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Natali Vera Pimentel March 14, 2024

Exploring the Driving Factors of Human Astrocyte Development

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Abstract

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Human brain development is a complex and highly orchestrated process involving multiple components. Early brain development involves neural stem cells, also known as radial glia, generating neurons and eventually switching to generating astrocytes, a glial cell, in a time-dependent manner. This cell fate transition is termed the “gliogenic switch”. Disruption to this switch may contribute to neurodevelopmental disorders, such as autism spectrum disorder (ASD) and Down syndrome. Various intrinsic and extrinsic signals, such as transcription factor (TF) activity and cytokine signaling, are essential for initiating the gliogenic switch. We are interested in manipulating TFs to better understand the driving factors behind human astrocyte development. We first focused on knocking down neurogenic genes by using short-hairpin RNA (shRNA) lentiviruses. Knocking down master regulators of neurogenesis at different time points before the gliogenic switch can provide insight into the specific roles that neurogenesis has on astrogenesis. We chose to knock down NEUROGENIN2 (NGN2) by creating NGN2 shRNA lentiviruses and infecting human cortical organoids (hCOs) and human induced pluripotent stem cell (hiPSC)-derived NPCs. Due to complications with our positive control, we were unable to properly assess the impact of NGN2 knockdown. We identified gliogenic TFs through paired RNA-seq and ATAC-seq analyses and were interested in overexpressing these TFs to observe the effect on astrocyte production. We started with RFX4 and SOX21 overexpression lentiviruses and, unfortunately, ran into fluorescence issues that prevented further investigation of these two TFs. We next focused on LHX2 and began infecting hiPSC-derived NPCs and human fetal tissue at different time points. The hiPSC-NPCs were infected with LHX2 at different stages of differentiation and showed a suppression of neurogenic genes and a promotion of astrocytic genes at early time points. The human fetal tissue was infected with LHX2 at different time points before and after the gliogenic switch and presented significant morphological changes after the gliogenic switch. The combined trends from the LHX2 infections suggest this TF assumes different neuronal and glial responsibilities based on timing. This project provides insights into the role of these specific TFs on the gliogenic switch, further developing our understanding of human astrocyte development.

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Chapter 1: General Introduction

Human development from fertilization to adulthood is a precisely regulated process wherein temporal accuracy is imperative. Of particular importance is the intricate orchestration of events during brain development. In the developing central nervous system (CNS), neural stem cells, called radial glia, generate both neurons and glial cells (Anthony et al., 2004; Kriegstein and Alvarez-Buylla, 2009). Aligned along the ventricular surface, radial glial processes provide a structural scaffold facilitating neuronal migration and cortical formation. Later in fetal development, radial glia switch to producing glial cells, specifically astrocytes followed by oligodendrocytes, that will continue this cortical formation. This cell fate transition is called the gliogenic switch and is characterized by concurrent genomic and signaling changes at both the extracellular and intracellular levels (Takouda et al., 2017; Pavlou et al., 2019).

Astrocytes possess significant developmental responsibilities in the brain, such as synapse formation, maintenance, and elimination (Chung, Allen, and Eroglu, 2015). They also hold several crucial responsibilities during injury and disease. Due to their critical roles, disruptions to the development and production of astrocytes can contribute to a number of neurodevelopmental disorders, including autism spectrum disorder and Down syndrome (Chung, Allen, and Eroglu, 2015). Therefore, the timing of astrocyte development is critical for proper function and physiology of the brain. Understanding the mechanistic switch between neurogenesis and the onset of astrocyte production can provide insight into how these disorders progress.

Various intrinsic and extrinsic signals are essential for proper human brain development. These include changes in transcription factor activity, chromatin accessibility, gene expression, histone modifications, and signaling pathways, all of which are required for the gliogenic switch

(Lattke & Guillemot, 2021). Extrinsic factors, such as changes in cytokine signaling, can directly activate astrocytic genes. For example, interleukin (IL)-6 cytokines can activate the JAK-STAT signaling pathway, namely STAT3, which has been shown to directly activate the canonical astrocyte gene *GFAP* (Namihara and Nakashima, 2013). Additionally, neuronally secreted ligands can drive astrocyte differentiation in radial glial cells (Voss et al., 2023). Intrinsically, key transcription factors, such as NFIA/B (Deneen et al., 2006; Tiwari et al., 2018; Tchieu et al., 2019; Yeon et al., 2021) and SOX9 (Kang et al., 2012; Caiazzo et al., 2015; Li et al., 2018; Neyrinck et al., 2021), alongside their interacting partners like Zbtb20 (Nagao et al., 2016) and PITX1 (Byun et al., 2020), play pivotal roles in initiating the early specification of astrocytes. Additional pro-gliogenic factors, including STAT3 (Fukuda et al., 2007; Deb et al., 2023), REST (Kohyama et al., 2010), and ETV5/ERM (Li et al., 2012), orchestrate signaling cascades critical for instigating astrocyte differentiation.

While decades of research have been critical to our current understanding of astrocyte development, the specific mechanisms that drive the gliogenic switch are not entirely understood. These studies have been predominantly conducted in rodent models and tend to investigate predetermined candidates. Moreover, *GFAP* is widely used as a canonical astrocyte marker, but it is also expressed in other cell types in the brain, such as radial glia (Jurga et al., 2021), making it critical to expand the criteria for what defines an astrocyte.

To better understand the mechanisms governing human astrocyte development, we utilize human induced pluripotent stem cell (hiPSC)-derived neural stem cells (NSCs), human fetal tissue, and human cortical organoids (hCOs) as model systems. NSCs and hCOs are stem cell-derived, while human fetal tissue is primary tissue which provides more relevancy and accuracy as a model system to human functions. NSCs and isolated human fetal tissue cells are

considered 2D model systems, which have the advantage of higher transfection and infection efficiencies compared to 3D models (de Caro et al., 2023). NSCs also rapidly proliferate, granting increased cell numbers for experimental conditions and downstream analyses. However, these 2D cell cultures are limited in cell-type diversity, tissue organization, and cellular maturation (Lanjewar & Sloan, 2021). To enhance their functionalities, hiPSCs can be formed into 3D organoids. Organoids more accurately replicate tissue cytoarchitecture, facilitate endogenous cell-cell interactions, and can sustain growth for extended periods (Amin and Paşca, 2018; Lee et al, 2007). This enables heightened cellular diversity and prolonged cell maturation as opposed to 2D cultures.

hiPSCs can be directly differentiated into hCOs using patterning molecules that mimic the developmental cues present during early brain development (Paşca et al., 2015). This process is particularly advantageous for studying astrocyte development, as hCOs remarkably recapitulate the timing of the gliogenic switch observed in human brain development (Sloan et al., 2017). Notably, they are devoid of other cell types typically present in the brain, such as microglia, endothelial cells, or blood vessels, other than radial glia and neurons. Additionally, astrocytes are produced within hCOs even without the presence of exogenous molecules at the time of their development. Thus, hCOs are a powerful reductionist model system for studying human astrocyte biology and the gliogenic switch. Similar to 2D cultures, hCOs also have limitations. Due to their 3D structure, hCOs tend to have a lower transfection and infection efficiencies compared to 2D cultures, especially when targeting cells within the core of the organoid. However, conducting experiments using both 2D and 3D cultures provides a comprehensive approach, leveraging the advantages of each system to address limitations inherent in either model.

Table 1: Advantages and Disadvantages of 2D and 3D model systems

	Advantages	**Disadvantages**
Neural Stem Cells (NSCs)	● Higher transfection & infection efficiency ● Rapid proliferation ● Stem cell derived	● Limited cell-type diversity ● Minimal tissue organization ● Altered cell maturation
Human Cortical Organoids (hCOs)	● Replicate cytoarchitecture ● Cell-cell interactions ● Longer periods of cell growth ● Cell-type diversity ● Recapitulate gliogenic switch ● Stem cell derived	● Lower transfection & infection efficiency ● Absence of cell types like microglia and cellular structures like vasculature
Human Fetal Tissue	● Higher transfection & infection efficiency ● Cell-type diversity ● Cell-cell interactions ● Primary tissue	● Limited resource ● Limited cell number ● Less proliferative

Utilizing human model systems, the research conducted in this thesis aims to uncover some of these gaps in knowledge of human astrocyte development using two main approaches: knockdown of neurogenic genes and the identification of candidate transcription factors driving the gliogenic switch.

Chapter 2: Approaches for Knocking Down Neurogenic Genes

Transcription factors are potent regulators of cell fate decisions. While the driving TFs of astrocyte development are not fully understood, essential TFs have been identified in neuronal development. Neurogenesis is partially controlled by these master regulators, which include NEUROG2 (NGN2), ASCL1 (Mash1), and NEUROD1 (Berninger et al., 2007; Boutin et al., 2010; Gao et al., 2009; Chanda et al., 2014; Gascón et al., 2016; Araújo et al., 2018). Radial glia activate these lineage-specific TFs to produce neurons, which then migrate away from the stem cell towards their final destination in the cortex (Molofsky and Deneen, 2015). These TFs are even potent enough to force a fibroblast cell to become a neuron (Zhang et al., 2013; Bocchi et al., 2022).

Studies have shown that overexpression of these potent TFs during a gliogenic timepoint suppress or inhibit gliogenesis (Cai et al., 2000; Sun et al., 2001; Miller & Gauthier, 2007). On the other hand, the Nieto et al. study showed that the absence of Ngn2 in both in vitro and in vivo models prevented NSCs from differentiating into neurons and instead produced astrocytes (Nieto et al., 2001). The Cai et al. study also showed that inhibiting the neurogenic TFs by inducing Id1 and Id2 suppressed neurogenesis and promoted gliogenesis (Cai et al., 2000). Based on that information, our hypothesis is that in order for astrocyte development to occur, there must be a downregulation of neurogenesis. To test this, we utilized short-hairpin RNA to target neurogenic master regulators. Targeting neurogenic master regulators may allow us to better understand the role of these components in relation to the onset of the gliogenic switch. We investigated the use of short-hairpin RNA delivered by lentiviruses to knockdown neurogenic master regulators and the effects on the gliogenic switch.

Short-hairpin RNA (shRNA) is a gene silencing technique that uses RNA interference to degrade target mRNA. The shRNA sequence is 19-22 base pairs long and contains a hairpin loop sequence for stability. Once the shRNA sequence is transcribed into mRNA, it travels to the cytosol and Dicer proteins process it into silencing RNA (siRNA) where it is loaded onto the RNA induced silencing complex (RISC). Then, the mRNA of the target gene binds to RISC and gets degraded. Hence, the target gene is silenced (Moore et al., 2010). One of the main advantages of using shRNA over siRNA is its stability. Additionally, shRNAs can be cloned into lentiviruses, which have the capability to survive proliferation since they get integrated into the genome (Rao et al., 2009). On the contrary, siRNAs are non-integrating, which means the knockdown is transient and weakens over time as the cell undergoes multiple divisions (Rao et al., 2009).

Leveraging the ability to clone shRNAs into lentiviruses, this project focuses on knocking down NGN2, a master regulator of neurogenesis, to test the effects on astrocyte development.

Chapter 3: Overexpression of Transcription Factors

Transcription factors (TFs) are proteins that can manipulate gene expression by binding to specific or non-specific binding sites where they either recruit or block RNA polymerases (Maston, Evans, and Green, 2006). TFs do not work alone; they are involved with many other elements such as enhancers, insulators, silencers, repressors, and more. All of these working pieces form a transcriptional network that can regulate and manipulate gene expression based on what factors are in the network and/or timing of the transcriptional activity.

Transcriptional networks play a crucial role in determining cell fate within the developing central nervous system (CNS). Many of the canonical transcription factors associated with neurogenic and gliogenic roles demonstrate versatile regulatory effects. For instance, NFIA and SOX9, known for their astrogenic functions, also influence oligodendrocyte and neurogenic fates (Stolt et al., 2003; Deneen et al., 2006). Conversely, the neuronal master regulator ASCL1 is also essential for oligodendrocyte development and the control of glial cell numbers (Parras et al., 2007; Sugimori et al., 2008; Vue et al., 2014). The ability of a single transcription factor to direct various cell fates relies on the dynamic temporal and environmental factors (Knijnenburg et al., 2008; Dennis et al., 2019; Işıldak et al., 2020) and the genetic state of a given progenitor cell (Voss & Hager, 2013; Amiri et al., 2018; Yoon et al., 2018; Katada et al., 2021).

Given the complexity of transcriptional networks and cell fate decisions, we aimed to identify key transcription factors driving astrocyte development. We performed a cross-analysis of RNA-seq and ATAC-seq data from hCOs pre- and post-gliogenic switch and identified LHX2, RFX4, and SOX21 as regulators of astrocyte development (Lanjewar et al., unpublished data). RFX4 and SOX21 have previously been implicated in glial specification and development and are both expressed in astrocytes (Zhang et al., 2006; Cahoy et al., 2008; Wang et al., 2014;

Tiwari et al., 2018; Trevino et al., 2020 and 2021). Surprisingly, LHX2 is canonically known for its involvement in forebrain and neuronal development (Roy et al., 2014; Chou & Tole, 2019). However, its role in gliogenesis is more unclear and may be region-dependent (Subramanian et al., 2011; de Melo et al., 2016; Muralidharan et al., 2017).

Using a combination of genomic manipulations and transcriptional profiling, we investigate the roles of LHX2, RFX4, and SOX21 in astrocyte development using human fetal tissue samples and NPCs.

Chapter 4: Methods

shRNA Design

shRNA oligonucleotide sequences for NEUROGENIN2 (NGN2) were designed using VectorBuilder's shRNA target design tool (https://en.vectorbuilder.com/tool/shrna-target-design.html). 3 sequences were chosen for *NGN2* based on their high knockdown scores. A scrambled shRNA sequence was also designed as a control. A functional shRNA sequence includes a hairpin loop, an antisense strand, and a linker chain.

Table 2: shRNA Oligo Sequences

shRNA Name	shRNA Anti-Sense Sequence (5' 3')
NGN2 shRNA1	ATGGCTGGCATCTGCTTTATT
NGN2 shRNA2	CATCTGTCTCTTATGATTTAT
NGN2 shRNA 3	GGTTAGAAGTCATTGTATAAT
Scrambled shRNA	ATTCGTCTACCGCTAAGCACT

The shRNA oligo sequences were cloned into the p.LKO.3G_X7 lentiviral plasmid backbone (Addgene, Cat. 171213) under a U6 promoter with an EGFP tag using AgeI (New England Biolabs, Cat. R3552) and EcoRI (New England Biolabs, Cat. R3101) restriction enzymes. NGN2 and scrambled shRNA sequences were annealed and ligated into the digested plasmid backbone using T4 DNA ligase (New England Biolabs, Cat. M0202), according to the manufacturer's protocol, to create the shRNA plasmids. The ligated shRNA plasmids were transformed into 5-alpha Competent *E. coli* cells (New England Biolabs, Cat. C2987), following

the manufacturer's protocol. After a clone was selected and expanded, shRNA plasmids were purified using a miniprep kit (QIAGEN, Cat. 27014) then sent off for Sanger sequencing (GENEWIZ from Azenta Life Sciences) to confirm proper insertion of the shRNA. Lentiviral shRNAs were made following the lentiviral production protocol outlined below.

TF Overexpression Design

Candidate TFs involved in astrocyte development were identified via paired ATAC-seq and RNA-seq analysis of human cortical organoids across neurogenic and gliogenic timepoints (Lanjewar et al., unpublished). Out of the identified candidate TFs, we selected RFX4, SOX21, and LHX2 to perform additional experiments on due to their high gliogenic potential. We designed overexpression lentiviruses for each of these TFs, which included cloning hLHX2 (NM_004789.4), hRFX4 (NM_001206691.2), or hSOX21 (NM_007084.4) into a lentiviral backbone under an EF-1α promoter with a T2A-TurboGFP tag. Due to issues with minimal fluorescence, a second lentiviral construct was designed for RFX4 and SOX21, with TurboGFP under its own (hPGK) promoter. All plasmids were designed and obtained from VectorBuilder. Lentiviral TFs were made following the "Lentiviral Production" protocol.

To validate these overexpression lentiviruses, we infected HEK293T cells and performed fluorescence-activated cell sorting (FACS) to collect GFP- and GFP+ cells. We then performed qRT-PCR to test for overexpression. We also used a CMV-GFP lentivirus, pLenti CMV GFP Hygro (Addgene, Cat. 17446-LV; Campeau et al., 2009), as a control to ensure that we were observing effects due to overexpression instead of infection with lentivirus.

Lentiviral Production and Validation

To create the viral vectors, we used HEK293T cells and followed the Thermo Fisher "Improve lentiviral production using Lipofectamine 3000 reagent" protocol. Briefly, we cultured HEK293T cells in a T-75 flask until they were about 75% confluent and passaged them into another flask with lentiviral packaging media (Opti-MEM, 5% fetal bovine serum, and 1 mM sodium pyruvate). The next morning, we transfected the HEK293T cells with the purified shRNA plasmids, along with Lipofectamine 3000, P3000, and a ViraPower lentiviral packaging mix (Invitrogen, Cat. L3000015 and K497500), then waited 6 hours to exchange the media with pre-warmed lentiviral packaging media. The morning after, we harvested the first batch of media which contains the lentivirus. We harvested the second batch of lentivirus the following afternoon and concentrated the viruses (Millipore Sigma, Cat. UFC510024).

To validate the lentiviruses, we infected a neuroblastoma cell line (SHSY5Y) or HEK293T cells and performed reverse transcription quantitative polymerase chain reaction (qRT-PCR). Briefly, we cultured the neuroblastoma cells or HEK293T cells until they were at least 50% confluent and infected them with the lentiviruses with a multiplicity of infection (MOI) of at least 5. We waited about 2 days post-infection and collected either unsorted cells or GFP sorted cells via fluorescent-activated cell sorting (FACS) for qRT-PCR.

qRT-PCR

RNA was extracted from the collected cells using the RNeasy kit (QIAGEN, Cat. 74106) based on the manufacturer's protocol. cDNA was synthesized using reverse transcriptase (Thermo Fisher Scientific, Cat. 18-090-200) with random hexamers (Thermo Fisher Scientific, Cat. N8080127) and oligodT primers (Thermo Fisher Scientific, Cat. 18-418-020). cDNA was amplified for 40 cycles with each sample ran in triplicates. The efficiency and specificity of all

primers (Table 3) were validated via serial dilutions using qRT-PCR. We normalized the CT value of each gene to the CT value of the housekeeping gene (GAPDH) of the same sample and then calculated fold change compared to control samples. The primers used in this study include:

Table 3: qRT-PCR Primers

	Forward	Reverse
GAPDH	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT
LHX2	GGGTCCTCCAGGTCTGGTT	AAGACGGACGTCACAGTTGG
NGN2	CACAGTTAGAGCCAACTAAGATGT	GCACTAACACGTCCTCTTCCT
RFX4	CACCCAATTATATCAGGAGTTTGAC CAT	ACCTTCACAACACAGCGGTC
SOX21	GCACAACTCGGAGATCAGCA	CACCGGGAAGGCGAACTT

Neural Progenitor Cell (NPC) Culture

hiPSCs (8858.3, 1208-2, and NS C9) were differentiated into NPCs using the Leng et al., 2022 protocol with slight modifications. All lines were genotyped by SNP array to confirm genomic integrity and regularly screened for Mycoplasma prior to differentiation. Briefly, iPSC colonies at 80-90% confluency were detached from culture plates using Accutase and formed into 3D spheroids using AggreWell plates. From days 1-13, Neural Induction Media (NIM, DMEM/F12, 2% B27 -A, 1% N2) was supplemented with 2.5 uM Dorsomorphin (R&D Systems, Cat. 3093/50) and 10 uM SB-431542 (Selleck Chemicals, Cat. S1067). Following neural rosette selection, NPCs were maintained on Matrigel-coated plates (VWR, Cat. BD354277) in NIM supplemented with 20 ng/mL FGF2 (R&D Systems, Cat. 233-FB-01M) with media changes every 2-3 days. NPCs did not undergo purification via FACS.

NPCs were differentiated on growth factor reduced Matrigel (VWR, Cat. 80094-330) in NIM supplemented with BDNF (20 ng/ml; PeproTech, Cat. 450-02-1mg) and NT-3 (20 ng/ml; R&D Systems, Cat. 267-N3-005/CF). Removal of FGF2 marked day 1 of differentiation.

Plasmid Transfection

Due to problems with lentiviral fluorescence, we tested TF overexpression via plasmid transfection. Cells were cultured to ~80% confluency. 2.5 ug of plasmid DNA was incubated with Lipofectamine 3000 reagents, according to the manufacturer's protocol (Invitrogen, Cat. L3000015). The plasmid-lipid complex was added to the cells and left for 2-3 days prior to changing media. Cells were analyzed about 7 days post-transfection.

Human Tissue

Fetal human brain tissue (GW12-21) was obtained with informed consent following elective pregnancy termination in compliance with all Emory University-approved protocols and in compliance with the Emory Institutional Review Board. No compensation was provided to any individuals, and samples were collected only in the absence of specific pathologies or pharmaceutical interventions.

Immunopanning

Primary fetal tissue samples were dissociated and immunopanned following the Zhang et al., 2016 protocol. Briefly, the tissue was chopped and enzymatically dissociated using a papain digestion (20U/mL, Worthington Biochemical, Cat. LS003126) at 34°C for 45-60 min. The digest was inhibited via ovomucoid then triturated to obtain a single-cell suspension. The cell

suspension was added to Petri dishes pre-coated with cell-type specific primary antibodies for about 10 minutes each. Unbound cells were transferred to the subsequent Petri dish until the final positive selection plate. Bound cells were washed 8X with PBS and then incubated in a trypsin solution for 7 min at 37°C. After spinning, immunopanned cells were plated on poly-d-lysine-coated coverslips or cell culture plates in a serum-free medium. Media was changed every 3 days. The following antibodies were used in this sequence: anti-THY1 (BD Biosciences, Cat. 550402) to deplete neurons, anti-HepaCAM (R&D Systems, Cat. MAB4108) to pull down astrocytes, and anti-CD15 (BD Biosciences, Cat. 559045) to enrich for neural stem cells.

Immunocytochemistry

Cells grown on PDL-coated coverslips were fixed with 4% PFA for 15 min at room temperature. Blocking buffer, 5% donkey serum and 0.15% Triton X-100, was added for 30 minutes, followed by the addition of primary antibodies (listed below) overnight at 4°C. The next day, after 3X washes in PBS, the cells were incubated with secondary antibodies and DAPI (1:1000, Invitrogen, Cat. D3571) for 1 hour at room temperature. Following 3 additional washes with PBS, the coverslips were mounted onto slides for imaging. Primary antibodies: GFAP (1:1000, BioLegend, Cat. PCK-591P), GFP (1:1500, Aves, Cat. GFP-1020), GLAST (1:100, Miltenyi Biotec, Cat. 130-095-822), HOPX (1:50, Santa Cruz, Cat. sc-398703), LHX2 (1:500, Abcam, Cat. ab184337), SOX9 (1:200, 2 nights incubation, R&D Systems, Cat. AF3075), TBR1 (1:2000, Abcam, Cat. ab31940), TUJ1 (1:1000, BioLegend, Cat. 801201), and TurboGFP (1:500, Origene, Cat. TA150075).

Western Blot

Cultured cells were dissociated using Accutase, spun down, then lysed in protein extraction buffer (150 mM NaCl, 20 mM Tris–HCl (pH 7.5), 5 mM MgCl2, 1% NP-40, supplemented with protease and phosphatase inhibitor cocktail (Sigma-Aldrich, Cat. PPC1010; dilution 1:100)). Cells were further lysed by pipetting and using a pellet pestle motor (DWK Life Sciences, Cat. 749540-0000 and 749521-0590). A BCA protein assay (Thermo Fisher Scientific, Cat. 23227) was used to determine protein concentrations. Protein lysates (25 μg) were loaded into 4–12% Bolt Bis–Tris Plus Gels (Invtirogen, Cat. NW04120BOX), separated by SDS-PAGE (Invitrogen, Cat. NW04120BOX), and then electro-transferred onto nitrocellulose membranes (Invitrogen, Cat. IB23001) using the iBlot 2 Dry Blotting system. Membranes were blocked in 5% milk in PBS and probed with primary antibody overnight at 4°C. The following antibodies were used: Actin (1:2000, Sigma-Aldrich, Cat. MAB1501), RFX4 (1:1000, LSBio, Cat. LS-C109967-100), and SOX21 (1:1000, R&D Systems, Cat. AF3538). Secondary antibodies conjugated to horseradish peroxidase were incubated with the membranes at room temperature for 1 hour. Proteins were visualized by enhanced chemiluminescence (Thermo Fisher Scientific, Cat. 34577) and imaged using a Bio-Rad ChemiDoc Imaging System.

RNA-sequencing

Infected cells were lysed in Qiazol (Qiagen, Cat. 79306) and RNA was extracted using the RNeasy kit (QIAGEN, Cat. 74106) with DNase-I digestion (Qiagen, Cat. 79254), according to the manufacturer's protocols. Samples were sent off to Admera Health for bulk RNA-sequencing using the NEB Next Ultra II kit with poly-A selection (New England Biolabs, Cat. E7805S) at 40 million paired end reads per sample.

The following sequence was used to process files: (1) Trimmomatic to trim Fastq files, (2) STAR aligner with the paired-end option to map to hg38, (3) FeatureCounts to assemble transcripts and create raw count matrices, and (4) EdgeR to normalize counts and determine differentiation gene expression. Differentially expressed genes were determined as having a $\log_2$(fold change) > 2 and adjusted P value < 0.05. Normalized transcript counts were used for analyses and data visualization via GraphPad Prism (10.1.0) or ggplot2 in RStudio.

Branch Length Measurements

SNT's semiautomated tracing method (Fiji plug-in) was used to trace and analyze the branches of CMV-GFP+ (control) and LHX2-GFP+ cells. Branches were measured by selecting the longest primary branch(es) for each cell and using the SNT software to quantify the total path length (μm) for each branch. A few representative cells were selected per image. This experiment was performed blinded to minimize bias; the analysis was performed without knowledge of the condition (control or LHX2) or age (GW) of the images.

Chapter 5: Results

shRNA Knockdown of *NGN2*

We first wanted to understand how knockdown of neurogenesis affects astrocyte development. We chose to target NGN2, a master regulator of neuronal development. To create lentiviral knockdown constructs, we designed 3 oligonucleotide sequences for the *NGN2* gene, as well as a scrambled control. Next, we performed molecular cloning to clone the shRNA sequence into a lentiviral plasmid backbone containing a GFP marker. Once the shRNA plasmids were purified, we transfected HEK293T cells to create shRNA lentiviruses (Figure 1A and 1B).

To validate the shRNA lentiviruses, we used SHSY5Y cells because these neuroblastoma cells endogenously express *NGN2*. We infected SHSY5Y cells with the shRNA lentiviruses (Figure 1C) and sorted based on fluorescence for virus-containing cells (GFP+) and non-virus-containing cells (GFP-). We performed qRT-PCR and found that *NGN2* expression was downregulated in GFP+ cells infected with NGN2-shRNA (Figure 1D). Surprisingly, we also observed a knockdown in the control GFP+ cells infected with scrambled shRNA. To address this problem, we redesigned the scrambled shRNA, but we continued to see *NGN2* knockdown in the control. We also used different scrambled shRNA plasmids but saw no improvements. Due to repeated perplexing and inconsistent results, we were unable to investigate downstream effects on astrocyte production due to NGN2 knockdown. Thus, we continued to Overexpression of Transcription Factors

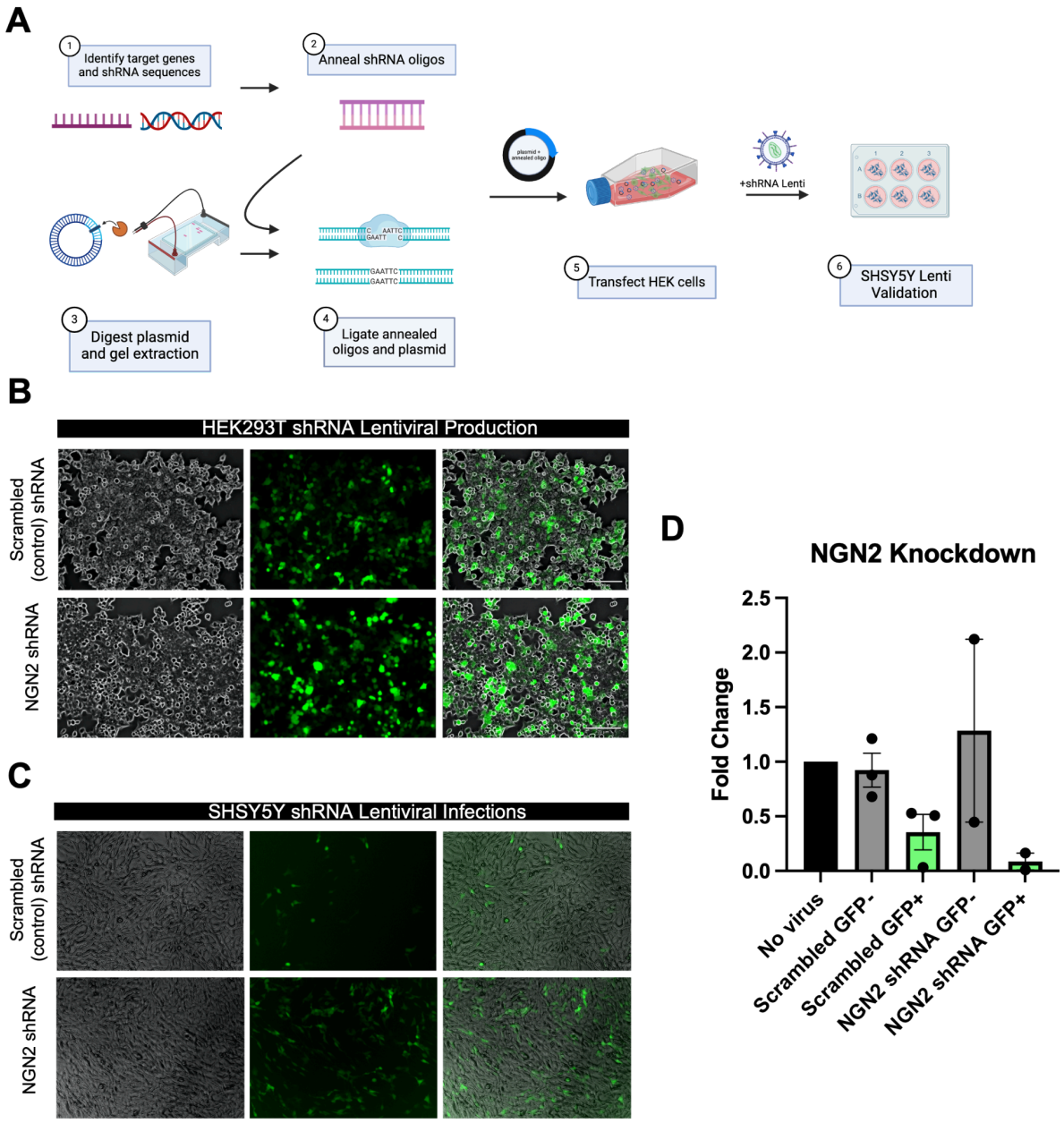


Figure 1. Knockdown using shRNA Lentiviruses. A Schematic of shRNA lentiviral construction, production, and validation. **B** Images of scrambled (control) and NGN2 shRNA lentiviral production in HEK293T cells. Left panels show cell density. Middle panels show GFP fluorescence. Right panels show overlay. **C** Images of shRNA lentiviral infections in SHSY5Y cells. Left panels show cell density. Middle panels show GFP

fluorescence. Right panels show overlay. **D** qRT-PCR data of NGN2 knockdown in GFP-sorted SHSY5Y cells.

Overexpression of Transcription Factors

We next wanted to identify transcription factors (TFs) involved in driving astrocyte development. To do this, we performed paired ATAC-seq and RNA-seq analyses of human cortical organoids across neurogenic and gliogenic timepoints. RNA-seq analysis provides information on the genomic changes occurring at these time points, whereas ATAC-seq analysis provides information on chromatin accessibility. Utilizing a Paired Expression and Chromatin Accessibility (PECA) computational algorithm (Duren et al., 2020), we utilized these sequencing analyses to identify candidate gliogenic TFs that have a regulatory potential for astrocyte genes. We identified 8 candidate TFs: EMX1, EOMES, FOXG1, LHX2, POU3F3, RFX4, SOX21, and TBR1 (Figure 2A). These TFs had a high predicted regulatory potential for astrocyte genes and lacked regulatory potential for randomly selected genes, meaning these candidate TFs are predicted to specifically regulate astrocytic genes (Figure 2A). However, many of these candidate TFs, including EMX1, EOMES, FOXG1, POU3F3, and TBR1, are canonically involved in neuronal development (Kobeissy et al., 2016; Englund et al., 2005; Hanashima et al., 2002; Hanashima et al., 2004; Arnold et al. 2008; Hashizume et al., 2018). LHX2 has been implicated in both neurogenesis and gliogenesis, and its regulatory capabilities may be region dependent (Subramanian et al., 2011; de Melo et al., 2016; Muralidharan et al., 2017). In contrast, RFX4 and SOX21 have previously been implicated in glial cell fate specification and are expressed in astrocytes (Zhang et al., 2006; Cahoy et al., 2008; Tiwari et al., 2018; Trevino et al., 2020 and 2021). Due to their known involvements in glial development, we chose to further understand the roles of RFX4 and SOX21 in driving the gliogenic switch.

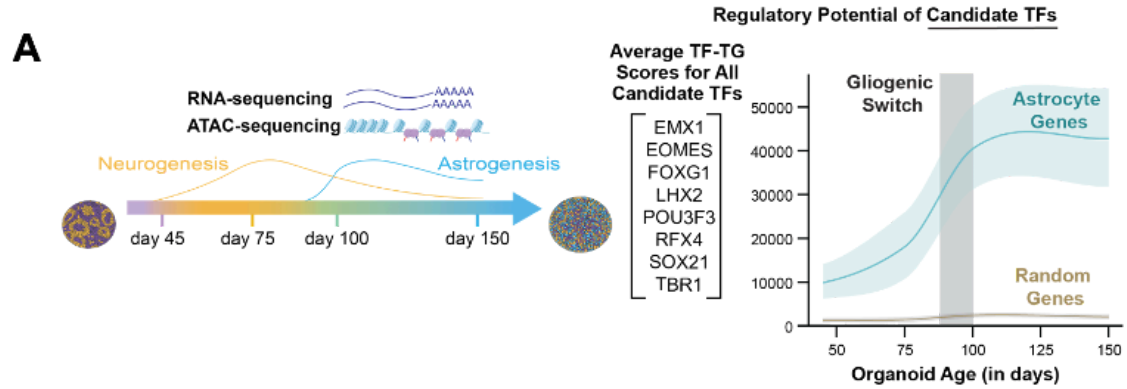
RFX4 and SOX21:

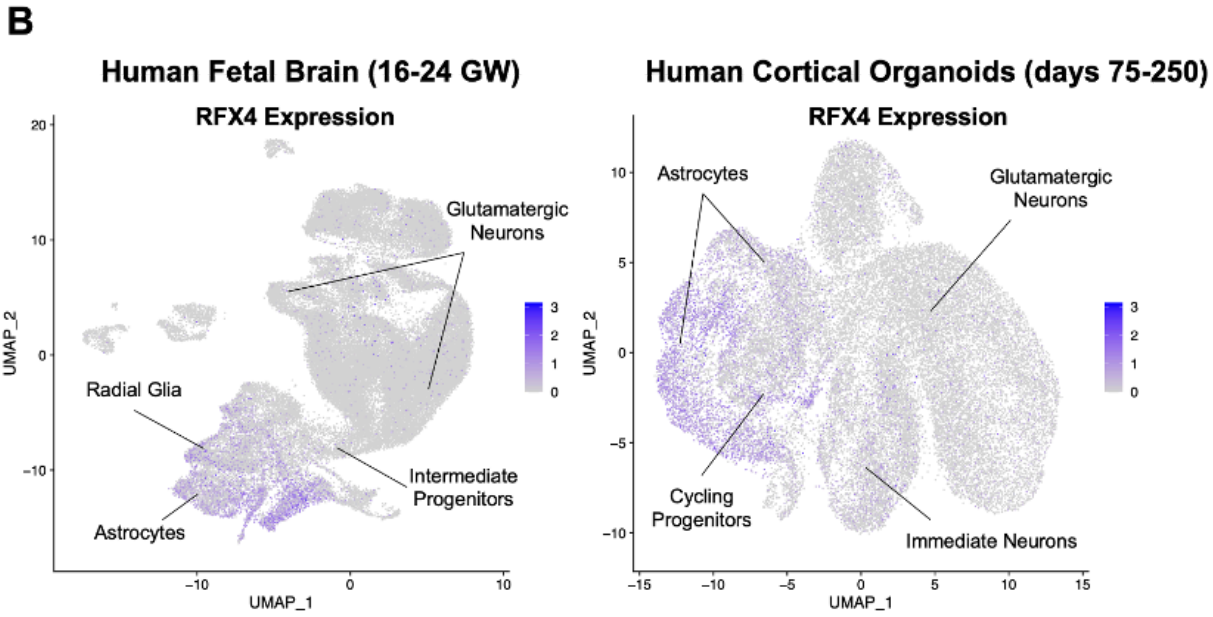
RFX4 is a TF from the regulatory factor x family that has been characterized into 5 different proteins, *RFX1-5*. Furthermore, *RFX4* has been broken down into variants, *v1-v6* where these variants are characterized based on the exons they span or omit and different DNA binding locations. For example, *RFX4_v1* and *RFX4_v2* are heavily expressed in testis while *RFX4_v3* and *RFX4_v4* are distinct to the brain (Zhang et al., 2007). *RFX4_v4* has been identified as a gene that is expressed by astrocytes (Cahoy et al., 2008) and *RFX4_v3* has been characterized in patterning and development for other parts of the brain such as the midbrain (Zhang et al., 2007). Importantly, *Rfx4* is known to maintain a stem cell state for progenitor cells by interacting with and regulating musashi proteins (Msi1) (Kawase et al., 2014).

SOX21 is a TF that belongs to the *SOXb* family and plays crucial roles in neuronal development, specifically maintaining progenitors and repressing neuronal growth by blocking *NGN2* function (Ohba et al., 2004; Mallanna et al., 2010; Whittington et al., 2014). Previous studies also produced conflicting results that show *Sox21* promotes neuronal differentiation in different regions in the CNS (Sandberg et al. 2005; Matsuda et al., 2012) and that it is expressed in mature astrocytes (Trevino et al., 2020). These combined findings highlight that *SOX21* has different roles in early brain development, which may be dependent on its binding partners and/or the timing of expression (Keifer, 2007; Whittington, 2014).

We first confirmed whether RFX4 and SOX21 are expressed in human astrocytes. We re-analyzed single cell sequencing data of the human fetal cortex (GW16-24; Trevino et al., 2021) and human cortical organoids (days 78-252; Andersen, unpublished) and found expression of both RFX4 and SOX21 in human astrocytes (Figure 2B and 2C). Next, to confirm the presence of RFX4 and SOX21 at the protein level, we performed immunopanning on GW19

human fetal tissue samples and collected HepaCAM+ astrocytes (Zhang et al., 2016). Given the challenge of distinguishing between radial glial and astrocyte identities at this stage, we selected two distinct astrocyte markers, GFAP and GLAST for immunocytochemistry (ICC). Glial fibrillary acidic protein (GFAP) is often used as a canonical astrocyte marker (Yang & Wang, 2015) and glutamate aspartate transporter (GLAST) is a glutamate transporter predominantly expressed in astrocytes (Pajarillo et al., 2019). Our stains revealed co-localization of RFX4 with both astrocyte markers (Figure 3A). Due to unavailability of antibodies, we were unable to confirm SOX21 co-localization.


A
RNA-sequencing
ATAC-sequencing
Neurogenesis
Astrogenesis
day 45
day 75
day 100
day 150
Average TF-TG Scores for All Candidate TFs
EMX1
EOMES
FOXG1
LHX2
POU3F3
RFX4
SOX21
TBR1
Regulatory Potential of Candidate TFs
Gliogenic Switch
Astrocyte Genes
Random Genes
Organoid Age (in days)


B
Human Fetal Brain (16-24 GW)
RFX4 Expression
Glutamatergic Neurons
Radial Glia
Intermediate Progenitors
Astrocytes
UMAP_1
UMAP_2
Human Cortical Organoids (days 75-250)
RFX4 Expression
Astrocytes
Glutamatergic Neurons
Cycling Progenitors
Immediate Neurons

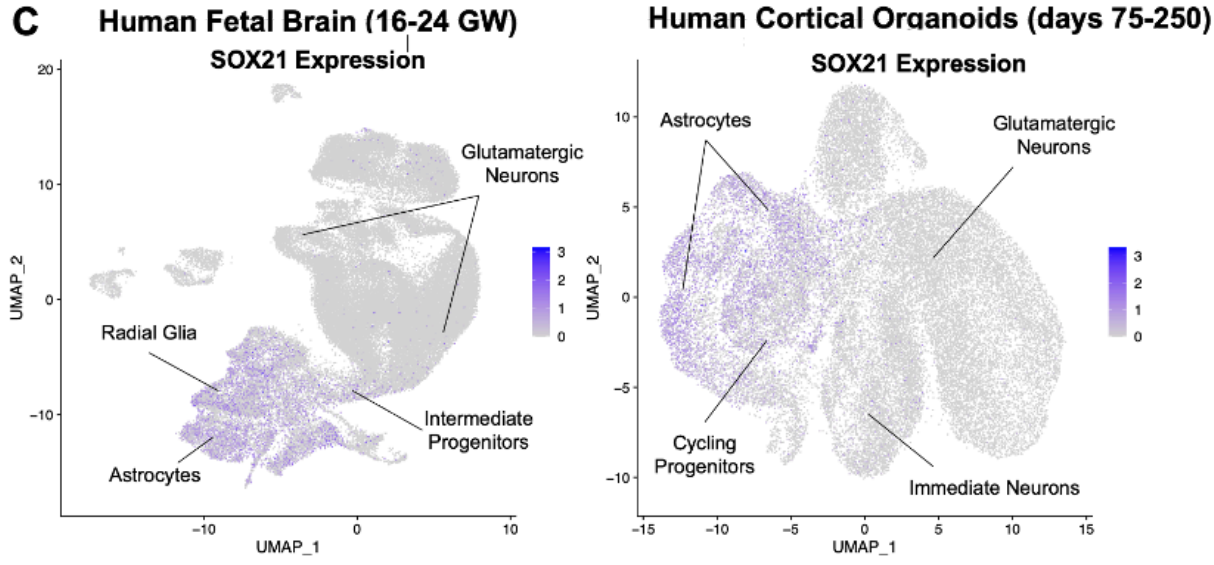

C
Human Fetal Brain (16-24 GW)
SOX21 Expression
Glutamatergic Neurons
Radial Glia
Intermediate Progenitors
Astrocytes
UMAP_1
UMAP_2
Human Cortical Organoids (days 75-250)
SOX21 Expression
Astrocytes
Glutamatergic Neurons
Cycling Progenitors
Immediate Neurons

Figure 2. Identification of Gliogenic TFs and Single Cell Sequencing Data from Fetal Tissue and hCOs. A Schematic of paired RNA-seq and ATAC-seq analysis of human cortical organoids to identify candidate TFs of the gliogenic switch. **B** RFX4 expression in single cell sequencing data from human fetal brain tissue (16-24 GW) on the left and hCOs (d75-250) on the right. **C** SOX21 expression in single cell sequencing data from human fetal brain tissue and hCOs.

Given the presence of RFX4 and SOX21 in astrocytes, we proceeded with assessing their functional impact on the gliogenic switch. We hypothesized that overexpressing our gliogenic TFs, RFX4 and SOX21, at different time points in NPCs and hCOs would enact genomic and structural changes driving a push towards an astrocyte lineage. We designed overexpression lentiviruses for RFX4 and SOX21, each tagged with a GFP reporter. We also used a CMV-GFP lentivirus as a control to ensure viral infection did not induce expression changes. To validate these overexpression lentiviruses, we infected HEK293T cells. We then sorted for GFP+ cells by fluorescence activated cell sorting (FACS) and performed qRT-PCR on the GFP+ cells. We observed an upregulation of *RFX4* and *SOX21* gene expression in GFP+, whereas there was no upregulation in control or GFP- cells (Figure 3B). There was only a slight upregulation in unsorted cells, revealing the importance of sorting for GFP+ cells specifically.

To investigate the effect of overexpressing these TFs in neural progenitor cells (NPCs), we infected hiPSC-derived NPCs with the validated overexpression lentiviruses. However, NPC infection with either RFX4 or SOX21 lentivirus showed minimal GFP fluorescence (Figure 3C). Without sufficient GFP signal, we could not collect enough GFP+ cells, meaning any downstream genomic analysis (qRT-PCR, RNA-seq, ATAC-seq) would be skewed by cells that did not uptake the lentivirus (GFP- cells). However, we performed qRT-PCR on the unsorted

NPCs and observed an upregulation of both *RFX4* and *SOX21*, especially in RFX4-infected cells, which showed a 35000-fold upregulation of RFX4 compared to the control (Figure 3D). This discrepancy between lack of fluorescence and acute mRNA overexpression indicates there may be a problem with GFP translation and protein expression in the infected hiPSC-derived NPCs. Since we observed GFP+ cells while generating lentiviruses in the HEK293T cells, we explored alternative transfection techniques such as plasmid transfection and nucleofection instead of lentiviral infection. However, these efforts resulted in similar fluorescence issues. To troubleshoot, we redesigned both RFX4 and SOX21 overexpression plasmids by placing TurboGFP under a separate promoter (hPGK) than the target TF (EF1a). We hypothesized that placing TurboGFP under a separate promoter would improve fluorescence by increasing the chance of TurboGFP transcription. We repeated infection experiments but did not see any significant differences in fluorescence after these adjustments. Given these problems, we pivoted our focus to testing the effects of LHX2 overexpression on astrocyte development due to successfully producing lentivirus.

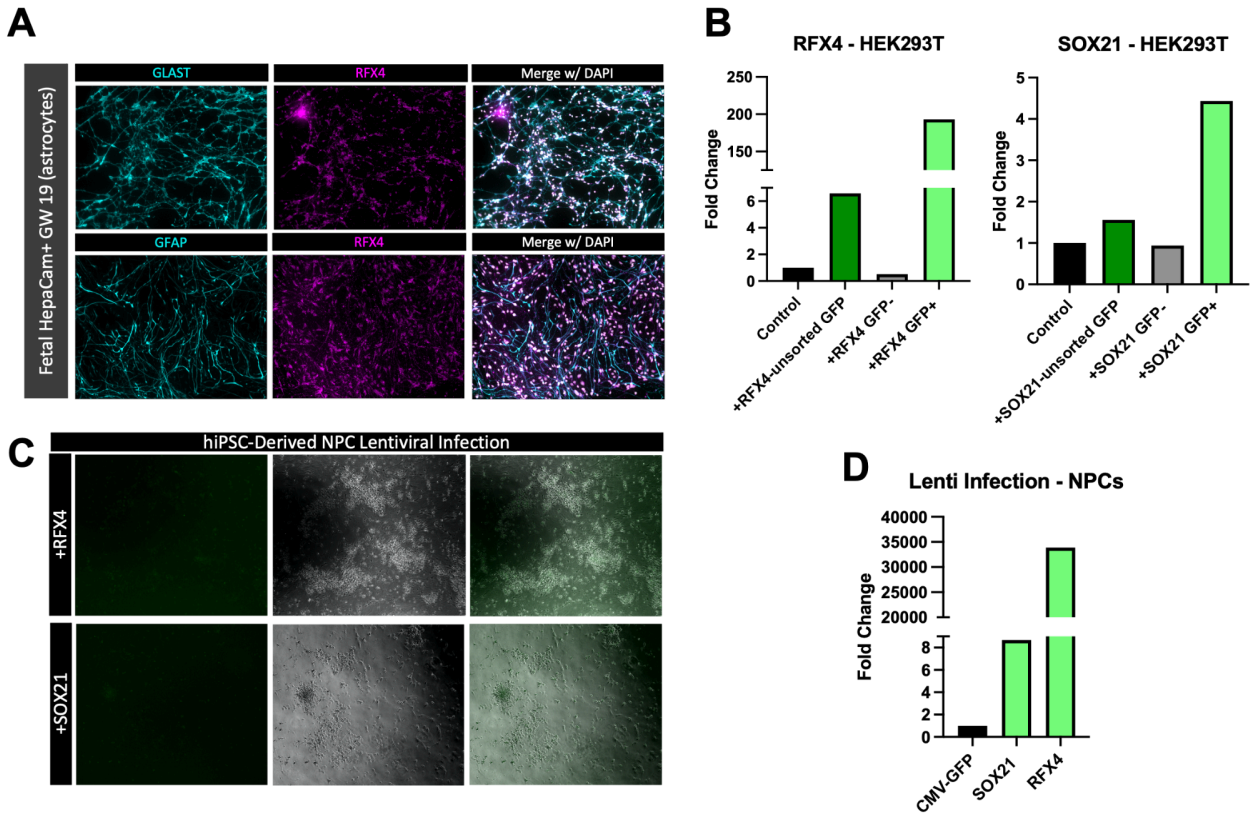


Figure 3. RFX4 and SOX21 Overexpression Lentivirus Infection in HEK293T and hiPSC-Derived NPCs. A Images of fetal astrocytes (HepaCam^{+}, GW19) stained with astrocytic markers (GLAST and GFAP; left), RFX4 (middle), and a merge with DAPI (right). **B** qRT-PCR data of GFP-sorted HEK293T cells infected with RFX4-GFP, SOX21-GFP, or CMV-GFP (control) overexpression lentiviruses. **C** Image of GFP fluorescence in hiPSC-derived NPCs infected with RFX4-GFP and SOX21-GFP overexpression lentiviruses. Left panels show GFP fluorescence. Middle panels show cell density. Right panels show overlay. **D** qRT-PCR data of hiPSC-Derived NPC lentiviral infections.

LHX2:

Lhx2 is a TF that has been associated with cell fate determination and patterning in the brain, especially in early development (Chou and Tole, 2018). *LHX2* has been implicated in the regulation of signaling pathways, most notably NOTCH and Wnt signaling, that are directly responsible for the rise of cell types and/or cortical and retinal structures (de Melo et al., 2016; Kinare et al., 2018). Lhx2 has been shown to promote neurogenesis while suppressing gliogenesis in the developing hippocampus by interacting with Pax6 and inhibiting NOTCH signaling (Subramanian et al., 2011). However, Lhx2 has also been found to be involved in astrocyte maturation in the cortex by influencing chromatin remodeling (Lattke et al., 2021). Given these conflicting reports and our paired analyses revealing a role of LHX2 in cortical astrocyte development (Figure 2A), we wanted to further investigate how overexpression of LHX2 might influence the gliogenic switch.

The lentiviral production and validation of LHX2 mirrored the same process as the previous two TFs. NPCs infected with *LHX2* overexpression lentiviruses did not exhibit the same fluorescence issues as *RFX4* and *SOX21*, allowing us to move forward with lentiviral infection. We tested LHX2 overexpression in both fetal and hiPSC-dervied NPCs.

We collected $CD15^+$ NPCs from human fetal brain samples via immunopanning. We specifically collected NPCs before (GW12-16) and after (GW19-21) the gliogenic switch in order to test whether the stage of the progenitor cell (neurogenic versus gliogenic) could influence the ability of LHX2 to drive astrocyte development. We infected the NPCs with either CMV-GFP (control) or LHX2-GFP lentivirus and measured branch length using a Fiji plug-in (Figure 4A). Measuring branch length is a simple way to visualize astrocytic structural changes due to LHX2 overexpression because shortening of processes or branches is a characteristic of immature astrocyte development (Freeman, 2016). LHX2-GFP+ GW12-16 $CD15^+$ cells showed

no significant difference (Two-sided Mann-Whitney U-test: $P = 0.2743$, $P = 0.0087$, $P = 0.2293$, $P = 0.4612$) in branch length compared to CMV-GFP+ (control), except for GW15 (Figure 4B and 4C). Interestingly, LHX2-GFP+ GW19-21 $CD15^+$ cells showed a significant decrease (Two-sided Mann-Whitney U-test: $P < 0.0001$, $P < 0.0001$, $P = 0.0001$) in branch length compared to CMV-GFP+ (Figure 4D and 4E). This decrease in branch length suggests that overexpressing LHX2 post-gliogenic switch promotes astrocytic morphological characteristics. As astrocytes mature, the ends of their processes or branches thin out and shorten, appearing more star-like (Freeman, 2016).

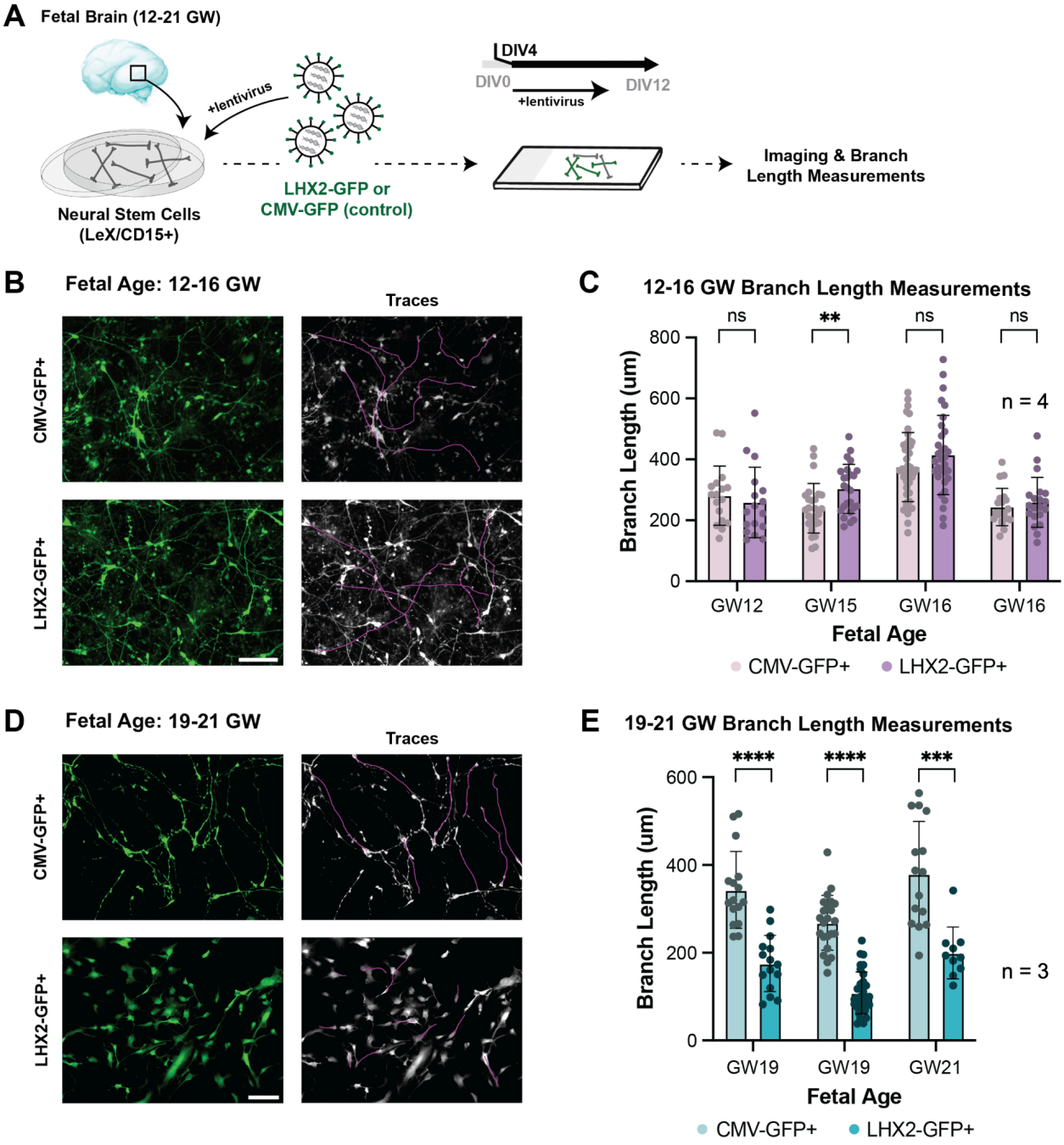


Figure 4. LHX2 Overexpression Promotes Astrocytic Characteristics Post-Gliogenic Switch. A Schematic of lentiviral infection and analysis. **B** Images and branch length traces of infected GW12-16 $CD15^{+}$ cells. Scale bar = 220 μm **C** Branch length (μm)

quantification of infected GW12-16 $CD15^+$ cells. Two-sided Left to right: P = 0.2743, P = 0.0087, P = 0.2293, P =1490.4612. n = 104 CMV-GFP+ cells and n = 94 LHX2-GFP+ cells across 4 fetal samples. **D** Images and branch length traces of infected GW19-21 $CD15^+$ cells. Scale bar = 100 μm **E** Branch length (μm) quantification of infected GW19-21 $CD15^+$ cells. Left to right: P < 0.0001, P <0.0001, P = 0.0001; n = 54 CMV-GFP+ cells and n= 57 LHX2-GFP+ cells across 3 fetal samples. All statistical tests are Two-sided Mann–Whitney U test. ns: not significant, P > 0.05; ** P < 0.01;*** P < 0.001; **** P < 0.0001. Data are presented as mean values. Error bars indicate standard error of the mean

Given the interesting differences in branch length when LHX2 is overexpressed post-gliogenic switch but not pre-gliogenic switch, we wanted to further understand how LHX2 influences astrogenesis at different stages of development. To test this, we designed an experiment where we infected hiPSC-derived NPCs at different time points during differentiation. We first cultured hiPSC-derived NPCs without differentiating them (d0, FGF2+). When the hiPSC-derived NPCs were about 50% confluent, we infected them with LHX2-GFP or CMV-GFP lentivirus. After 8 days in culture, we sorted for GFP+ cells and performed transcriptomics analyses. The timeline experiment consists of 3 more time points after d0. In order to generate a complete timeline, we cultured more hiPSC-derived NPCs, and when they were about 50% confluent, we started differentiation (d1, -FGF2). Following FGF2 withdrawal to induce differentiation, the cells were cultured for 8, 22, or 36 days and then infected with LHX2-GFP or CMV-GFP lentivirus. The cells were collected 8 days post-infection, sorted for GFP+ cells using FACS, and analyzed via bulk RNA-sequencing (Figure 5A). Each timepoint

was analyzed to observe the transcriptional changes occurring upon LHX2 overexpression across time.

To understand the influence of LHX2 overexpression on both neurogenesis and gliogenesis, we categorized the volcano plots based on changes in neuronal and astrocyte genes across the 4 time points (Figure 5B). In the neuronal volcano plots, there seems to be a stronger downregulation at earlier time points compared to later in differentiation. In the astrocytic volcano plots, there seems to be a stronger upregulation at earlier time points compared to later in differentiation. This may indicate that LHX2 has a stronger influence in hiPSC-derived NPCs early in development, compared to when the hiPSC-derived NPCs are being differentiated.

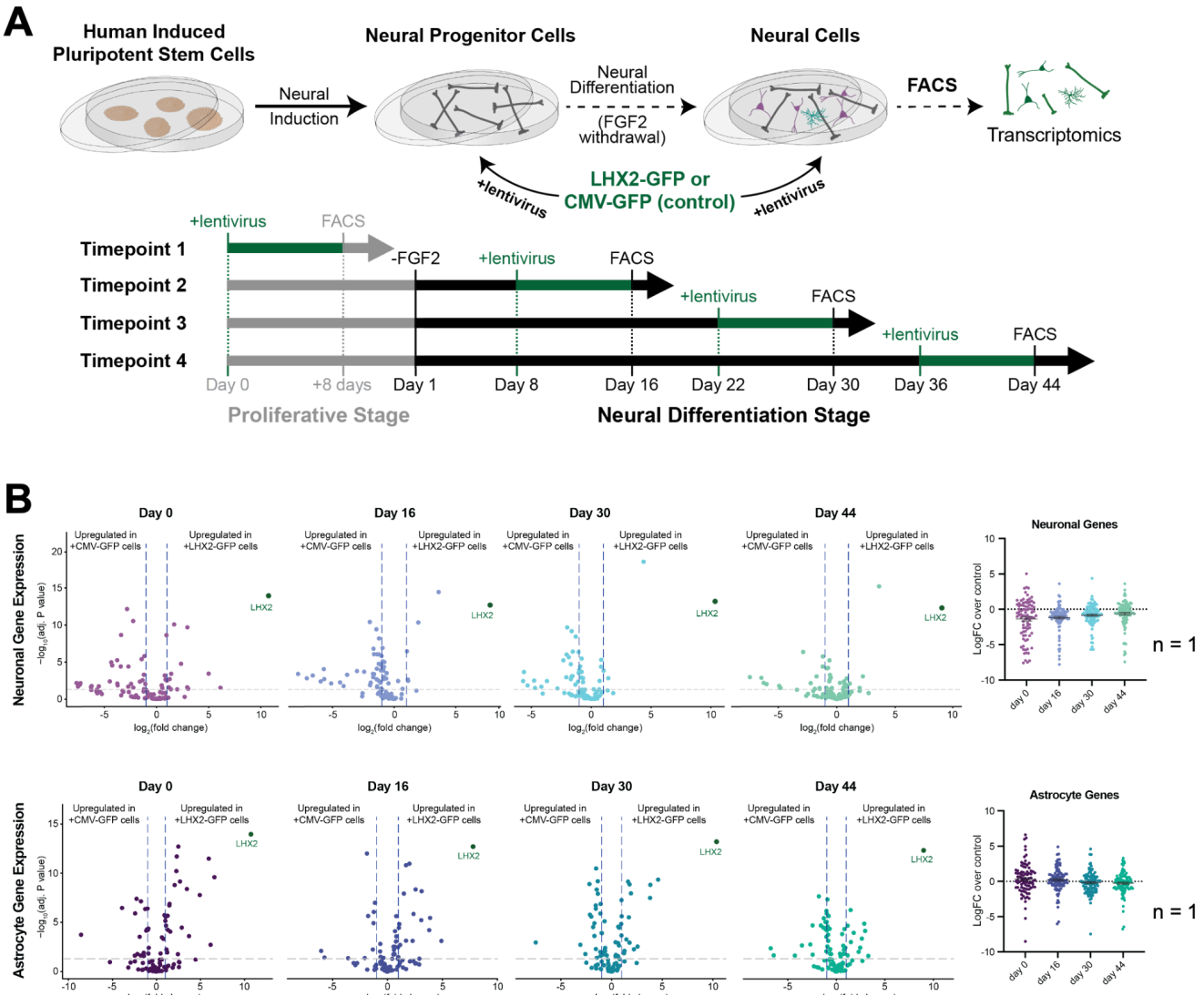


Figure 5. Effect of LHX2 overexpression across NPC differentiation. A Schematic of NPC differentiation and timeline of lentiviral infections. n = 1 hiPSC line **B** Volcano plots of bulk RNA-seq data throughout timeline experiment in neuronal and astrocyte genes in LHX2-GFP+ versus CMV-GFP+ (control) cells. Quantifications (far right) are depicted as Log(Fold Change [FC]) over control.

Chapter 6: Discussion and Future Directions

Astrocyte development involves numerous intricately coordinated events, all working together to generate functional astrocytes. This process begins with radial glia giving rise to neurons with influences from master regulators and signaling pathways. After the bulk of neurogenesis is completed, radial glia transition into producing astrocytes (Anthony et al., 2004; Kriegstein and Alvarez-Buylla, 2009; Takouda et al., 2017; Pavlou et al., 2019). We first investigated how knocking down NGN2, a master regulator of neurogenesis, affects the gliogenic switch by using shRNA lentiviruses. Our results showed that the NGN2 shRNA lentivirus successfully knocked down NGN2 expression. However, our results also showed the scrambled shRNA lentivirus knocking down NGN2 expression, making our data less convincing. We tried redesigning the scrambled shRNA but we still had issues with the control shRNA seemingly knocking down NGN2 expression. These results indicated that we had an unreliable positive control that would have invalidated any potential results with the experimental NGN2 shRNA. Due to the lack of confidence in the shRNA knockdown of NGN2, this prevented us from investigating the downstream effects on astrocyte production.

In effort to understand the driving factors of astrocyte production, we shifted to investigating the effect of overexpressing gliogenic TFs on the gliogenic switch. We identified candidate TFs from paired RNA-seq and ATAC-seq analyses and infected hiPSC-derived NPCs and fetal NPCs with overexpression lentiviruses of the candidate TFs. While RFX4 and SOX21 overexpression lentiviruses showed upregulation of gene expression, they also showed minimal fluorescence in cell culture. Due to issues with fluorescence, we were not able to assess the impact of overexpressing these TFs. To address this, we redesigned the plasmids by placing the fluorescent protein on a separate promoter and recreated the lentiviruses. We also explored other

infection and transfection methods, however, our efforts did not improve the fluorescence issue. Without fluorescence, there is no way to distinguish cells that received the virus through imaging or sorting. Without sorting, we would not be able to isolate and analyze the cells specifically with TF overexpression. Before moving forward, we will need to address and rectify the fluorescence issue.

LHX2 is another candidate gliogenic TFs we identified. Based on the high LHX2 regulatory potential for astrocyte genes and the ability to produce functional overexpression lentiviruses, we were able to infect human fetal NPCs and hiPSC-derived NPCs with the lentivirus. We collected human fetal NPCs at different gestational weeks before and after the gliogenic switch. We looked at branch length after LHX2 overexpression to determine any structural changes and found that LHX2 induces astrocytic characteristics after the gliogenic switch. Considering the extensive literature on how Lhx2 promotes neurogenesis and suppresses gliogenesis (Subrmanian et al., 2011; de Melo et al., 2016; Kinare et al., 2018), these were intriguing results because it potentially indicates that LHX2 has the ability to induce astrocytic morphological changes depending on the timing of when it is upregulated. To better understand the role of LHX2 at different stages of development, we infected hiPSC-derived NPCs at different time points in differentiation. We observed two opposing trends where neuronal genes were downregulated at earlier time points and gliogenic genes were upregulated at earlier time points. These combined trends may potentially point to an upstream effect that may be allowing for other TFs to influence astrocyte development. LHX2 is a TF that has been shown to highly regulate chromatin accessibility during development (Zibetti et al., 2019). Sox2 is a transcriptional target of LHX2 (Zibetti et al., 2019) and has been implicated in regulation of neurogenesis and gliogenesis by interacting with the Notch1, RPB-J, and Hes5 genes (Bani-Yaghoub et al, 2006). Overexpression of LHX2 could potentially promote chromatin

accessibility of Sox2, a transcriptional target, leading to an upregulation of the Notch pathway and inhibition of neurogenesis.

As it stands, this work provides a launching point to look into the inner workings of astrocyte development through manipulating TFs. Gaining insights into the driving factors of human astrocyte production contributes to the understanding of the early development of neurodevelopmental disorders. Despite the technical issues with the shRNA viral vectors, this could have provided insight into the role of NGN2 on the gliogenic switch. It is understood that NGN2 prevents early astrocyte production during neurogenesis (Sun et al., 2019), but it is not as well understood how NGN2 manipulates astrocyte production during the gliogenic switch. Technical issues with RFX4 and SOX21 lentiviruses prevented us from adequately exploring the role of these candidate TFs in astrocyte development. Without the fluorescence issue, we could have further assessed the direct role of these TFs on the gliogenic switch and astrocyte production. Luckily, the LHX2 overexpression lentiviruses did not pose fluorescence issues nor other technical problems. We were able to observe morphological changes after infecting human fetal NPCs at different time points before and after the gliogenic switch, suggesting that LHX2 takes on different responsibilities based on context. We were also able to observe genomic changes after infecting hiPSC-derived NPCs at different stages of differentiation, further supporting the different contextual roles of LHX2 in astrocyte development and potentially suggesting an upstream effect based on the neurogenic and astrocytic genomic trends.

Future Directions

If we are able to rectify the scrambled shRNA knockdown, we would begin infecting and knocking down NGN2 in hiPSC-derived NPCs, hCOs, and human fetal tissue. This would allow us to understand the structural and genomic changes of gliogenesis due to NGN2 knockdown.

We are interested in performing another timeline experiment where we would knockdown NGN2 at an early time point and collect at different time points leading up to the gliogenic switch. This would allow us to look at snapshots of the structural and genomic effects of knocking down NGN2 in hCOs. Since NGN2 is a master regulator of neurogenesis, we hypothesized that we would observe a lack of structural neuronal formation and potentially an early onset of astrocyte production.

If we are able to fix the fluorescence issue in the RFX4 and SOX21 overexpression lentiviruses, we would begin infecting our model systems to further understand the genomic and structural impact of these TFs. We are interested in carrying out similar experiments to LHX2 using hiPSC-derived NPCs and human fetal tissue.

Moving forward with LHX2, we would like to pinpoint other TFs and signaling pathways involved in the genomic and structural changes we observed by overexpressing LHX2. To do so, we would perform Western blots and co-immunoprecipitation to identify changes in signaling pathways or binding partners of LHX2. Specifically, we are interested in SOX2 as a potential downstream target of LHX2 due to its known role in Notch signaling and its relationship with LHX2. By identifying these other TFs and signaling pathways, we can further characterize the role of LHX2 and relate it to other known pathways and transcriptional networks. We are also interested in collecting human fetal astrocytes and staining for known astrocyte markers, like SOX9 and GLAST, after LHX2 infection to visually understand the role of LHX2 in astrocytes.

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