Investigating the Role of Pseudouridylation in Neuronal Development

BACKGROUND

RNA modifications, also known as epitranscriptomics, are emerging as a novel layer of dynamic gene regulation [1]. RNA modifications alter existing RNAs' structure and function to influence various cellular pathways via RNA processes such as transcription and translation [2, 3]. Pseudouridine (Ψ) was the first RNA modification discovered [1]. Despite being the most abundant and widespread RNA modification in living organisms, little is known about its function. **In this proposal, I will establish an approach to systematically investigate the biological roles of pseudouridine by focusing on the unique activities that this modification imparts to RNA.**

The isomerization of uridine to pseudouridine by pseudouridine synthases (PUS enzymes) structurally stabilizes RNAs via the formation of an extra hydrogen bond donor [3]. Ψ was previously thought to primarily stabilize tRNA and rRNA; however, new developments in modern-sequencing techniques reveal the more interesting downstream effects of pseudouridylation. For example, there is evidence that H/ACA RNPs, RNA-dependent PUS enzymes convert stop codons into sense codons in yeast [2]. Interestingly, PUS7-mediated pseudouridylation has been found to regulate stem cell growth and fate determination partly through tRNA modification, which activates tRNA-derived fragments to inhibit protein synthesis [3]. This provides evidence that Ψ modifications may be critical in cell lineage commitment.

Specifically, mutations associated with several pseudouridylating enzymes, namely PUS1, PUS3, and PUS7, are associated with neuronal disorders and intellectual disability [4]. PUS1 mutations are associated with cognitive impairment [5]. Additionally, PUS1 acts on the steroid RNA activator, a co-activator of the nuclear estrogen receptor α regulating neuronal survival [5]. Truncated PUS3 and reduced levels of ψ U39 in tRNA were detected in patients with intellectual disability [5]. Lastly, mutations in PUS7 can cause intellectual disability and microcephaly in humans [4]. These studies suggest that Ψ can substantially impact neuronal differentiation and function; however, the mechanisms regarding pseudouridylation in neurogenesis are poorly understood**. My results will contribute to our understanding of the importance of pseudouridylation in neurogenesis and neuron function.**

PROPOSED RESEARCH

My overarching goal is to elucidate the molecular and functional roles of Ψ in neuronal cells. Based on the reported links to human brain function, **I hypothesize that Ψ is a critical modification for neuronal differentiation and function. I further propose that the Ψ landscape between stem cells and neurons is unique and specific.** I will test the above hypotheses with the following aims:

Aim 1—To identify pseudouridylating enzymes for investigation. There are 13 known PUS enzymes in human cells; however, only a few predicted human PUS enzymes have been studied to date. In addition to PUS1, PUS3, and PUS7, I will predict other PUS enzymes associated with neuron function by first performing weighted gene co-expression network analysis (WGCNA). This analysis will reveal sets, or modules, of highly correlated genes. To perform WGCNA, I will use multiple human tissue RNA sequencing datasets from the GTEx project. I expect PUS1, PUS3, and PUS7 to appear in one module because these genes share connections to neuronal function. Other PUS enzymes that are expected to correlate with neuronal genes will fall in this module. I will then conduct gene ontology enrichment analysis to verify that the PUS candidates in that module are associated with neuron processes. *These experiments will predict key pseudouridylating enzymes in addition to PUS1, PUS3, and PUS7 that will be investigated in neurons.*

Aim 2—To investigate the effects of PUS enzymes on neurogenesis. To define the impact of PUS enzymes on early neurogenesis, I will use CRISPR/Cas9 to knock out each candidate gene identified in aim 1 in iNGN cells. iNGN cells are a human induced pluripotent stem cell line which has been engineered to be readily induced into neurons within four days by doxycycline [6]. I will design a gRNA that targets the N-terminal coding exon of each gene to induce nonsense-mediated mRNA decay and validate that the enzyme is no longer expressed via Western Blot.

I will explore two different methods of inducing edited-iNGN cells into neurons to gather more comprehensive results. For the first method, I will supplement cell media with doxycycline to induce the

formation of neurons. Since this procedure only takes four days, it will allow for efficient generation of easily reproducible data. However, the rapid induction of robust neuronal morphology by doxycycline may limit the resolution of detectable phenotypic changes in these cells. Thus, the second, slower method enables me to mark differences at each stage during differentiation. I will differentiate successfully edited colonies of iNGN cells using a slow differentiation method following a previously published protocol [7]. I will use an inducible Cas9 system to knock out the PUS enzyme at different time points to determine the most critical points of pseudouridylation during neurogenesis [8]. To detect potential morphological alterations, I will use a neurite outgrowth assay and monitor the expression of neuronal markers via immunofluorescence. Rescue of the phenotypes by ectopic expression of the wild-type protein will control for the specificity of the effects. I will select the cell lines with the strongest phenotypes for additional study in aim 3. *These studies will reveal how PUS enzymes impact neuronal maturation.*

Aim 3—To determine neuron-specific RNA targeting by PUS enzymes. To determine the positions of RNA binding by PUS enzymes at single-nucleotide resolution, I will perform UV cross-linking and immunoprecipitation, followed by high-throughput sequencing (iCLIP-seq) in iNGN-derived neurons expressing Flag-tagged PUS enzymes. I will corroborate these results by using specific antibodies to pull down endogenous PUS complexes. Mock-infected cells will be used as a control to exclude non-specific RNA binding. I will complement these results with Ψ-seq to confirm that the PUS-bound sites are catalyzed. A comparison of the sites bound in stem cells and neurons will reveal neuron-specific Ψ sites. Because PUS enzymes may have multiple substrates, it may be unclear how to assign mutant phenotypes to loss of modification in specific RNA species. I will control for this variable via rescue experiments by transfecting specific synthetic pseudouridylated RNA substrates identified by Ψ-seq experiments. *These experiments will reveal the Ψ landscape in neurons and identify RNA targets for future study.*

Summary: Successful completion of these aims will shed new light on the poorly understood but critical roles that pseudouridylation plays in neuronal development. A potential follow-up study to investigate the function of Ψ in the identified RNA targets is an RNA pull-down assay. To determine the role of Ψ on the complex composition of RNA species, Ψ and non-Ψ RNA probes tagged with biotin can be pulled down, and the interactome can be analyzed by mass spectrometry. Future research into the biological roles of RNA modifications will reveal novel causes of neurological disorders.

Intellectual Merit: In Dr. Murn's lab, I've gained the necessary training in molecular biology techniques and RNA biochemistry to carry out this project. I am currently optimizing pseudouridine-seq and will use my experience in this technique for this proposal. I will receive the bioinformatic training necessary to carry out my proposal through future mentoring from Dr. Chaolin Zhang. Dr. Zhang's lab at Columbia University is an ideal fit because of his focus on RNA-protein interactions, RNA regulatory networks in neural development, and expertise in high-throughput transcriptomic data analysis.

Broader Impacts: As a graduate student and later as a professor, I will mentor undergraduate women of color and encourage them to pursue research careers. I will continue to empower young high school women by establishing additional chapters of Queens of STEAM. The support of the NSF GRFP will enable me to carry out my research while continuing STEM outreach.

[1] I. A. Roundtree, M. E. Evans, T. Pan, and C. He, "Dynamic RNA Modifications in Gene Expression Regulation," *Cell*. 2017. [2] J. Karijolich and Y. T. Yu, "Converting nonsense codons into sense codons by targeted pseudouridylation," *Nature*, 2011. [3] N. Guzzi *et al.*, "Pseudouridylation of tRNA-Derived Fragments Steers Translational Control in Stem Cells," *Cell*, 2018. [4] H. Darvish *et al.*, "A novel PUS7 mutation causes intellectual disability with autistic and aggressive behaviors," *Neurology: Genetics*. 2019. [5] M. T. Angelova *et al.*, "The emerging field of epitranscriptomics in neurodevelopmental and neuronal disorders," *Frontiers in Bioengineering and Biotechnology*. 201. [6] V. Busskamp *et al.*, "Rapid neurogenesis through transcriptional activation in human stem cells.," *Mol. Syst. Biol.*, Nov. 2014. [7] Y. Shi, P. Kirwan, and F. J. Livesey, "Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks," *Nat. Protoc.*, Oct. 2012. [8] K. I. Liu *et al.*, "A chemical-inducible CRISPR-Cas9 system for rapid control of genome editing," *Nat. Chem. Biol.*, 2016.