LSCI rats could walk. I found T13-L1 to have severe atrophy, and it is hard to tell what functional test can be suitable for them besides histological tests mentioned earlier. For L1-L2 LSCI rats, since they could walk, footprint analysis with toe spread measurement would be a good way. However, there are other information in addition to toe spread that could be examined, such as gait that the van Gorp’s group have used. Other than that, it is important to identify which segment of
lumbosacral spinal cord is the target, and what specific type of functional damage it causes. Since rat’s spinal cord is still different from human’s, the proper mapping of segment and function is key to determine which functional test to use. Here I divided the foot placement positions into plantar, side and dorsal, but there might be better ways to show functional recovery in LSCI.

E. Stimulation or exercise might be needed for functional recovery

From our results, it seems that cell transplantation itself might not be enough to achieve functional recovery, and it might even hinder the recovery. Cell toxicity might be one of the reasons why the cell group had the worst toe spread. We could perform apoptotic assays such as terminal deoxynucleotidyl transferase dUTP nick end labeling staining (TUNEL) staining to see whether it was the case. It was possible that although the cells survived, migrated and grew long processes, but they did not have the proper guidance about where to innervate into and what correct host cells to connect to form the right synapses and neuromuscular junctions. They might even compete and disrupt the existing host connections. About the performance of the medium group, some studies have suggested that since the medium contained many nutrients and growth factors, it might be beneficial alone already, even without any cells.

In order to give transplanted cells the proper guiding signals, exercise or electrical stimulation could be possible solutions. Clinically, walking exercise and electrical stimulations have been found beneficial to injured patients. Future studies such as giving animals treadmills or other exercising equipment would be promising to obtain better functional recovery. Hwang’s group did treadmill
training on T9-contused SD rats transplanted with E14 SD rat spinal cord-derived NSCs for 9 weeks, and found treadmill training helped functional recovery (Hwang et al., 2014).

For human, devices such as exoskeletons could promote better recovery. Device training with exoskeleton to assist mobility has shown positive results (Platz et al., 2016). Electrical stimulation can help motor recovery (Young, 2015).

F. Timeline of recovery

The time frame of observation after injury and transplantation might be an important factor, too. Like mentioned earlier, the intrinsic factors from the donor cell could influence the time course of cell type and maturation (neurons first, and then astrocytes and oligodendrocytes). It depends on where the cells are from: mouse, rat or human. Because of different life span between species, human cells mature slower than that of mouse and rat. The life span ratio between human and rat is around 34.8:1, so one month in human is comparable to one day in rats (Sengupta, 2013). We may need to wait a lot longer for the transplanted NSCs become mature and functional neurons.

Even the cells survived, migrated, differentiated and matured, they also need time to make the right connections (synapses and neuromuscular junctions). This might take an extremely long time, especially for human cells, such as the human iPSC-NSCs we used. Lu’s group examined C5-hemisectioned RNU rats transplanted with human ESC-derived NSC (H9 ESC line) at different time points: 1, 3, 6, 12 and 18 months. Their results show that human cells require a long time for maturation (Lu et al., 2017). Therefore, since we only kept our
animals for 3 months, there might not be enough time for the cells to mature and connect properly.

So far, most SCI studies on rats only kept animals for 4 weeks to 12 weeks, with the majority of 7 to 9 weeks. This might be enough if the cells were from mice or rats, but not enough for cells from human. Also, if considering the distance that the newly differentiated need to migrate and make connections, the growing rate is relatively very slow. For astrocytes differentiated from ESC, the growing/migration speed was 2-3 mm per month. Neurons might also fall into that range. The neurons need to grow long distance: up to the brain to form cortical tract and down to innervate the muscles. To innervate the muscles, the transplanted cells will need to grow more than many centimeters, thus will probably require years to reach.

Although the human iPSC-NSCs did not help functional recovery 10 weeks after transplantation, I have established the LSCI model in RNU rats and found that these cells are a good source for transplantation. We established the first step for treating LSCI.
## Table

### 1. Primary antibodies

**Table 1.** Information of primary antibodies.

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Figures

1. Rat neonatal NSCs survived and migrated in uninjured LSC

Figure 1. Rat neonatal NSCs survived and migrated after transplanted into uninjured LSC. Rat neonatal (P1) NSCs isolated from GFP F344 rats were transplanted into WT F344 rats. Mosaic overlay of GFAP (red) and GFP (green) on horizontal sections (A) 1 week, (B) 4 weeks and (C) 7 weeks after transplantation. Left: rostral; right: caudal.
2. Rat neonatal NSCs differentiated and grew long processes
**Figure 2.** Rat neonatal NSCs transplanted into uninjured LSC expressed markers for NSCs and astrocytes but not for neurons. (A) Overlay of Nestin (red), GFP (green) and Hoechst (blue) 1 week (A1) and 7 weeks (A2-A4) after transplantation. (B) Overlay of PSA-NCAM (red) and GFP (green) 4 weeks after transplantation. (C) Overlay of Tuj1 (red) and GFP (green) 1 week (C1) and 7 weeks (C2-C3) after transplantation. (D) Overlay of neurofilament (red) and GFP (green) 1 week after transplantation. (E) Overlay of GFAP (red) and GFP (green) 1 week (E1), 4 weeks (E2) and 7 weeks (E3) after transplantation.
3. Rat neonatal NSCs in culture with lithium

A. Total cell count

B. Nestin%

C. PSA-NCAM%

D. Tuj1%

E. A2B5%

F. GFAP%

G. GalC%
**Figure 3.** The effects of lithium on rat neonatal NSCs in culture. (A) Total cell count. The percentage of (B) Nestin, (C) PSA-NCAM, (D) Tuj1, (E) A2B5, (F) GFAP, and (G) GalC positive cells under different concentration of lithium (0, 1, 2, and 4 mM). 0 mM was as non-treated control. Left panel of each marker: without FBS; right panel of each marker: with 5% FBS.
4. Rat neonatal NSCs in culture with PTEN U1 adaptor

A. U1 adaptor (+)

B. Nestin (+)

C. PSA-NCAM (+)

D. Tuj1 (+)

E. A2B5 (+)

F. GFAP (+)

G. GalC (+)
Figure 4. The effects of PTEN U1 adaptor on rat neonatal NSCs in culture. (A) The percentage of PTEN U1 adaptor positive cells in different concentration of treatment in culture. The percentage of (B) Nestin, (C) PSA-NCAM, (D) Tuj1, (E) A2B5, (F) GFAP, and (G) GalC positive cells among PTEN U1 adaptor positive or negative cells after decision tree analysis. U1 (-) indicates PTEN U1 adaptor negative cells as internal control U1(-)Marker(+), while U1 (+) indicates PTEN adaptor positive cells U1(+). Marker(+). (H) Diagram of decision tree analysis for a marker. H1, decision tree; H2, an example field with 10 cells under the microscope. One circle indicates one cell. Red circles: U1(+) cells; filled green circles: Marker(+) cells. All cells are first divided into U1(+) or U1(-), then each are further divided into Marker(+) or Marker(-). U1(-)Marker(-) will be the internal control, as shown in (B) to (G) as U1(-), while U1(+). Marker(+) is the target, as shown in (B) to (G) as U1(+).
5. Human iPSC-NSCs in culture

**Figure 5.** Human iPSC-NSCs showed neuronal potential in culture. (A) Percentage of positive cells of markers for different cell types. (B) Diagram of NSC lineage and summary of proliferation and differentiation with markers for each cell type.
6. Human iPSC-NSCs transplanted into injured RNU LSC

![Image of cells and markers]
**Figure 6.** Survival, migration and characterization of human iPSC-NSCs after transplanted into RNU rats after LSCI. (A) Mosaic image of horizontal LSC section. Left: rostral; right: caudal. A1: red, green and blue channels. The same signals showing on all three (red, green and blue) channels are autofluorescent macrophages. Macrophages (yellow overlay) are abundant around the injury site (the middle) and formed lines both rostrally and caudally. A2: red and blue channels. Red channel is enhanced to show the color difference between human cells and autofluorescent macrophages (red overlay). Human iPSC-NSCs are pink. The iPSC-NSCs appeared in the center (white arrow) and some entered the dorsal roots (white arrowheads). The 4 asterisks indicate the 4 injection sites. (B) Ki67 signals. Cell cluster near the injection sites (C) Human specific Nestin signals. C1, dorsal root: transplanted human cells (STEM101) formed bundles and expressed Nestin; C2, near the injection site: human cells (STEM121) and showed Nestin; C3, midway rostrally: human cell processes (STEM121) lined up parallel to each other and showed no Nestin; C4, near the rostral end of the section: the process has no Nestin signal. (D) NeuN signals of a cell cluster near the injection site. (E) Tuj1 signals. E1, near the injection site; E2, midway away from the injection site rostrally. Both showed strong colocalization between Tuj1 and STEM121. (F) MAP2 expression with strong colocalization with STEM121. (G) Neurofilament expression with very little colocalization (only one process) with STEM121. (H) GFAP colocalized very little with STEM121. (I) Olig2 did not colocalize with STEM101. (J) Overlay of synaptophysin and STEM121 near the injection site. J1: regular confocal setting; J2-J3: Airyscan setting. (K) VGluT1 and STEM121 near the injection site.
7. Animal conditions after LSCI

![Images of animals](A, B1, B2, C1, C2, C3, C4, D1, D2, D3)

![Graph showing autotomy progression](E)

- **T13-L1**
- **L1-L2**
Figure 7. Animal conditions after LSCI. (A) Animals with injury at T13-L1 had autotomy with plantar and dorsal foot placement. (B) Animals with injury at L1-L2 had callus with plantar foot placement. B1, at early stage; B2, at late stage. (C) Animals with T13-L1 LSCI showed dragging without stepping or walking. C1, dorsal and side foot placement; C2, dorsal foot placement on both feet; C3, side and plantar foot placement; C4, plantar and dorsal foot placement. (D) Animals with plantar placement on both feet that can walk. D1, parallel feet placement with shorter toe spread; D2, parallel feet placement with longer toe spread; D3, inward feet placement. (E) Autotomy rate throughout the 12 weeks after injury. (F) Percentage of
each type of foot placement throughout time in animals with T13-L1 LSCI. (G)

Percentage of each type of foot placement throughout time in animals with L1-L2 LSCI.
8. Human iPSC-NSCs transplanted RNU static footprint analysis

A. SSI (0)

B. SSI (0) (T13-L1)

C. SSI (0) (L1-L2)
D

SSI (2)

Index

Week

E

SSI (2) (T13-L1)

Index

Week

F

SSI (2) (L1-L2)

Index

Week
**Figure 8.** Static sciatic index (SSI) as functional recovery analysis of human iPSC-NSCs transplantation into LSCI RNU rats. (A-C) SSI normalized to week 0. A, from all animals with different treatments (injury only, medium injection or cell transplantation); B, from animals with post-hoc T13-L1 laminectomy; C, from animals with post-hoc L1-L2 laminectomy. (D-F) SSI normalized to week 2. D, from all animals with different treatments; E, from animals with post-hoc T13-L1 laminectomy; F, from animals with post-hoc L1-L2 laminectomy.
**Conclusion**

Rat neonatal NSCs isolated from the SVZ could survive, migrate, and grew processes, but they remained as NSCs or differentiate into astrocytes instead of neurons. Neither lithium nor PTEN suppression could promote neurogenesis of these cells. On the other hand, human iPSC-NSCs could survive, migrate, differentiate to neurons, grow long processes and make synapses in RNU rats after LSCI. Although the transplanted human iPSC-NSCs did not help functional recovery, they are a better source to replace damaged or lost neurons. We have established the first step of treatment for LSCI.
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References


