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USING SMALL MOLECULES TO CONVERT HUMAN ASTROCYTES INTO FUNCTIONAL NEURONS

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ASTRACT

Astrocytes are the most abundant cells in our central nervous system and play a critical role in neurodevelopment and the regulation of neuronal functions. Previous study in our lab has shown that overexpressing single transcription factor NeuroD1 can reprogram astrocytes into neurons both in vitro and in vivo. Here, we have found that small molecules are able to replace the transcription factor and successfully convert human astrocytes into functional neurons in vitro. These small molecules are known to be involved in normal developmental pathways such as Wnt and sonic hedgehog pathways. Based on immunocytochemistry and gene analysis, we were able to use a group of small molecules to inhibit astroglial phenotype and promote neuronal phenotype over two-week period. About 67% of the human astrocytes were successfully converted into neurons based on immunocytochemistry analysis. The converted neurons were fully functional and consisted of mainly glutamatergic neurons. This chemical reprogramming method may potentially be developed into drug therapy for brain repair and provide a new and less invasive approach in brain repair.

Note: Due to conflict with patent application process, the names and specific function of each small molecule compound was not included in this Honors Thesis. The small molecules were grouped by general function and influence on neurodevelopmental pathways.
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Chapter 1

Advantages of using small molecules in the human brain

Brain damage or neurodegenerative diseases eventually lead to irreversible neuronal loss. The human brain has limited capacity in regeneration of neurons via adult neurogenesis, which is known to produce neurons throughout adulthood in the hippocampus and subventricular zone. In patients with neurodegenerative diseases, it is unlikely the newly generated neurons due to adult neurogenesis in dentate gyrus or subventricular zone will be able to achieve global repair of neuronal loss. In Alzheimer’s disease mouse model, adult neurogenesis was impaired and reduction in proliferation rate was observed (Winner et al, 2011). Although adult neurogenesis can regenerate neurons, the process is restricted to specific brain regions and requires time for new neurons to migrate to injured areas (Ming et al., 2011). Thus, researchers have been studying a variety of methods to repair brain damage and regenerate neurons in the brain.

Currently, there are some cell transplantation studies using induced pluripotent stem cell (iPSC) or embryonic stem cell derived cells (ESCs) in neurodegenerative disease models (Dimos et al, 2008; Kriks et al, 2011). However, such approaches with exogenous cell transplantation have certain disadvantages, including complications with immunorejection, tumorigenesis, and instability in the differentiation process. In this study, we have developed an alternative and novel approach in regenerating neurons directly from astroglial cells using a combination of small molecules that could avoid such complications.
The use of small molecules in generating iPSCs

Recently, Takahashi and Yamanaka achieved a breakthrough in the stem cell field by demonstrating that the overexpression of four transcription factors—Oct4, Sox2, Klf4, and C-Myc—can convert somatic fibroblasts to pluripotent stem cells, also known as induced pluripotent stem cells (iPSCs) (2006). This technique could potentially provide more personalized treatments for patients and reduce the risk of immunorejection. Tissues can be obtained from the patient and transformed into stem cells in vitro, which then can be further differentiated to specific types of cells through defined cultures. This process can avoid using a donor’s tissue that is likely rejected by the patient’s immune system, which could lead to additional complications.

The major problems with iPSC are the low efficiency (0.05% to 20%) and problems with partial reprogramming, which refers to failure to achieve complete reprogramming and achieve transgene silencing. Yamanaka suggested that the low efficiency could be explained by the stochastic model, which suggests that most cells initiate the reprogramming effort, but only a few could achieve complete reprogramming (2009). Gene delivery methods are critical in the success of iPSC generation. The inconsistent proviral integrations of retroviruses and lentiviruses can explain the incomplete reprogramming of some cells.

In addition, it requires a long time to reprogram somatic cells into stem cells, and then further differentiate stem cells into neuronal cells. First, it may take numerous trials to generate iPSC from somatic cell that would take up to months. Then, it would take at least a couple more months for the stem cells to differentiate into specific cells. Although evidence shows that the inclusion of demethylation-promoting small molecules can promote iPSC generation and accelerate the process, it would still require months to obtain enough neurons for transplantation.
Another alternative in generating pluripotent stem cell from somatic cells is using combinations of small molecule compounds. Hou et al. showed that using a combination of seven small molecule compounds can reprogram mouse embryonic fibroblasts into pluripotent stem cells at a frequency up to 0.2% (2013). They used small molecules that were epigenetic modulators that promoted reprogramming efficiency.

The small molecule approach may have some limitations to overcome such as lack of tissue specificity, problem with individual response to drug treatment, and difficulty in determining the toxicity and side effects of small molecules in the long run. Despite these limitations, compared to using transcription factors, the small molecules have advantages of being non-immunogenic, more easily synthesized and may be less invasive because it could avoid the use of retrovirus or lentivirus that issues for clinical applications. The use of small molecules also offers a potential for gradient chemical therapy that is more similar to neurodevelopment gradient for specific signals such as WNT and sonic hedgehog signaling.

**The use of small molecules for neural differentiation from stem cells**

Recently, researchers have been able to generate specific neuronal cell types for different neurodegenerative diseases such as Parkinson’s disease and ALS. Dimos et al. was able to generate motor neurons from patients with ALS from induced pluripotent stem cells using Shh agonists and retinoic acid (2008).

Kriks et al. have engrafted dopamine neurons derived from human pluripotent stem cells in Parkinson’s disease animal models (2012). Kriks et al. derived human dopamine neurons using small molecules that promote sonic hedgehog and canonical WNT signaling (2012). However, the differentiation process requires at least 25 days for the cells to become
Kriks identified three different induction conditions for dorsal forebrain, ventral/hypothalamic and midbrain dopaminergic (DA) identify. The dorsal forebrain condition consisted of dual SMAD inhibition small molecules. The ventral/hypothalamic condition included dual SMAD inhibitors, sonic hedgehog agonists and FGF8. Lastly, the midbrain DA condition included small molecules used in ventral and hypothalamic condition and small molecules that inhibit glycogen synthase kinase 3β and promote WNT pathway signaling. The DA condition resulted in the highest expression of Ascl1 and Lmx1a at day 25 compared to other two conditions. Despite the improvement in behavior after engrafting the differentiated dopamine neurons into adult rhesus monkeys, immunocytochemistry results indicated incomplete immunosuppression even with immunosuppressant drugs, which could become a complication in human patients later on.

Research with human embryonic stem cells remains controversial due to practicality and ethical issues. A major concern in using stem cell as a stepping-stone in neuronal differentiation is the possibility of tumorigenesis due to uncontrollable proliferation of remaining undifferentiated cells. Recently, the transplantation of fetal neural stem cell into an ataxia telangiectasia patient caused tumor formation originating from transplanted tissues 4 years later (Jung et al, 2011). The use of human embryonic stem cells for disease-related treatments may be impractical because of the laborious culture conditions required for maintenance, low efficiencies by gene-targeting and dramatic heterogeneity in differentiation among different human ESC lines (Hanna et al, 2010).
Direct conversion from somatic cells to neurons via overexpression of transcription factors and small molecules

Our study focuses on in situ reprogramming from somatic cells to neurons that could avoid such safety problems and be more direct. Many studies have shown transdifferentiation of somatic cells to neurons across different cell lineages. Reprograming efforts have been demonstrated in converting human or mouse fibroblasts to neurons by overexpressing neural-lineage-specific transcription factors including Ascl1, Brn2, and Myt1 (Vierbuchen et al., 2009). However, it remains unclear if the induced neural cells have silenced fibroblast-specific genes and maintain in the neural state independent of expression of the three transcription factors (Hanna et al, 2010). Other studies have differentiated human embryonic cells into neurons. Motor neurons were generated from human stem cells using small molecules that target dual activating-like kinase (ALK) inhibition and sonic hedgehog signaling (Amoroso et al., 2013).

Another study combined both transcription factors Ascl1 and Ngn2 and small molecules SB-431542 and GSK-3β inhibitor CHIR99021 to convert human fibroblasts and cord blood-derived stem cells to neurons (Ladewig et al., 2012). SMAD and glycogen synthase kinase-3β have been used in neural differentiation of human embryonic stem cells. Ladewig et al. were successful in converting fibroblasts into both inhibitory GABAergic (20%) and excitatory glutamatergic (35%) neurons, and a small fraction of serotonin- and very small population of tyrosine hydroxylase positive neurons. The inhibition of transforming growth factor-β (TGF-β)-SMAD pathway promotes mesenchymal-to-epithelia transition that is implicated in the initial stages of iPSC development. Ladewig et al. hypothesized that MET may play a major role in direct neuronal conversion of human postnatal fibroblasts cells.
Small molecules along can be used to accelerate the neural differentiation of human stem cells. Chambers et al. used a combination of five small molecules to differentiate human pluripotent stem cells into functional nociceptors within 10 days of differentiation compared to at least 30 days in vitro differentiation without small molecules (2012). In addition to the use of SMAD inhibitors, Chambers et al. also added Notch inhibitors that are known to be involved in neuronal differentiation and maturation.

**Why human astrocytes in the brain**

In the central nervous system, there are a limited amount of cell types that can be converted into neurons. Astrocytes, which are the most abundant cells in the central nervous system, are readily accessible and responsive to treatments including drug therapy and gene therapy. Astrocytes play a major role in protecting neurons and become proliferative when injury occurs in the brain. No cell transplantation is needed if one is able to convert the available astrocytes into neurons in vivo.

Furthermore, adult rat astrocytes isolated from the hippocampus retain the potential to promote neurogenesis in stem cell culture (Song et al., 2002). According to Song et al, adult astrocytes along with fibroblast growth factor were able to direct stem cells to differentiate to neurons (2002). In addition, neurons and astrocytes can be differentiated from the same neural progenitor cell type (Reynolds & Weiss, 1992). Together, these results suggest that astrocytes are ideal candidates for the conversion process.

In previous studies, researchers have been able to generate neurons from astroglial cells in vitro and in vivo using overexpression of transcription factors such as NeuroD1, Ngn2, and Ascl1 (Guo et al., 2014; Arlotta and Berninger, 2014). Astrocytes can be first converted to
neuroblast cells and further differentiated into neurons. Kang et al. demonstrated the generation of iPS-like cells and neural stem cell- like cells from mouse astrocytes by the overexpression of BMi1, which is known to be involved in cellular reprogramming. Kang et al. was also able to replace Bmi function with small molecules to reprogram astrocytes to partially reprogrammed pluripotency (2014). Shh agonists were able to replace Bmi1 and generate iPSC-like cells under 2i/LIF culture conditions.

Lastly, we reasoned that glial cells would be more accessible at nearby injury sites. The first response to brain damage is the activation of glial cells. Glial scars consisting of reactive astrocytes and microglia cell form around the injury site. Previously, we have demonstrated that reactive astrocytes and NG2 can be reprogrammed into neurons using the single transcription factor NeuroD1 in adult mouse cortex (Guo et al., 2014). Although the reprogramming efficiency remains fairly high, the single transcription factor method can pose health risks due to the use of virus vectors. In my honors thesis, I substituted NeuroD1 with a combination of small molecules that target similar pathways as NeuroD1 in human astrocytes. To mimic neurodevelopment and reduce cell death, the small molecules are added sequentially according to normal neurodevelopment pathways.
Chapter 2

Methods

Human and Mouse Astrocyte Cell Culture

Mouse astrocytes were isolated from wild type C57/BL6 mice. Experimental protocols were approved by the Pennsylvania State University IACUC and in accordance with guidelines of the National Institutes of Health. Human astrocytes were purchased from ScienCell (HA1800, California). Both human and mouse astrocytes were cultured on poly-D-lysine coated coverslips in 24 well plates. The cells were cultured in DMEM/F12 (Gibco) medium consisting of fetal bovine serum, penicillin/streptomycin, 3.5 mM glucose, and B27 supplemental, 10 ng/ml epidermal growth factor (EGF) and 10 ng/ml fibroblast growth factor 2 (FGF2). Cells were maintained in 37°C in humidified air with 5% CO₂.

Reprogramming astrocytes into neurons

The cell culture conditions are optimized for the reprogramming process by manipulating serum concentration, withdrawal of growth factors, confluence of cells, and B27. Different coating of glass coverslips including PDL and laminin are also tested for the human astrocytes reprogramming method. Combinations of small molecules were selected and tested based on the pathways known to be involved in the conversion process in previous hESC to neurons studies including TGFβ, GSK3β, Wnt, BMP, and Shh pathway, which were collectively referred as master conversion molecules (MCM) in this study. Different lengths of treatment are tested as well. Small molecules were added to the medium and refreshed or changed every other day. For
maturation of neuronal cells after conversion, the medium was replaced weekly and supplemented with BDNF, NT3, and NGF.

To examine expression differences between reprogrammed cells and human neuroprogenitors, we derived NPCs from human pluripotent stem cells gifted from Dr. Fred Gage. The NPCs were cultured on poly-L-ornithine and laminin-coated coverslips with neuronal proliferation medium including DMEM/F12, antibiotics, B27 and N2 supplements, and FGF2.

**Substitution Drug Experiment**

Analogous small molecules are used in substitution of original combination of small molecules to use alternate drugs to inhibit pathways involved in the reprogramming process. Based on previous experiment, the concentration did not affect efficiency but rather toxicity. Thus, the concentrations of alternate small molecules are not focused on given that no massive cell death occurred. The ratios of the number of cells expressing NeuN to DAPI are quantified to determine the efficiency of reprogramming.

**Characterizing Cells and Immunocytochemistry**

In order to characterize initial human astrocyte conditions and ensure no stem cells or progenitor cells contaminate the human astrocyte cell cultures, immunocytochemistry is performed. The cultures were fixed at 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature. Cells were washed three times by PBS and then incubated in blocking buffer consisting of 2.5% normal goat serum, 2.5% normal donkey serum and 0.1% Triton X-100 in PBS. Cells were incubated with primary antibodies for a variety of protein markers overnight at 4°C. After additional washing in PBS, the cells were incubated with secondary antibodies conjugated with Alexa Fluor 488, Alexa 546, Alexa 647 (1:1000, Molecular Probes) for 1 hour at
room temperature, followed by washing in PBS. Coverslips were then mounted onto microslides with anti-fading mounting solution with DAPI (Invitrogen). Images of the samples were taken with epifluorescent microscope (Keyence BZ-9000) or a confocal microscope (Olympus FV1000). Z-stacks of digital images were acquired and analyzed using FV10-ASW 3.0 Viewer software (Olympus).

To understand the effects of small molecules and reprogramming mechanism more clearly, the expression of certain genes including Oct4, GFAP, S100b, SOX2, Tuj1, doublecortin (DCX), nestin, NeuroD1, and NeuN are monitored from the initial to last day of small molecule addition by immunocytochemistry. Also, glia marker, GFAP, is monitored as well to detect the presence of glia cells in response to the small molecules. The neuronal expression will be analyzed by randomly selecting and measuring the fluorescence level of cells using ImageJ and testing for significance compared to control group with only DMSO over the days of treatment using Two-way ANOVA test.

When reached certain maturity around 2 months after initial small molecule addition, the cells in the small molecule group are tested for functionality by electrophysiology whole-cell patch clamp recording using Multiclamp 700A patch clamp amplifier (Molecular Devices, Palo Alto, CA) as described previously (Guo et al., 2014). Functional neurons are detected by recording spontaneous events, resting potential, and action potentials. For voltage-clamp experiments, the membrane potential was typically held at -70 mV resting membrane potential, except when recording inhibitory post-synaptic potentials, which were recorded with cells held at 0 mV. Data were acquired using pClamp 9 software (Molecular Devices, Palo Alto, CA). Na+ and K+ currents were analyzed using MiniAnalysis software (Synaptosoft, Decator, GA). All experiments were conducted at room temperature (22-24°C).
Chapter 3

Results

One of the most common methods to identify effective small molecules is to screen a large chemical compound pool. However, this process may take a long time and require resources we do not have yet. Instead, we searched the literature for small molecules that facilitate dedifferentiation of somatic cells and neural differentiation. The two major selection criteria for small molecules for this conversion process were to inhibit astrogliogenesis and promote neurogenesis and neural differentiation. Table 1 shows the function of small molecules and relative order in conversion strategy. After our initial screening, 20 small molecule compounds met our selection criteria. The 20 small molecule compounds were then tested on both mouse and human cortical astrocytes in vitro.

Table 1. List of small molecule groups and function

<table>
<thead>
<tr>
<th>Small Molecule Group</th>
<th>General Function</th>
</tr>
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<tbody>
<tr>
<td>Inhibit gliogenesis</td>
<td>• Inhibition of SMAD pathway, BMP pathway</td>
</tr>
<tr>
<td>Initiate neurogenesis</td>
<td>• Inhibit Notch pathway and activate WNT pathway</td>
</tr>
<tr>
<td>Promote neural differentiation</td>
<td>• Increase sonic hedgehog signaling.</td>
</tr>
</tbody>
</table>

Reprogramming mouse astrocytes into neurons by small molecules

As seen in Figure 1, morphological changes occur mostly around day 6 to day 8 after initiation of treatment. In the drug groups, the cells become more dimensional and less flat compared to the control group throughout the conversion process. At the end of the conversion, the cells become more elongated and more fibroblast-like.
The conversion efficiency is fairly low in mouse astrocytes. There is no significant difference between the control group and the drug groups. The primary mouse astrocytes culture seem to contain some contamination of neuroprogenitor cells that can eventually differentiate after the withdrawal of serum, as seen in Figure 2 of Control group of positive Tuj1 and NeuN markers.

**Figure 1.** Phase image taken every other day starting at day 2 to day 8 of conversion process. In all drug groups, elongated and fibroblast-like observed after day 6. (DIT stands for days in treatment referring to how many days since first day of small molecule addition.)

The most significant result from the mouse astrocyte experiment is the treatment time for each small molecule group. Experiments were performed to determine the effects of different lengths of different drug function group, as indicated by Table 1. Control group was only cultured with DMSO vehicle. Drug Group 1 had longer treatment of GSK3b inhibitor. Group 2 had only 2 days of drug treatment consisting of all molecules. Group 3 had longer treatment of SMAD inhibitors. Based on results from Group 3, it seems that longer treatment of SMAD
inhibitors lead to more elongated and fibroblast-like cell morphology, which is not our goal. Longer neurite processes were seen in Group 1. Thus, longer initiation time for neurogenesis seems to promote neural differentiation. Since we observed the least fibroblast-like cells and longer neurite processes in Group 1, we decided to focus on testing the efficiency of using Group 1 as the original protocol and referred as Master Conversion Molecules (MCM) throughout this study.

Figure 2. Conversion results after 11 days of drug treatment with different timing of SMAD, GSK3 inhibitors and Shh agonists. Control group was only cultured with DMSO vehicle. Drug Group 1 had longer treatment of GSK3 inhibitor. Group 2 had only 2 days of drug treatment consisting of all molecules. Group 3 had longer treatment of SMAD inhibitors. Conversion efficiency was low for most groups. Some neuronal signals observed in control group most likely due to contamination of neural progenitor cells.
Human Astrocytes Conversion

Initially, small molecules were tested with similar concentrations used in previous literature and mouse astrocytes, but massive cell deaths occurred. To reduce cell deaths, we decreased the concentration and number of compounds added each day of the conversion process. A series of different concentrations were tested to find out the optimal concentration for reprogramming. It seemed concentration differences mainly affected the toxicity and reprogramming efficiency. We tested hundreds of small molecule compound combinations, and found the optimal combination with least toxicity. The sequential addition of the compounds seemed to be better because it minimized cell death throughout the conversion process as well as after treatment. Astrocytes that were treated with more than 5 compounds per day seem to have low cell survival rate. The cell density 1 month after initiation addition of compounds was very low and cell debris was observed.

Compared to mouse astrocytes with the same small molecule protocol, we observed more neuron-like morphological changes throughout the process. We did not observe elongated and fibroblast-like cell morphology throughout the process, as seen in Figure 3. Group 1 had the similar treatment in the mouse astrocyte experiment Group 1. Group 2 lacked SHH agonist throughout the conversion process. Group 2 and 3 had longer treatment time of dual SMAD inhibitors.

Human astrocytes were infected with 1 µl retroviruses encoding EGFP. Few cells per coverslips were infected and followed throughout the period. Images were taken using an epifluorescent microscope every other day tracking GFP+ cells on entire coverslips by Grace Lee, see Appendix A. Due to migration of cells, it is difficult to follow the same exact cell over time. But the specific cells can be followed based on proximity to other GFP+ cells. The rate for cell
survival of the GFP+ cells was fairly high in both group based on constant number of GFP+ cells throughout the treatment. In the drug group, the GFP+ cells transformed from large astroglial morphology to bipolar neuronal-like cells. The GFP signal seems to condense into smaller soma in the treatment group, whereas the GFP signal remained fairly distributed throughout the cell in the control group.

Figure 3. Phase image taken every other day starting at day 2 to day 8 of conversion process.

Prior to small molecule treatment, the human astrocytes were characterized by immunostaining for glial markers such as GFAP and S100β to confirm the identity of the cells.
After the small molecule treatment, we performed immunocytochemistry that stained for neuronal markers to assess the reprogramming progress and efficiency. Immature neuronal signal DCX was observed throughout the two week period of treatment. During the later days of the treatment period, we observe neuronal processes protein markers such as β-tubulin III (Tuji1), MAP2, and NeuN. We have found that about 67% of our human astrocytes have been successfully converted into neurons based on the ratio of Tuji1 to nuclear DAPI (analysis provided by Jiuchao Yin; control, 3.3 ± 0.5%, n = 4 batches of cultures; MCM, 67.1 ± 0.8%, n = 4 batches of cultures; p < 0.0001, Student’s t test).

**Figure 4.** Immunocytochemistry results: conversion efficiency based on Tuj1+ cells (scale bar size is 20 μm, images provided by Jiuchao Yin).

**Substituting selected small molecule compounds with analogous compounds**

Different compound may have different selectivity and toxicity. Here, we replaced the original selected small molecule compounds with drugs with same function and similar selectivity. The results show that the replacement of SMAD inhibitors and neurogenesis drugs decreases the reprogramming efficiency (Fig. 8). However, the conversion efficiency seemed to have decreased compared to previous
efforts, suggesting later passages of human astrocytes may have less reactivity to the small molecules and decreased capability to be converted to neurons.

**Functional characterization of small molecule-converted human neurons**

One of the most important aspects of neurons is their ability to communicate with other neurons and glial cells. Thus, we investigated the functionality of the converted-neurons using whole-cell recordings. Electrophysiology data provided and analyzed by Lei Zhang. After 1 month since initial treatment, patch clamp recording revealed some firing of action potential but not repeatedly, suggesting immature neurons. After 3 months since initial treatment, we observed significant sodium and potassium currents and firing of repetitive action potentials. We also found robust spontaneous synaptic events, including both excitatory postsynaptic currents
(EPSCs; frequency = 0.66 ± 0.14 Hz; amplitude = 24.8 ± 8.2 pA, n = 15), and inhibitory postsynaptic currents (IPSCs; frequency = 0.48 ± 0.21 Hz; amplitude = 23.3 ± 6.3 pA, n = 2).

**Determination of specific cell type after small molecule conversion**

After further testing for specific type of neurons, the majority of cells were excitatory glutamatergic cells (VGlUT1, 88.3 ± 4%, n = 4 batches) and minority of inhibitory GABAergic cells (8.2 ± 1.5%, n = 4 batches). The immunocytochemistry results were negative for other neuronal subtypes, including for cholinergic marker (VAcT), dopaminergic marker (TH), or spinal motor neuron marker (ISI1). The quantitative results were provided by Lei Zhang and Jiuchao Yin.

![Figure 6. Quantification results of neuron subtype conversion based on mature neuronal marker MAP2. Majority of converted cell using protocol with longer inhibition of GSK3 and promotion in neurogenesis was glutamatergic and a small fraction of GABAergic cells. Results from replacing original small molecule compounds with analogous drugs. Asterisks symbol (*) refers to level of significance, **** reaching p<0.0001 using one way ANOVA statistical test comparing with group 1 protocol, also known as Master Image and data provided by Lei Zhang (VGlUT1, 88.3 ± 4%, n = 4 batches; GAD67, 8.2 ± 1.5%, n = 4 batches using Student’s t test).]

**Gene expression progression over time**

To monitor gene expression changes during the small molecule addition period, cells were fixed and stained for genes such as DCX, neurogenin2 (Ngn2), NeuroD1 every other day. Glia marker GFAP was analyzed from first day of treatment to day 10, as seen in Fig 5. Based on
quantification results, GFAP expression is suppressed in the drug group, whereas GFAP expression elevated over time in control group.

Figure 7. GFAP expression from day 0 to day 10 in control and small molecule group. A) Confocal images of GFAP comparing the control group to the drug group. B) Quantification results using 2-way ANOVA test for significance. (Significant time and interaction effect with p<0.0001, suggesting significant in suppressed GFAP expression in drug group (MCM) compared to control group (DMSO) using two-way ANOVA comparison test. D4, 6, 8, 10 had significant suppressed in GFAP expression compared to matched control GFAP expression with p-values 0.0006, 0.0017, 0.0028, and <0.0001 respectively using Sidak’s multiple comparison test.)

To understand more about the conversion process, we assessed if the cells dedifferentiate into the neural stem cell phase. Increase DCX signaling can be observed early on of the process (Fig.8). Despite the increase DCX that indicate effective small molecule treatment, no stem cell
Oct4 signaling detected (some faint signaling was due to cell debris that did not co-localize with DAPI staining).

Also, we tested other markers such as nestin and Sox2, which are both neural stem cell markers from day 0 to day 10 (Fig. 9). Compared to neural progenitor cells derived from human stem cells, the Sox2 and nestin protein expression was very low and insignificant. Nestin remained fairly low throughout the process, suggesting a more direct conversion from astrocytes to neurons. There was a greater variability between individual cells in the drug group for the expression of Sox2. In general, there was a slight increase in Sox2 at day 4-6, but much lower and insignificant compared to the neural progenitor Sox2 expression.

In group 1, also known as MCM, Ascl1, Ngn2, and NeuroD1 expression was also assessed with the help of Lei Zhang. We observed early upregulation of Ascl and Ngn2 since day 2, whereas neuronal gene NeuroD1 was upregulated later from day 4 to day 8, which was consistent with the drugs added to promote neurogenesis. The most significant increase in protein expression

Figure 8. Stem cell marker Oct4 and immature neuronal gene DCX tested for MCM group to determine if cells treated by drug group go through stem cell phase from day 4 to day 12 of treatment. Lack of Oct4 signal in drug group suggest more of direct conversion rather than complete dedifferentiation to neural stem cells. Images taken by epifluorescent microscope for drug group.
occurred around day 4 for Ngn2 and day 6 for NeuroD1.

Figure 9. Neural stem cell protein expression from day 0 to day 10 since first day of drug addition in neural progenitor cells derived from human stem cell, control and drug group 1 (MCM). Nestin and sox2 was tested in neural progenitor cell derived from human stem cell as positive control signaling. The lack of neural progenitor cell gene nestin and sox2 suggest a conversion pathway that is more direct rather than dedifferentiation from astroglial cell to neural progenitor or stem cell.
Chapter 4

Discussion

Based on our results, the sequential addition of a combination of small molecules can effectively convert human astrocytes, but not mouse astrocytes, into functional neurons in vitro. The converted cells are positive for neuronal markers and functional in terms of ability to communicate with other cells via action potential firing and spontaneous synaptic events. The decreased reprogramming efficiency may reflect the need for additional inhibition of glial fate determinants because of less proliferative astrocytes and more mature human astrocytes. The majority cell type after conversion is excitatory with a small fraction of inhibitory neurons. These results are consistent with normal development that most cells in our central nervous system are excitatory.

The failure to convert mouse astrocytes to neurons can be most likely due to the difference in response to small molecules. First, the selection of small molecules was mostly based on previous studies that mainly used human samples rather than mouse samples. The effects of the small molecule compounds used in this Thesis on mouse astrocytes remains unclear. The effective concentration may differ from what we have tested. Thus, future studies may use analogous drugs that are known to be effective on mouse astrocytes or vary the concentrations more drastically with varying time lengths of treatment, and then used to study the effectiveness in vivo by directly injecting small molecules into the mice brain.

On the fundamental basis, mouse and human astrocytes are very different. When human astrocytes were transplanted into mouse brain, researchers observed the formation of gap-junctions with mouse astrocytes and showed humanlike physiological phenotypes, such as fast
propagation of calcium signals, enhanced long-term potentiation and improved learning and memory behavior (Pekny & Pekna, 2014). For example, TGF-β inhibitors are known to impede human iPSC formation and promote cell differentiation. But on the other hand, TGF-β inhibitors are known to enhance iPSC formation in mouse models because TGF-β destabilizes the naïve pluripotent state of mouse embryonic stem cells (Hanna et al., 2010). However, the conversion was successful in human astrocytes, although the reason for the differences in conversion efficiency remains unclear.

**Possible mechanisms of small molecule-mediated conversion process**

In neurodevelopment, neurons and glia both arise from neural progenitor cells. At first, the precursor cells mainly differentiate into neurons that migrate to developing cortical areas. The differentiation of neural progenitor cells into glial cells occurs later in neurodevelopment depending on environmental signals (Gapp et al, 2014). The low expression of GFAP demonstrates the effectiveness of our SMAD inhibitors and neurogenesis compounds in suppressing glial gene expression. The lack of stem cell marker Oct4 expression throughout the conversion process indicates that our method is independent of complete dedifferentiation to the ground state of pluripotent stem cell. This direct transdifferentiation process may reduce the risk for unstable differentiation and tumorigenesis. The lack of expression of nestin and Sox2 suggest that human astrocytes could have only slightly, not completely, dedifferentiated to astroglial precursor-like cell or neural precursor-like cell to the point it was capable of differentiating or directly converting to neurons.

Although unlikely and not supported by our results, another hypothesis is that the small molecules may enable the human cortical astrocytes to become reactive astrocytes, in which the
reactive astrocytes have stem cell properties, and the astrocytes differentiate into neurons. After brain injury, astrocytes become reactive and have the capability to form neurospheres if isolated from the injury site (Buffo et al., 2008). Reactive astrocytes are indicated by an increase in the synthesis of GFAP or re-expression of progenitor markers such as nestin. But our data showed no increase in either GFAP or nestin, which suggest that our combination of small molecules does not make the human astrocytes become reactive astrocytes and behave like neural stem cells.

Glial fate determination is regulated by both Notch and BMP signaling (Buffo et al., 2008). Notch and BMP signaling are inhibited by the small molecule combination we selected. The generation of glutamatergic neurons suggests the role of Ngn2 and NeuroD1 in subtype of cell fate determination. Ngn2 has been reported to play a major role in cell specification for glutamatergic neurons in the embryonic brain development (Schuurmans et al., 2004). Ngn2 and NeuroD1 are regulated by WNT signaling (Urban and Guillemot, 2014). The neurogenesis compounds promote neurogenesis by activation of WNT signaling, suggesting the role of small molecules in determining specific subtype neuronal cell fate.

Epigenetics also play a role in reprogramming efforts. The cell identity is often regulated by epigenetic changes. The stability of cell identity often is determined by modifications to DNA and histones. Thus, we included a small molecule compound that targets histone deacetylase to promote the efficiency. During neurogenesis, astrogliogenesis genes are methylated more and neuronal genes are demethylated (Hirabayashi & Gotoh, 2010), which is expected to be observed in our direct conversion process using small molecules as well. Other epigenetic mechanisms such as histone modification may affect the conversion efficiency as well. Epigenetic analysis
such as DNA methylation assay, bisulfate sequencing, or ChIP-Seq can be performed to identify genes downregulated and upregulated by the sequential addition of small molecules.

Further studies may include modifying the protocol to generate different types of neurons other than glutamatergic or GABAergic neurons for specific diseases. In Parkinson’s disease, loss of dopaminergic neurons in the midbrain leads to motor deficits. As mentioned in Introduction, different culture conditions and factors led to different differentiation process of human embryonic stem cells. The use of FGF8 and Shh agonists pushed the cells toward more dopaminergic cell fate, whereas Shh agonist and retinoic acid promotes the motor neuron cell fate.

The cell culture conditions are also critical for human clinical applications. The use of xenogeneic serum replacement and feeder cells are sources of non-human salic acid, which causes immunological reactions involving human antibodies. Hu et al demonstrated that the xeno-free culture system increases the reprogramming efficiency by 10-fold, which about 0.1% efficiency, after converting human adult dermal fibroblasts into iPSCs (2015). The integration-free hiPISC could also be differentiated into motor neurons. Thus, our next step could also use xeno-free culture system to further increase our reprogramming efficiency.

Interesting, in human brain studies, there may be a compensatory mechanisms indicated by increased hippocampus neurogenesis in patients with AD, which was suggested by increased DCX and neurogenic differentiation (NeuroD) protein expression (Winner et al., 2011). Thus, another possible use of small molecules is to promote adult neurogenesis in the human brain and prevent neuronal loss.
References


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