GSX1 PROMOTES PROLIFERATION OF NEURAL STEM CELLS AND
INFLUENCES DIFFERENTIATION TOWARDS NEURONAL CELL FATES IN VITRO

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ABSTRACT OF THE THESIS

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Spinal cord injury effects over 300,000 people in the U.S. and the prevalence increases each year by 17,000 cases. SCI causes the formation of a complex injury environment due to the initial impact and secondary signaling cascades. Currently, there are no cures for SCI and treatments focus on decreasing inflammation and using surgical decompression to limit the spread of injury. The loss of neuron function in the spinal cord causes paralysis and highlights neurogenesis as a key factor in promoting recovery. Endogenous cell responses during SCI have suggested various cell populations as potential targets for new therapeutics. The recent advances in gene therapy are promising for introducing transcription factors into the cells after SCI to promote growth and recovery. Transcription factors Gsx1 and Nkx6.1 have been used in previous studies by our lab due to their role in spinal cord development. Transduction of Gsx1 in mouse models of SCI has been shown to promote functional recovery, while Nkx6.1 did not promote functional recovery. In this study, a neural stem cell culture model was used to further determine the effect of Gsx1 on a stem cell population. Here, we show that Gsx1 transduction increased proliferation in mouse and human stem cell culture models. Lentivirus mediated transduction of Gsx1 followed by a 14-day differentiation of NSCs resulted in increased Map2 positive neurons and decreased GFAP positive astrocytes. Control of gene expression was increased by cloning into a TetON inducible lentivirus vector that can be induced by doxycycline. This study shows the inducible lentivirus expressing Gsx1 also
increased the percentage of Map2 positive neurons while decreasing GFAP positive astrocytes in the NSC differentiation model. Gsx1 expression increased proliferation marker Ki67 in mouse and human stem cells while the transcription factor Nkx6.1 did not significantly alter proliferation in either cell line. The inducible lentivirus allowed for easier transduction and selection of cells in culture and created stable transduced cell lines for future studies. This study supports the role of Gsx1 in promoting neuronal cell fate and the potential for Gsx1 gene therapy for spinal cord injury.
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Chapter 1

Introduction

1.1 Spinal Cord Injury

Spinal cord injury (SCI) effects approximately 300,000 people in the United States [1]. The incidence of SCI grows each year in the US by an estimated 17,000 people. Major causes for SCI include vehicle crashes, falls, violence, and sports injuries [1]. Spinal cord injury can be categorized into primary and secondary injury. Primary injury is due to the mechanical trauma that compresses or impacts the spinal cord. Secondary injury includes the resulting cell necrosis, blood spinal cord barrier damage, ischemia, and a complex inflammatory response [2, 3]. SCI stimulates proliferation of astrocytes to create a scar border around the lesion [4]. The astrocyte scar border creates a barrier for axon regrowth after injury. Production of inhibitory extracellular matrix by reactive astrocytes also contributes to the negative growth environment after injury [5]. Inflammatory molecules and chondroitin sulfate proteoglycans are upregulated to block regeneration of neurons in the glial scar [5, 6]. While there are many negative aspects of the glial scar, there are some benefits during acute stages of injury [7]. The scar border limits the spread of injury and separates spared neural tissue from the inflammation at the lesion core [4]. SCI also disrupts the balance of excitatory and inhibitory interneurons which creates a barrier to neuron integration [8]. This complex injury environment created a major challenge for treating SCI. Current treatments for SCI focus on decreasing inflammation with medication or surgical methods to decompress the spinal column [9]. These treat some symptoms of SCI but do not repair the injury or stimulate the growth of new neurons after injury.

1.2 Neurogenesis After Spinal Cord Injury

Neurogenesis in adult mammals can occur in neurogenic regions of the brain under normal conditions while the spinal cord is non-neurogenic under normal conditions [10, 11]. Endogenous neural precursors have been induced in situ to differentiate into mature neurons in the neocortex of adult mice [10]. After
SCI, endogenous cell division occurs which mainly develop into oligodendrocytes and astrocytes [12]. The generation of new cells after SCI has been targeted as a potential recovery mechanism if the cell fates could be directed to a neuronal fate. A recent study by Tai et al. used SOX2 to reprogram NG2 glia into neurons to promote functional recovery in animal models [13]. While previous studies have shown proliferation of astrocytes and NG2 glia after SCI, other studies have suggested the proliferation of neural progenitors [14]. Neural progenitors have been shown in the periventricular and parenchyma regions of the spinal cord and were able to proliferate in vivo after spinal cord injury [14]. This highlights the potential role of endogenous neural stem/progenitor cells in SCI repair.

1.3 Transcription Factors for Neural Development

Neural stem/progenitor cell function and development can be influenced by the expression of transcription factors. Transcription factors Genomic screened homeobox1 (Gsx1 or Gsh1) and NK6 homeobox 1 (Nkx6.1) have been previously shown to regulate neurogenesis during development [15-18]. Development of the neural tube utilizes morphogens to pattern neural progenitors and drives the expression of specific transcription factors [19]. Gsx1 is expressed in the early and late neurogenic phases in the dorsal spinal cord [19]. Gsx1 has been shown to control the inhibitory and excitatory cell fate of interneuron progenitors through interactions with a basic helix-loop-helix transcription factor Ascl1 [17]. Previous studies by our lab have shown that Gsx1 expression upregulates Notch, Nanog, and WNT signaling pathways [20]. Notch 1 has been shown to promote proliferation in the adult mouse spinal cord [21]. Nanog and WNT signaling pathways are involved in maintaining stem cell phenotypes [22, 23]. During development, class B progenitor cells that express Gsx1/2 mature into excitatory neuronal fates [19]. Gsx1 expression also inhibits oligodendroglia specification in the telencephalon and promotes telencephalic progenitors to a mature neuronal fate [15, 16]. Studies with the Gsx1 homolog Gsx2 have suggested Gsx2 controls neurogenesis after injury in the adult subventricular zone after injury, highlighting the potential use of Gsx transcription factors for therapeutic use in the nervous system [24].
Transcription factor Nkx6.1 is expressed in the ventral spinal cord and plays a role in neural patterning and neurogenesis [18, 25, 26]. Nkx.61 promotes MN and V2 interneuron generation in the developing ventral neural tube [26]. The role of Nkx6.1 extends into astrocyte development in the ventral spinal cord by controlling specification and maturation of astrocytes [18]. Previous studies by our lab have shown Nkx6.1 interacting with Notch1 to regulate expression of Notch signaling [27]. Notch has a significant role in spinal cord development and ability for neural stem cells to self-renew and control differentiation between neuronal and glial fates [28].

Previous studies in our lab have suggested the use of Gsx1 to promote functional recovery after SCI [20]. Nkx6.1 was also tested and but did not promote functional recovery. A lentiviral vector was used to transduce Gsx1 to the injury site and promoted proliferation, increased neural stem progenitor cells, increased the number of glutamatergic and cholinergic interneurons, and attenuated glial scar formation [20]. The previous work suggests Gsx1 may act through influencing endogenous neural stem/progenitor cells. The cytomegalovirus (CMV) promoter used targets a broad spectrum of cell types and not specifically neural stem cells in the spinal cord models previously studied. Therefore, a cell culture model of neural stem cell response to Gsx1 expression was used to further test the function of Gsx1.

1.4 Neural Stem Cells

Neural stem cells (NSCs) are capable of proliferation and differentiation into neurons, astrocytes, and oligodendrocytes. This capability makes NSCs a useful model for studying the influence of factors throughout the differentiation process. The mouse neuroectodermal cell line NE-4C was used as a neural stem cell population in this study. The NE-4C cell line was established through the immortalization of early embryonic forebrain vesicles by p53 deficiency [29]. NE-4C cells have been established as an in vitro model of neural stem cell growth and neuron production [30], [31], [32]. Differentiation of NE-4C induced by retinoic acid has been shown to result in approximately 57% GABAergic neurons and 44% glutamatergic neurons while neurotransmitter phenotypes catecholaminergic and serotonergic were not
detected [33]. NE-4C cells therefore make a suitable model for testing the effect of Gsx1 on these neurotransmitter phenotypes. NE-4C cells can further differentiate into astrocytes after extended culture time, creating a mixed population of neuron and glia on top of a basal layer of cells [30].

SH-SY5Y cells are a human neuroblastoma cell line that was originally derived from a human bone tumor biopsy [34]. The use of a human cell line increases relevance of the results for clinical applications and could confirm mechanisms between the mouse and human studies. SH-SY5Y have seen widespread use with many studies differentiating the cells into dopaminergic neurons for Parkinson’s disease research [35]. The undifferentiated SH-SY5Y grow as both adherent and floating cells. These cells have been shown to proliferate continuously and express immature neuronal markers [36].

1.5 Current Study

In the current study, a population of neural stem cells in cell culture were transduced with Gsx1 to further evaluate the effects on Gsx1 on NSC proliferation and differentiation. In vitro neural stem cell differentiation induced by retinoic acid resulted in significantly higher percentages of neurons in Gsx1 transduced cells and significantly reduced the percentage of astrocytes in Gsx1 transduced cells compared to control cells. Mature neuronal populations did not have significant changes in GABAergic or glutamatergic neuron subtypes in this study. An inducible TetON lentivirus vector was created with to increase control over Gsx1 expression in vitro and create stable transduced cell lines for future work. NSC differentiation with the inducible TetON lentivirus expressing Gsx1 also increased the percentage of neuronal cells while decreasing the percentage of astrocytes. Gsx1 promotes proliferation in both mouse and human neural stem cell populations. This study contributes to data supporting the role of Gsx1 in promoting neurogenesis in neural stem cells and the potential for therapeutic use in SCI treatment.
Chapter 2

Methods

2.1 Cell Culture

NE4C

NE4C (CRL-2925) were maintained in Eagle’s Minimum Essential Medium with L-Glutamine (EMEM, ATCC 30-2003) supplemented with 10% fetal bovine serum (FBS), 2mM GlutaMax (Gibco GlutaMax 100x), and 1% pen-strep at 37 °C temperature with 5% CO₂. Sub-confluent cultures were split using TrypLE Express (Gibco) diluted 4-fold with 1X Phosphate-Buffered Saline (PBS) and transferred into poly-L-lysine (PLL) coated dishes. Dishes were coated with PLL for 15 minutes at room temperature and allowed to dry for at least 2 hours before plating cells.

For neural differentiation, NE-4C were cultured in EMEM 5% FBS with 2mM GlutaMax and 1% pen-strep. Retinoic acid (RA) was used to induce neuron formation. RA treatment (10⁻⁷M) was added in media every other day starting one day after plating NE-4C (Day in Vitro 0, DIV0) and continuing until DIV8. RA was then removed and NE-4C culture was continued until DIV14.

For lentivirus transduction, NE-4C cells were seeded one day prior to transduction in a PLL coated 24-well plate. NE-4C were coated with polybrene (4μg/mL) in EMEM + GlutaMax for 1 hour at 37°C with 5% CO₂. Lentivirus was then added to cells and incubated overnight. After incubation, media with virus was removed and replaced with EMEM + GlutaMax supplemented with 5% FBS, 1% pen-strep, and 2mM GlutaMax. The next day cells were checked for GFP expression and transduced cells were selected using 0.5μg/mL puromycin for 48-hours. NE-4C culture media was refreshed and cells were expanded for one day before plating. NE-4C were plated at 50,000 cells per well in PLL coated 24-well plates in EMEM supplemented with 5% FBS, 1% pen-strep, 2mM GlutaMax, and RA treatment (10⁻⁷M). NE-4C cells were then cultured according to the neural differentiation protocol described previously.
NE-4C cells transduced with the inducible lentivirus were seeded one day before adding virus in PLL coated 24-well plates in EMEM supplemented with 5% FBS, 1% pen-strep, and 2mM GlutaMax. The day of transduction, NE-4C were coated with polybrene (4µg/mL) for 1 hour before adding virus. Lentivirus was added and cells were incubated overnight at 37°C. Media was then refreshed with standard culture media. One well of cells transduced with each virus were tested for inducible GFP expression by adding 1 µg/mL of doxycycline to the culture media. Cells were checked for GFP expression the next day and were observed to have minimal GFP expression. Cells were then selected with 0.5 µg/mL puromycin for 48-hours. NE-4C were then transduced a second time to increase GFP expression following the previously described method. After the second transduction, cells were selected again with 0.5 µg/mL puromycin before expanding for use.

Proliferation staining: NE-4C cells were seeded only poly-L-lysine coated plates in EMEM 5%FBS 1% p/s with 2 mM GlutaMax. The next day DOX (1µg/ml) was added to wells to induce gene expression. After 24 and 48-hour DOX exposure timepoints, cells were fixed with 4% paraformaldehyde (PFA) for 15 mins at room temperature.

**SH-SY5Y**

SH-SY5Y cells were maintained in DMEM/F12 supplemented with 10% heat inactivated FBS, 1% pen-strep, and 2mM GlutaMax. Sub-confluent cultures were split using TrypLE Express diluted in 1X PBS. Cells were transduced as previously described above. Transduced cells were selected for a minimum of 5 days with 1 µg/mL puromycin before expanding. Proliferation tests of transduced cells was performed as previously described. SH-SY5Y cells were induced with 1 µg/mL DOX for 48-hours and fixed to stain with 4% PFA.

**2.2 Lentivirus Constructs**

Lentivirus containing Gsx1 was purchased from Origene. PS100121 pLenti-EF1a-C-mGFP-P2A-Puro (lenti-Gsx1) cloning vector with custom gene synthesis of coding region only from NM_008178.2.
Lentivirus containing Nkx6.1 was cloned into PS100121 pLenti-EF1a-C-mGFP-P2A-Puro with custom gene synthesis of coding region only from NM_144955.2. Control lentivirus (Lenti-Ctrl) was also purchased with the same PS100121 plasmid and GFP reporter, but no gene inserted.

Genscript performed a site directed mutagenesis on the pLenti-EF1a-Gsx1 and pLenti-EF1a-Nkx6.1. Site-Directed mutagenesis was used to add a STOP codon and an XmaJI sequence. The mutated pLenti-EF1a-Gsx1 was then subcloned into pCW57-GFP-2A-MCS (Addgene 71783). pCW57-GFP-2A-MCS was a gift from Adam Karpf [37]. The final plasmids inserted into the inducible backbone were then packaged in HEK293T cells to create inducible Lenti-Gsx1-rtTA-Tag2GFP and Lenti-Nkx6.1-rtTA-Tag2GFP.

Figure 1. Lentivirus constructs. Lentivirus constructs used during this study. (A) Lenti-EF1a-Gsx1 construct from Origene. (B) Inducible TetON plasmid with Gsx1 cloned into pcW57 to make Lenti-Gsx1-rtTA-Tag2GFP.

**Lentivirus Production**

Lentivirus production followed previously established protocols (Addgene). HEK293T cells were plated on 10cm dishes at 200,000 cells/cm two days before transfection. HEK293T cells were transfected with a mixture of target vector (lenti-Gsx1-rtTA-Tag2GFP or lenti-Nkx6.1-rtTA-Tag2GFP), envelope
plasmid (pMD2.G/VSVG, Addgene 12259), and 2nd generation packaging plasmid (psPAX). HEK293T cells were cultured in Dulbecco’s Modified Eagle Media/Nutrient Mixture F-12 (DMEM/F-12 + GlutaMax) supplemented with 10% fetal bovine serum (FBS), and 1% pen-strep. Pen-strep free media was used during transfection. HEK293T cells were transfected once reaching a minimum of 60% confluency. Transfection was performed using PEI. One day after transfection 4mM Caffeine supplement was added to the media. Viral supernatant was collected on day 2 and day 3 after transfection. Supernatant was centrifuged at 25,000 x g and re-suspended in PBS with 15mM HEPES, 5% Trehalose, and 2mM Magnesium Chloride (MgCl₂) to make 100x concentrated storage solution. Lentivirus aliquots were then stored at -80°C.

Lentivirus titer was calculated using a standard fluorescence tittering assay (Addgene). 50,000 HEK 293T cells were seeded onto a 12-well plate and grown for 48-hours. Lentivirus was then added in serial dilutions to growth media with 4ug/ml Polybrene and incubated overnight. Cells were estimated at 40 – 50% confluent at the time of transduction. The next day, media was refreshed and DOX (1ug/ml) was added. After 48-hours of DOX exposure, cells were imaged for counting. Viral titer was calculated using \( \text{TU/mL} = \frac{\text{Number of cells transduced} \times \text{Percent fluorescent} \times \text{Dilution Factor}}{\text{Transduction Volume in mL}} \) (Addgene).

2.3 Immunostaining and Imaging

Cell cultures were fixed with 4% Paraformaldehyde in PBS at room temperature for 15 minutes. Cells were then washed 3x with PBS before adding blocking buffer (0.05% Triton X-100, 2% donkey serum, and 3% bovine serum albumin (BSA) in PBS) for 1 hour at room temperature. All antibodies were diluted in PBS. Cells were incubated with primary antibodies overnight at 4°C and washed 3 times for 5 minutes with PBS. Cells were incubated with secondary antibodies for 1 hour 30 minutes at room temperature followed by washing 3 times for 5 minutes with PBS. Cell nuclei were stained with 4’,6-diamidino-2-phenyindole (DAPI; 200ng/mL) and mounted to coverslips with Vectashield Plus antifade
mounting medium (Vector Laboratories, H-1900), or were mounted with Vectashield plus antifade mounting medium with DAPI (H-2000).

**Imaging**

Images were captured at the same exposure and threshold at the same intensity using Zeiss AxioVision Imager A.1. ImageJ was used to count the number of cells. ImageJ automated cell counter was used to count total number of cells per image by the number of DAPI+ cells. Threshold intensity value was constant on all images and automated watershed tool was used to separate nuclei that were in contact. The analyze particles tool was then used to count total number of cells per image with a constant particle size. This method was also used to count Ki67+ cells and GFP+ cells per image. Map2 and GFAP positive cells were counted manually. Manual counting was based on DAPI+ nuclei to identify individual cells with the cytoplasmic markers of interest. Co-labelled Map2 and GABA or vGlut2 cells were manually counted. Co-labelled cells were only counted if they were positive for both markers and were again identified by DAPI as individual cells. Co-labelled GFP and Map2 or GFP and GFAP cells were counted manually. In ImageJ, channels tool was used to select only the GFP channel and all cells positive for GFP were selected. The channels tool was then used to switch to show DAPI and Map2 or GFAP. Here, cells were unselected if they were only DAPI+ and included in the final count if they were Map2+ or GFAP+. Three images per well were counted for each condition in Figure 2, 3, and 4. Three images each were counted for the proliferation staining and differentiation with the inducible virus construct (Figures 5-10). Images were selected as representations of the total field. NE-4C cells grow as differentiated cells over a basal layer of undifferentiated cells, therefore areas with more dense layers were chosen for imaging and counting. Statistical analysis was performed using GraphPad PRISM 5.01. Student’s T-test. Significant p < 0.05.
Chapter 3

Results

3.1 Lentivirus transduction of Gsx1 increases the number of neurons and decreases astrocytes during NE-4C differentiation.

NE-4C cells differentiated for 14-days resulted in significantly higher percentages of Map2+ neurons in Lenti-Gsx1 (Lenti-ef1a-Gsx1) transduced cells compared to the Lenti-Ctrl transduced cells. Lenti-Ctrl transduced cells had $33.53\% \pm 1.76$ Map2+ neurons while Lenti-Gsx1 transduced cells had $43.53 \pm 3.94\%$ Map2+ neurons (Fig. 2A,B,C). Lenti-Ctrl transduced cells had $34.78\% \pm 1.98$ GFAP+ astrocytes which decreased to $26.61\% \pm 2.41$ GFAP+ astrocytes in the Lenti-Gsx1 transduced cells (Fig. 2A,B,F). Immunostaining confirmed Gsx1 expression in Map2+ neurons (Fig. 2E) in the Lenti-Gsx1 transduced cells. Lenti-Ctrl transduced cells stained for Gsx1 had minimal positive staining. This data suggests that Gsx1 promotes neuronal development from a neural stem cell population while also decreasing astrocyte development.
Figure 2. Gsx1 transduction increases the number of Map2+ neurons and decreases the number of GFAP+ astrocytes. NE-4C cells cultured in EMEM with 5%FBS, 1% pen-strep and 4mM GlutaMax for 14 days. Differentiation induced by retinoic from DIV0 to DIV8. Viral transduction was performed 7 days prior to plating for differentiation. Transduced cells were selected with 0.5ug/ml puromycin for 48-hours and expanded to plate for differentiation. (A, B, D, E) representative of fluorescence images of the control lentivirus (Lenti-Ctrl) and the lentivirus carrying Gsx1 (Lenti-Gsx1). Arrowheads indicate GFAP+, MAP2+, and Gsx1+ cells. (C, F) histograms of the percentage of MAP2+ neurons and GFAP+ astrocytes. N = 3. Data shown as cell counts with mean ± SEM. Students’ T-test.
3.2 Changes were not detected in glutamatergic or GABAergic neuron subtypes after Gsx1 transduction and differentiation.

Previous studies have highlighted the role of Gsx1 in specifying excitatory or inhibitory cell fates [17]. Our lab’s previous data in mouse models showed increased glutamatergic and decreased GABAergic neurons [20]. After undergoing the defined 14-day differentiation protocol Lenti-Ctrl and Lenti Gsx1 did not show significant differences in GABAergic (Fig. 3A,B,C) or glutamatergic neurons (Fig. 3D,E,F). Neuron subtypes were counted by the number of Map2+ and neuron subtype marker+ co-labelled cells. GABAergic neurons transduced with Lenti-Ctrl comprised 12.08% ± 0.96 of the total population while Lenti-Gsx1 transduced cells had 13.99% ±1.25 GABAergic neurons. Lenti-Ctrl cells showed staining for 14.96% ±1.70 excitatory vGlut2+ glutamatergic neurons while Lenti-Gsx1 had 17.86% ±1.81 glutamatergic neurons. These results showed slight increases in both subtypes tested but were not statistically significant.
Figure 3. Gsx1 transduction did not significantly alter percentage of Map2+ and GABA+ or Map2+ and vGlut2+ co-labelled neurons. NE-4C cells cultured in EMEM with 5%FBS, 1% pen-strep and 4mM GlutaMax for 14 days. Differentiation induced by retinoic from DIV0 to DIV8. Viral transduction was performed 7 days prior to plating for differentiation. Transduced cells were selected with 0.5ug/ml puromycin for 48-hours and expanded to plate for differentiation. (A, B, D, E) representative of fluorescence images of the control lentivirus (Lenti-Ctrl) and the lentivirus carrying Gsx1 (Lenti-Gsx1). Arrowheads indicate Map2+ GABA+ cells or Map2+ vGlut2+ cells. (C, F) histograms of the percentage of GABA+ and vGlut2+ neurons. N = 3. Data shown as cell counts with mean ± SEM. Students’ T-test.
3.3 Lenti-Gsx1-TetON transduced cells increased neurons and decreased astrocytes in DOX activated cells.

The Tet-ON system was chosen to improve temporal control over Gsx1 expression and allow for easier cell culture dynamics. This system allowed for transduction and selection without activating the Gsx1 gene. The development of the Tet-ON Lentivirus was based on the pCW57 plasmid (addgene source). Gsx1-tag2GFP was removed from the Lenti-ef1a virus and mutated to include a stop codon before cloning into pCW57. After receiving this modified plasmid, lentivirus packaging was performed to make the Lenti-Gsx1-TetON used in this study. Doxycycline (DOX) exposure controls gene expression along with the GFP reporter. Transduced cells exposed to 1µg/ml DOX starting at day 0 of differentiation had increased neuronal differentiation and decreased astrocyte differentiation (Fig. 4A-F). Control cells without dox exposure (NO DOX) differentiated into 28.64% ±2.11 Map2+ neurons, while DOX activated cells (1µg/ml DOX) differentiated into 48.74% ±5.27 Map2+ neurons. This was a significant increase in neuronal cells that were DOX activated throughout the differentiation period. Control (NO DOX) cells expressed 14.09% ±0.65 GFAP+ cells while DOX activated (1µg/ml DOX) cells expressed 7.21% ±1.76 GFAP positive cells. Gsx1 activated cells had significantly fewer GFAP+ astrocytes then the control cells. Counting of co-labelled cells resulted in the majority of GFP+ cells co-labelled with neuron marker Map2 (59.32% ±6.43), with a much smaller population of cells co-labelled GFP and GFAP (10.28% ± 3.76) (Fig. 4G). This further suggests the DOX activated Gsx1 expressing cells increasing neuronal cell fates and decreases astrocytes.
3.4 Gsx1 increases proliferation in NE-4C after 48-hours of DOX exposure.

NE-4C cells previously transduced and selected were tested for proliferation at 24 and 48-hours of DOX exposure. After 24-hours, the GFP reporter is being expressed along with Gsx1+ staining compared to no GFP expression in the control (NO DOX) cells (Fig. 5A,B). Proliferation marker Ki67 was used to determine the effect of Gsx1 expression on proliferation in NE-4C cells. Control (NO DOX) cells were 41.00% ±2.64 positive for Ki67 and the DOX activated (1µg/ml DOX) cells were 26.80% ±4.74 positive for Ki67. There was no significant difference at the 24-hour timepoint. DOX exposure for 48-hours significantly increase Ki67 (Fig. 6A,B). Control cells seeded without DOX for 48-hours had 65.70% Ki67+ cells. Cells activated with DOX for 48-hours had 79.55% ±1.65 Ki67+ cells. This was a significant increase compared to the control cells at 48-hours.
Figure 4. DOX inducible Gsx1 increased Map2+ cells and decreased GFAP+ cells.

NE-4C cells cultured in EMEM with 5% FBS, 1% pen-strep and 4mM GlutaMax for 14 days. Differentiation induced by retinoic acid from DIV0 to DIV8. Viral transduction was performed twice, and transduced cells were selected with 0.5µg/ml puromycin for 48-hours. Transduced cells were expanded and seeded for differentiation. (A, B, E, F) representative of fluorescence images of the control (NO DOX) cells and the DOX activated (1µg/ml). (C, D) bar graphs of the percentage of Map2+/DAPI+ cells and GFAP+/DAPI+ cells. (G) bar graphs of percentage of co-labelled Map2+GFP+ and GFAP+GFP+ cells over the total number of GFP+ cells. N = 3. Data shown as cell counts with mean ± SEM.
Figure 5. Gsx1 transduction followed by 24-hour DOX exposure had no significant effect on proliferation. NE-4C cells cultured in EMEM with 5%FBS, 1% pen-strep. Viral transduction performed twice to ensure high transduction efficiency. Transduced cells were selected with 0.5µg/ml puromycin for 48-hours and expanded. Cells were cultured with EMEM plus 5%FBS, 1% pen-strep with no DOX or 1µg/ml DOX for 24-hours. (A, B) representative of fluorescence images of the control no DOX cells (NO DOX) and the DOX activated. (E) bar graph of the percentage of NO DOX and 1µg/ml DOX treated cells labelled with proliferation marker Ki67 over total DAPI+ cells. N = 3. Data shown as cell counts with mean ± SEM. Students’ T-test.
Figure 6. Gsx1 transduction followed by 48-hour DOX exposure significantly increased proliferation. NE-4C cells cultured in EMEM with 5%FBS, 1% pen-strep. Viral transduction performed twice to ensure high transduction efficiency. Transduced cells were selected with 0.5µg/ml puromycin for 48-hours and expanded. Cells were cultured with EMEM plus 5%FBS, 1% pen-strep with no DOX or 1µg/ml DOX for 48-hours. (A, B) representative of fluorescence images of the control no DOX cells (NO DOX) and the DOX activated. (E) bar graph of the percentage of NO DOX and 1µg/ml DOX treated cells co-labelled with proliferation marker Ki67. N = 3. Data shown as cell counts with mean ± SEM. Students’ T-test.
3.5 Lenti-Nkx6.1 transduction did not alter proliferation marker Ki67 in NE-4C cells.

Nkx6.1 transduced cells were tested for proliferation marker Ki67 at 24 and 48-hours of DOX exposure. Control (NO DOX) cells did not express any GFP at the 24-hour timepoint and had 57.14% ±6.12 Ki67+ cells. DOX activated cells (1µg/ml DOX) started to express GFP after 24 hours and had 55.00% ±4.98 Ki67+ cells. Continued DOX exposure for 48-hours did not significantly alter the Ki67+ cell percentage compared to the control (NO DOX) cells. DOX activated cells had 72.97% ±5.23 Ki67+ cells while NO DOX cells had 76.42% ±6.74 Ki67+ cells.

3.6 Gsx1 promotes proliferation in SH-SY5Y cells after 48-hour DOX exposure.

SH-SY5Y cells that were transduced with Lenti-Gsx1 exposed to 1µg/ml DOX for 48-hours had significantly increased the percentage of Ki67+ cells compared to the control (NO DOX) cells. DOX activated cells were 50.74% ±2.77 positive for Ki67 while the NO DOX cells were 25.06% ± 1.89 positive for Ki67 (Fig 9). SH-SY5Y cells expressed GFP reporter gene and were positive for Gsx1.

3.7 Nkx6.1 did not alter proliferation in SH-SY5Y cells after 48-hour DOX exposure.

SH-SY5Y cells transduced with Lenti-Nkx6.1 and treated with 1µg/ml DOX for 48-hours were 29.17% ±2.08 positive for Ki67 while the NO DOX treated cells were 28.59% ±1.39 positive for Ki67. GFP reporter was expressed in cells after DOX activation and co-labelled with Nkx6.1 expression (Fig 10. B)
Figure 7. Nkx6.1 transduction followed by 24-hour DOX exposure had no significant effect on proliferation. NE-4C cells cultured in EMEM with 5%FBS, 1% pen-strep. Viral transduction performed twice to ensure high transduction efficiency. Transduced cells were selected with 0.5µg/ml puromycin for 48-hours and expanded. Cells were cultured with EMEM plus 5%FBS, 1% pen-strep with no DOX or 1µg/ml DOX for 24-hours. (A, B) representative of fluorescence images of the control no DOX cells (NO DOX) and the DOX activated. (E) bar graph of the percentage of NO DOX and 1µg/ml DOX treated cells co-labelled with proliferation marker Ki67. N = 3. Data shown as cell counts with mean ± SEM. Students’ T-test.
Figure 8. Nkx6.1 transduction followed by 48-hour DOX exposure had no significant effect on proliferation. NE-4C cells cultured in EMEM with 5%FBS, 1% pen-strep. Viral transduction performed twice to ensure high transduction efficiency. Transduced cells were selected with 0.5µg/ml puromycin for 48-hours and expanded. Cells were cultured with EMEM plus 5%FBS, 1% pen-strep with no DOX or 1µg/ml DOX for 48-hours. (A, B) representative of fluorescence images of the control no DOX cells (NO DOX) and the DOX activated. (E) bar graph of the percentage of NO DOX and 1µg/ml DOX treated cells co-labelled with proliferation marker Ki67. N = 3. Data shown as cell counts with mean ± SEM. Students’ T-test.
Figure 9. Gsx1 transduction followed by 48-hour DOX exposure significantly increased proliferation. SH-SY5Y cells cultured in DMEM/F-12 with 10% FBS, 1% pen-strep. Lentivirus transduced cells were selected with 1µg/ml puromycin for 5 days and expanded. Cells were plated with or without 1µg/ml DOX for 48-hours. (A, B) representative of fluorescence images of the control no DOX cells (NO DOX) and the DOX activated (1µg/ml DOX). (E) bar graph of the percentage of NO DOX and 1µg/ml DOX treated cells co-labelled with proliferation marker Ki67. N = 3. Data shown as cell counts with mean ± SEM. Students’ T-test.
Figure 10. Nkx6.1 transduction followed by 48-hour DOX exposure had no effect on proliferation.

SH-SY5Y cells culture in DMEM/F-12 with 10%FBS, 1% pen-strep. Lentivirus transduced cells were selected with 1µg/ml puromycin for 5 days and expanded. Cells were plated with or without 1µg/ml DOX for 48-hours. (A, B) representative of fluorescence images of the control no DOX cells (NO DOX) and the DOX activated (1µg/ml DOX). (E) bar graph of the percentage of NO DOX and 1µg/ml DOX treated cells co-labelled with proliferation marker Ki67. N = 3. Data shown as cell counts with mean ± SEM. Students’ T-test.
Chapter 4

Discussion

Transduction of NE-4C with Lenti-Gsx1(ef1a) increased neurons and decreased astrocytes in the 14-day differentiation model (Fig. 2). Previous results in our lab using animal models and Gsx1 transduction increased neural stem progenitor cells, increased specific neuron subtypes, and attenuated glial scar [20]. This study shows that a population of neural stem cells transduced with Gsx1 also increases neuron differentiation and decreases astrocyte formation. Gsx1 has been shown to promote mature neuronal fates in the mouse telencephalon and misexpression of Gsx1 increased neurogenesis in vivo [15]. Previous in vitro work with Gsx1 expressing cultures did not see an increase in neurogenesis in a 2-day neurosphere culture [15]. Here, we show the Gsx1 increased neurogenesis in a neural stem cell culture after 14 days. This difference could be attributed to the extended differentiation period used in this study. In Fig. 3 and Fig. 4, immunostaining of neuron subtypes did not see any significant changes. Gsx1 expression has been previously suggested to play a role in controlling inhibitory and excitatory cell fates [17]. Previous in vivo studies by our lab also showed an increase in excitatory interneurons while decreasing inhibitory interneurons [20]. Varga et al. showed the differentiation of NE-4C cells induced by retinoic acid has results in approximately 57% GABAergic neurons and 44% glutamateergic neurons based on NeuN positive cell number co-labelled with each marker [33]. Here, we calculated neuron subtypes as a percentage of the total population in each well which reduces the overall percentage. Varga et al. also used a different differentiation protocol then this study which can cause changes in cell populations. Neural stem cell induction timing with retinoic acid treatment the influenced by the supplements added in culture media can vary results between studies and comparisons to in vivo work. One important limitation of the work done with the Lenti-ef1a-Gsx1 virus was the immediate onset of Gsx1 expression. NE-4C cells were transduced, selected with puromycin for multiple days, and expanded before being able to plate for differentiation. To increase control over the process we decided to clone the Gsx1 (and Nkx6.1) genes into an inducible plasmid system.
The inducible plasmid, based on the pCW57-GFP-2A-MCS backbone, allowed for transduction and selection of cells without gene expression. The gene expression was then turned on by adding DOX at the time of cell seeding. The following day differentiation was induced with RA and DOX was added throughout the culture period. This system greatly improved control over gene expression. NE-4C differentiation with the inducible plasmid significantly increased neurons while decreasing astrocytes in cell counts of Map2+ or GFAP+ cells as a percentage of the total number of DAPI+ cells. The GFP reporter gene was present throughout differentiation and was co-labelled with Map2 positive cells approximately 60% of the time while only 10% of GFP expressing cells were co-labelled with GFAP positive cells. Remaining GFP expression was seen in DAPI positive cells that may be in the undifferentiated basal layer of cells or was not clearly co-labelled with another cell marker and therefore not counted in those groups. While GFP expression was present at high levels before differentiation, it was at a lower percentage than expected in the final immunostaining. This could be due to an incomplete puromycin selection that left some untransduced cells in the cell population. The efficiency of the inducible construct could also be lower than expected. This would result in a portion of cells being transduced and selected with puromycin resistance but not turning on expression. The co-labelled GFP and Map2 cells combined with increases in the number of Map2 positive cells in DOX activated cells further confirms the role of Gsx1 can play in influencing neural stem cell fates.

Immunostaining for proliferation marker Ki67 shows that Gsx1 also promotes proliferation in NE-4C cells. This increase required 48-hours of DOX activation for full expression of the plasmid to take effect. Results after 24-hour DOX exposure was not sufficient to increase proliferation. Lenti-Gsx1 transduced cell counting of Ki67 appeared to show a decrease in the DOX activated cells in the first 24 hours but this change was not significant. Variability between wells of the same condition at the 24-hour timepoint could be due to the close proximity to the stress of cell seeding and the time needed for DOX exposure to activate expression. The 48-hour DOX timepoint was sufficient to significantly increase expression of proliferation marker Ki67 in NE-4C cells. Proliferation of neural stem cells after spinal cord injury could
play a role in generating the cells necessary for repair. NE-4C cells transduced with Nkx6.1 inducible plasmid did not impact the expression of proliferation marker Ki67. This is contrary to what has been previously seen in vivo [20]. It is not clear why transduction with Nkx6.1 did not promote proliferation. Nkx6.1 has been shown to interact with Notch1 signaling in ventral neural stem/progenitor cells [27]. Notch1 is involved in maintaining neural stem cell fate therefore future studies should investigate the Notch pathway in transduced NE-4C cells to determine changes in function.

To further establish the clinical relevance of the cell culture model, the SH-SY5Y human neuroblastoma cell line was transduced with the inducible lentivirus construct. Importantly, SH-SY5Y cells replicated the proliferation changes seen in the NE-4C cells. Gsx1 increased the expression of proliferation marker Ki67 after a 48-hour DOX treatment while Nkx6.1 did not increase Ki67 expression. This data supports role of Gsx1 in modulating proliferation with previous studies showing Gsx1/2 mutants having decreased proliferation in the SVZ and showing Gsx1 transduction after SCI increasing proliferation in mouse models [16, 20]. The confirmation of proliferation in a human neural cell population contributes to the potential of Gsx1 treatment for SCI.
Chapter 5

Conclusion and Future Work

This study shows that lentiviral-mediated Gsx1 transduction and lentiviral delivery of TetON Gsx1 expression promotes the differentiation of neural stem cells toward a neuronal cell fate while attenuating astrocyte development in vitro. Additionally, Gsx1 expression increased proliferation in both mouse and human neural stem cells. Transduction of the Nkx6.1 gene did not affect proliferation in mouse or human stem cells. The potential to increase proliferation and influence differentiation of neural stem cells could provide a mechanism for neurogenesis after spinal cord injury.

The findings of this study will allow future work to utilize the stable transduced cell lines to further test changes in gene expression and identify the effect of Gsx1 and Nkx6.1 activation on neuron subtypes. Single-cell sequencing analysis on transduced cells will help identify the effect of Gsx1 and Nkx6.1 on the differentiation of neuronal subtypes. The inducible expression of Gsx1 will allow for experimentation with transient expression in different phases of differentiation. The ability to control gene expression during potential spinal cord injury treatments is another important step for clinical relevance.
## APPENDIX

### Primary Antibody

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