Characterization of MuSK Transcripts and Proteins in Neural Stem Cells

By

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B.S., Cornell University, 2022

Thesis

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Abstract of Characterization of MuSK Transcripts and Proteins in Neural Stem Cells, by Sei Hyun Choi, ScM, Brown University, May 2024.

Population over the age of 65 is at a high risk of neurodegenerative diseases, especially Alzhiemer's disease (AD), but there are currently no effective therapeutics to treat AD. Adult Hippocampal Neurogenesis (AHN), a process in which Neural Stem Cells (NSCs) proliferate and differentiate into newborn neurons, is significantly decreased in AD patients. Bone Morphogenic Protein (BMP) signaling increases in these patients and inhibits AHN. Although blocking the BMP signaling pathway to increase AHN seems appealing, it is not ideal to completely abolish BMP because BMP is involved in several other pathways. One possible drug target for increasing AHN is the Muscle Specific Kinase (MuSK) protein. MuSK Immunoglobulin 3 domain interacts with BMP and enhances BMP signaling pathway to transcribe AHN-suppressing genes. The Fallon Lab aims to induce alternative splicing with antisense oligonucleotides (ASO) and skip MuSK Ig3 domain to improve AHN in AD patients. While the Fallon Lab successfully designed lead candidate ASOs and assessed their efficiency in myoblasts, we have limited knowledge on MuSK expression on our cell of interest, NSCs. To examine whether our ASOs have a valid target in NSCs, I profiled MuSK at the transcript and protein levels in NSCs. Previous studies in the Fallon Lab discovered the MuSK transcript variant T2 in the hippocampus, which lacks Ig1 and Ig2 domains. In this study, I extended this variant study and demonstrated that T2 transcript spans the Ig3 domain to tyrosine kinase domain. I also observed that T2 is the predominant transcript of MuSK in the hippocampus, but not in the rest of the brain or in NSCs. Using immunocytochemistry, I also found that MuSK protein encoded by the T1 transcript is expressed in NSCs, indicating that NSCs will be targeted by our ASOs and may induce AHN. Our future work will focus on assessing the effect of ASOs on the proliferation of NSCs.

Chapter 1: Introduction

1.A. Neurodegenerative Diseases

Over the past decades, researchers have made great progress in conquering lethal diseases, increasing human lifespan by 6 years between 1970 and 2020 [1]. However, as life expectancy increases, the risk of neurodegenerative diseases increase in tandem. Circuit-selective dysfunction and loss of synapses are the biggest hallmark of neurodegenerative diseases, often due to the accumulation of pathological aggregates in the brain parenchyma [2]. Aging is the most well known risk factor in many neurodegenerative diseases, especially jeopardizing the population over the age of 65 [3, 4]. Despite the large number of patients and the population at risk, the mechanism of many neurodegenerative diseases is still poorly understood, hindering drug development.

Alzheimer's disease (AD) is the most common form of dementia, characterized by progressive cortical and hippocampal shrinkage and expansion of lateral ventricles that consequently accompanies cognitive decline and memory loss [5]. Microscopically, extracellular amyloid beta plaque and intracellular neurofibrillary tau tangles are accumulated in the cortex of AD patients [3]. In familial Alzheimer's disease, missense mutations in amyloid precursor protein (APP) or presenilin (PSEN) 1 and 2 increase the penetrance of the disease [3]. The apolipoprotein E4 (apoE4) gene also exerts a gene-dosage effect in AD; homozygosity for apoE4 gene accelerates the rate of neuronal dysfunction [6]. Nevertheless, the aforementioned genes are merely the risk factors of AD and poor diagnostic criteria especially in sporadic AD.

Until recently, the majority of researchers strongly advocated the amyloid beta hypothesis - amyloid beta oligomerization and toxic plaque formation initiate the cascade of action that led

to AD [7]. However, the studies on the amyloid beta plaque and AD are contradictory; the amyloid beta plaques can be found in healthy individuals and is poorly correlated to sporadic AD [7]. Indeed, a myriad of attempts to target amyloid plaques and tau tangles have been reported unsuccessful [8]. Recent study points to the abnormal immune response to be the sheer cause of AD, where pathological amyloid beta plaques and tau tangles call forth microglial activation [9]. According to Pascoal, the disease-inducing aggregates cause pro-inflammatory reactions that damage the brain parenchyma and lead to AD phenotypes. The study indicates that toxic aggregates are not the only culprit of AD, complicating the drug development.

Last year, the CDC reported that 6.7 million Americans are suffering from Alzheimer's disease, the number which is predicted to triple in 2060; biopharmaceutical industries are in a pressing need to find a remedy for AD [5]. However, there are currently no effective therapeutics to treat AD. In fact, researchers are only beginning to unveil the mechanism of AD. Last year, Lecanemab was approved by FDA for treating early stage familial AD, but shows low clinical efficiency to treat severe AD or sporadic AD [10]. In late stage AD, once neural death occurs, clearing amyloid beta plaque may be insufficient to restore memory loss.

In this study, we aim to ameliorate the memory loss in neurodegenerative diseases by increasing adult hippocampal neurogenesis, which is largely decreased in aged individuals and AD patients. The Fallon Lab designed ASOs to activate neural stem cell niche in the hippocampus. Neural stem cells will proliferate and differentiate into functional adult neurons, aiding new memory formation. Our ASO may be simultaneously used with other drugs clearing amyloid beta plaques that were less effective in treating AD phenotypes.

1. B. Adult Hippocampal Neurogenesis

During embryonic development, neural stem cells (NSCs) actively proliferate and differentiate in the ventricular zone, mature into different components of the central nervous system (CNS), and migrate out to the intermediate zone [11]. It was widely believed that neurogenesis halts after early stages of life, and NSCs are depleted after development [12, 13]. The small population of quiescent NSCs in the brain was undetected to the researchers for a very long time. However, recent studies showed that neural stem cells are capable of neurogenesis in adulthood, often generating new neurons to compensate for brain damage [14]. Adult neurogenesis is most actively detected in the dentate gyrus (DG) of hippocampus and the subventricular zone (SVZ) of the lateral ventricles [15]. Our primary focus in this study will be the neurogenesis in the hippocampus, for the hippocampus is one of the affected brain regions in AD patients.

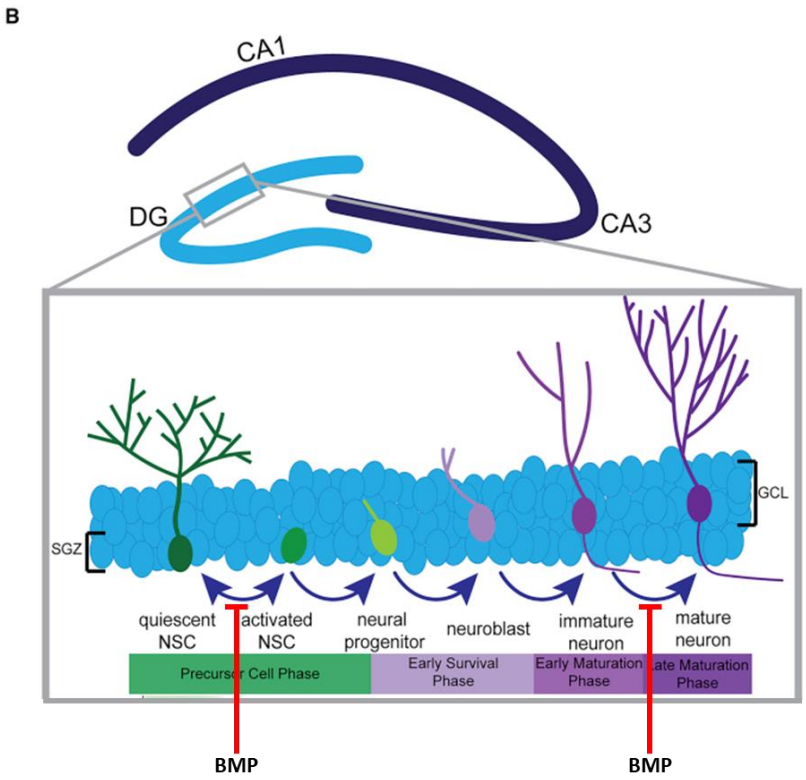

Figure 1. Adult Hippocampal Neurogenesis in Mice. Schematic representation of adult hippocampal neurogenesis in the mouse. Quiescent NSCs become activated to generate daughter cells that either return to quiescence for self-renewal of the NSC pool or differentiate into neurons. Neural progenitors that survive beyond the early survival stage mature into granule neurons and integrate into hippocampal circuitry. BMP inhibits AHN at two stages: NSC activation and integration/maturation of newborn neurons. Figure is modified from Babcock *et al.*, 2021, *Stem Cell Reports*. Figure legend is adapted from Babcock *et al.*, 2021, *Stem Cell Reports*.

The hippocampus plays a role in learning and memory formation, as well as regulation of emotion [16]. Adult hippocampal neurogenesis (AHN) is a process in which NSCs proliferate and differentiate into newborn neurons, which enter the hippocampus circuitry as granule cells and form new memories [16]. In mice, adult-born granule cells integrate into DG circuitry and help distinguish identical environmental cues in different places, or pattern separation [17]. NSCs residing in the subgranular zone (SGZ) interconvert between quiescent and activated states; generally, NSCs are largely quiescent in the adult brain (Figure 1). Upon activation, NSCs undergo either symmetric division to sustain the NSC pool or asymmetric division to generate newborn neurons [18].

A decrease in AHN is observed with natural aging, but the decline is more prominent in neurodegenerative disease patients [18]. In neurodegenerative disease patients, there is a higher threshold for NSC activation; the NSCs often remain in quiescent state or differentiate without proliferating, depleting the stem cell pool [18]. Significant drop of AHN in AD patients can macroscopically be observed by the shrinkage of the hippocampus [19]. Increasing AHN in 5xFAD mice, a widely used mouse model for AD, with exercise rescued them from the diseased phenotype, measured by increase in DCX-positive newborn neurons and improved performance on behavioral tasks [20]. This study is the first study to show the possibility that AHN can be a druggable target for AD.

Bone Morphogenic Protein, or BMP, plays a role in regulating stem cell activation. BMP suppresses NSC activation through BMP Receptor 1A (BMPR1A) in the hippocampus, preventing stem cell depletion (Figure 1) [15]. BMP signaling significantly increases with age, which may be the cause of decrease in AHN in the aged population [15, 18]. Study also reports

that BMP6 increases significantly in AD patients, keeping more NSCs quiescent and hindering AHN [21].

The study shows that BMP-treated NSCs were suspended in the quiescent state, whereas BMP antagonist Noggin activated NSCs and increased NSC proliferation [22]. However, it is nearly impossible to use BMP antagonist as a treatment, for BMP is involved in numerous pathways beside stem cell regulation, such as development and maintenance of bone and muscle [23]. To add on, Noggin was only transiently successful in increasing AHN because the initial increase soon depleted the stem cell pool [22]. We need to search for a better target that can increase both symmetric and asymmetric division of NSCs.

1. C. MuSK-BMP Pathway

Muscle-specific kinase, or MuSK, is a kinase protein mainly located in the neuromuscular junction (NMJ), involved in NMJ formation and maintenance [24]. It has three immunoglobulin (Ig) domains, frizzled cysteine-rich (CRD/FZ) domain, and tyrosine kinase (TK) domain. MuSK is known to play a role in the Agrin-LRP4 pathway by binding to LRP4 and phosphorylating intracellular Dok7, arranging NMJ structure [25]. The less known Wnt-MuSK signaling pathway regulates innervation of muscle and stem cell proliferation [26]. The Fallon Lab further demonstrated the role of MuSK in the BMP signaling pathway [27].

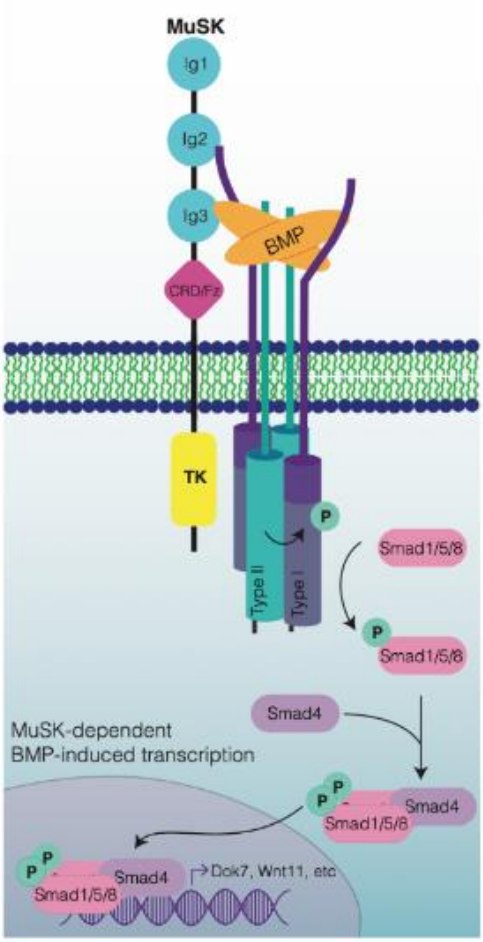

Figure 2. MuSK-BMP Pathway. BMPs bind MuSK via the Ig3 domain. MuSK-BMP signaling induces Smad1/5/8 phosphorylation and induces expression of a set of MuSK-BMP dependent transcripts in myoblasts and myotubes. MuSK also binds Type I BMP receptors via the Ig2 domain (Jaime, Fallon, unpublished). The MuSK-BMP pathway does not require MuSK tyrosine kinase activity. Figure is modified from Fish and Fallon, 2020, *Neurosci Lett*. Figure legend is adapted from Fish and Fallon, 2020, *Neurosci Lett*.

In the newly identified MuSK-BMP pathway, BMP-BMP receptor complexes phosphorylate Smad, which signals the cell to transcribe Dok7, Wnt11, etc (Figure 2) [25]. These

transcripts maintain the neural stem cells in quiescent state, as mentioned in chapter 1B. MuSK is a BMP co-receptor, where BMP binds to Ig3 domain, and BMP receptor type 1 binds to Ig2 domain to initiate the signaling cascade (Figure 2). Unlike the canonical MuSK signaling pathways, the MuSK-BMP pathway does not require a tyrosine kinase domain [27].

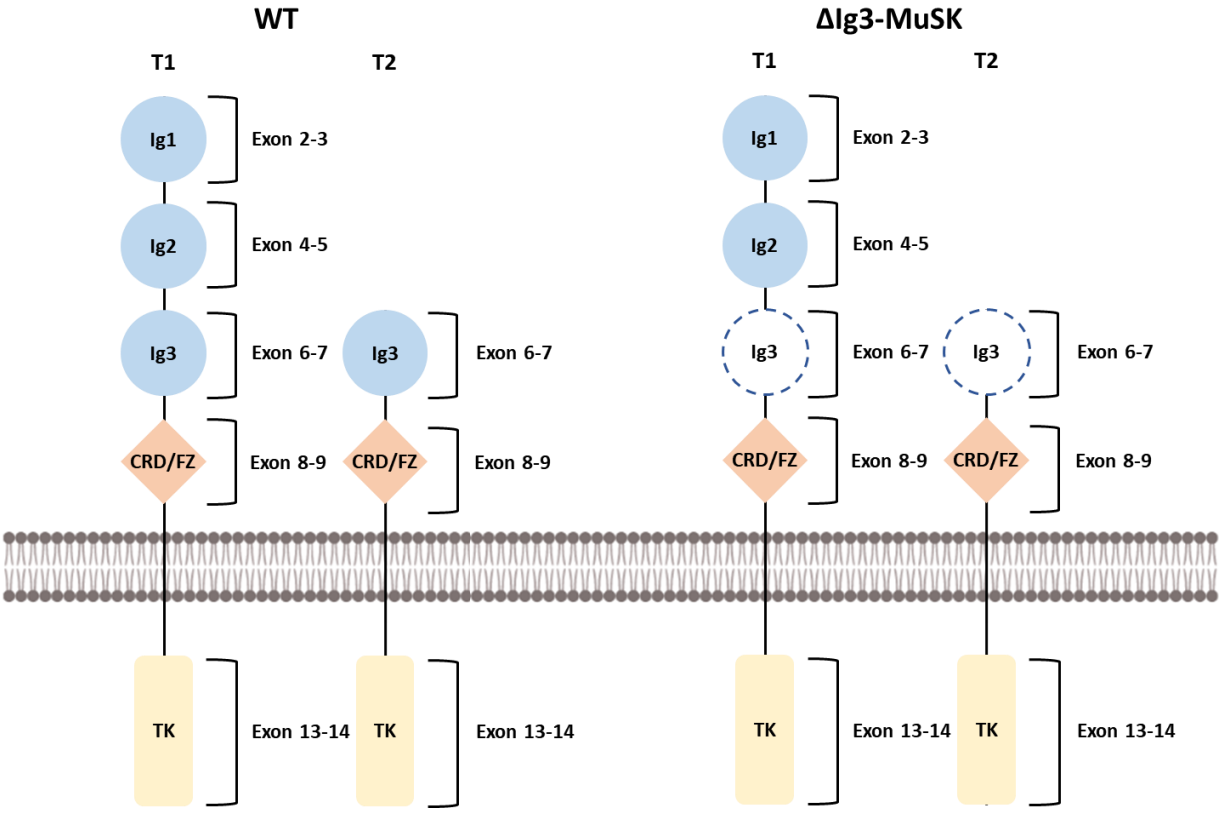

Figure 3. MuSK Transcript Variants in WT and ΔIg3-MuSK mice. T1, an abbreviation for transcription start site 1, is a conventional full-length MuSK. T2, an abbreviation for transcription start site 2, is a novel short-form transcript variant, identified by Julia Hecking. Julia Hecking confirmed that T2 is present in both WT and ΔIg3-MuSK mice. The protein domains and the corresponding exons are illustrated. In ΔIg3-MuSK protein, the absence of Ig3

domain is marked with the dotted lines. Exons 10-12 encode intermembrane polypeptides between the CRD/Fz and TK domains.

Julia Hecking, a former graduate student of the Fallon Lab, identified a novel transcript variant of MuSK unique to the brain and was not present in the muscle. This transcript variant was shorter than the conventional full length MuSK, excluding exons encoding the Ig1 and Ig2 domains (Figure 3). This short form of MuSK is annotated in the human genomic library, but not in mouse genomic library (Supplementary Figure 1). Our lab speculated that there is a distinct transcription start site in between exon 5 and 6 and named this transcript variant T2, an abbreviation for transcription start site 2. Until this study, we had limited knowledge of the structure of the T2 transcript. The RT-qPCR experiment with Taqman probe annealing to exons encoding different MuSK domains was performed to investigate the T2 RNA structure (Figure 5).

Previous study shows that Ig3 is not involved in the canonical MuSK pathways but only binds to BMP to initiate the signaling cascade [27]. Our lab thus engineered ΔIg3-MuSK mice using CRISPR-Cas9 system, which excised exon 6 and 7 that encodes for Ig3 domain. ΔIg3-MuSK mice had a lifespan comparable to that of the WT mice. Without the Ig3 domain to communicate with BMP, ΔIg3-MuSK showed reduced BMP responsiveness and enhanced AHN. Their increase in cognitive ability compared to their age-matched WT counterparts was confirmed with a novel object location experiment. However, we cannot use CRISPR-Cas9 to the AD patients to elicit the increase in AHN. Therefore, we designed antisense oligonucleotides to alternatively splice the Ig3 domain and mimic the ΔIg3-MuSK phenotypes.

1. D. Antisense Oligonucleotide

Antisense oligonucleotide (ASO) is an oligomer of approximately 25 nucleic acids, targeting mRNA to treat diseases. Although the ASO approach was first proposed in 1978 by Zamecnik, it took another twenty years for the ASO drug to be approved [28, 29]. In ASO therapeutics, mRNA is considered as a receptor; ASO binds to mRNA to modify gene expression. ASO is highly advantageous in that the 'receptor-binding' mechanism is well understood with Watson-Crick base pairing, which allows highly specific drug-target binding [29]. Furthermore, it is relatively easy to design ASO drugs once the gene sequence of the target mRNA is elucidated [29]. Together, ASO is efficiently used to yield desirable changes in gene expression.

There are ASO therapeutics already approved in the market to treat neurodegenerative diseases. For instance, Eteplirsen was designed to induce exon skipping to treat Duchenne muscular dystrophy (DMD), and Nusinerson triggers exon inclusion to treat Spinal Muscular Atrophy (SMA) [30, 31]. These precedents open the possibility of treating AD with ASOs. In our lab, we would like to imitate the outcomes of genetically modified ΔIg3-MuSK mice using ASOs.

The Fallon Lab designed ASOs to induce alternative splicing of the Ig3 domain, a binding domain for BMP. The effective ASOs will reduce BMP signaling and increase AHN while not interfering with other MuSK exons. Faith Keller, a former graduate student in the Fallon Lab, screened 25mers spanning intron 5 to exon 7 region. Faith detected successful skipping of Ig3 domain in ASO-treated myoblasts and selected three lead candidates. However, the effect of ASOs on our target cells, NSCs, remains unclear. Thus, we aim to profile MuSK

expression in NSCs before transfecting NSCs with our lead ASOs and investigate their efficiency in inducing alternative skipping.

Cell transfection is the traditional method to express foreign genes of interest *in vitro* [32]. However, previous research showed that NSC transfection with transfection agents were largely unsuccessful, the most successful transfection rate being under 20 % [33, 34]. Effective NSC transfection protocol is yet to be established. The Fallon Lab had not yet tested our lead ASOs in the relevant cell type, if not any non-muscle cells. Our first step will be developing the effective transfecting protocol for NSCs.

One possible strategy to transfect NSCs is gymnotic delivery, or transfection reagent-free uptake [35]. The advocates of gymnotic delivery suggest that gymnosis is ideal in predicting *in vivo* nucleic acid uptake, where foreign messages such as ASOs are delivered without transfection reagents [35, 36]. Moreover, gymnosis is rising as an alternative method to transfect the cells that show low efficiency with transfection reagents, namely NSCs [35]. In the future study, we will transfect NSCs with either RNA transfection reagent or gymnotic delivery.

1. E. Conclusion

The search for treatment of neurodegenerative diseases, especially AD, is in a pressing need. As life expectancy increases, more populations are at risk of neurodegenerative diseases, exerting high economic burden to the community. While BMP inhibition showed significant effect in increasing AHN, it is not a plausible treatment, for BMP is involved in many other pathways. We will thus induce AHN by blocking the MuSK-BMP pathway using ASO to induce

alternative splicing. This study is critical for the design and interpretation of the future studies using splice modifying antisense oligonucleotide (ASO) to promote the expression of delta Ig3-MuSK, which can be a potential treatment for neurodegenerative diseases. We expect our ASOs to alleviate memory loss in AD by reducing BMP responsiveness and increasing AHN, which will ultimately lead to increased cognitive ability.

Chapter 2: Methods

Euthanasia and Tissue Harvest

Animals were euthanized with CO_2 (2 L/min flow rate) and sacrificed by cervical dislocation. Whole brain dissection was performed immediately after euthanasia. Hippocampi from both hemispheres were harvested. The entire hemisphere excluding the hippocampus was harvested into the "Rest of Brain (RoB)" sample. The tissues were snap frozen in LN_2 and kept in -80 °C until usage.

Non-Adherent Cell Culture

The stock of WT and ΔIg3 neural stem cells (NSCs) were harvested from the mouse hippocampus by the lab of Ashley Webb at Brown University and stored in LN_2 until usage. NSCs were thawed and plated at 50,000 cells/mL on a 10 cm plate for expansion. NSCs were fed every other day with renewing media, which included NeuroBasal A (Gibco™), 1 % P/S/Q, and 2 % B27 (Gibco™), and supplemented with growth factors EGF (Gibco™) and bFGF (Gibco™). NSCs were incubated at 37 °C and 5 % CO_2. When NSCs proliferated and formed neurospheres, which typically takes 5-7 days, they were dissociated into single cells with 1 mL accutase (Corning™).

Adherent Cell Culture

Six-well plate was coated with 50 μg/mL Poly-D-Lysine (PDL) in PBS for 4 hours. The wells were washed with PBS after PDL coating, and 600,000 cells in renewing media were plated per well. NSCs were incubated at 37 °C and 5 % CO_2 until the full adherence was observed under light microscope (typically 24-48 hours after plating).

RNA Extraction and Reverse Transcription

RNA was isolated from both hippocampus and RoB tissue samples using the RNeasy® Lipid Tissue Mini Kit (Qiagen) according to the manufacturer protocol. RNA was isolated from NSCs using the RNeasy® Mini Kit (Qiagen) according to the manufacturer protocol. The RNA concentration was measured with a NanoDrop 2000 Spectrophotometer (ThermoFisher). For each sample, a maximum of 2500 ng RNA were reverse transcribed into cDNA with the SuperScript™ IV VILO™ Master Mix Kit without gDNA digestion. Both RNA and cDNA samples were kept in -80 °C until usage.

Quantitative RT-PCR

MuSK gene expression *in vitro* and *in vivo* was analyzed with RT-qPCR using TaqMan Gene Expression Assays (Applied Biosystems). Following probes were used for analysis: β-2-microglobulin (Mm0437762_m1), the exon 4-5 junction of MuSK transcript (Mm00448006_m1), the exon 6-7 junction of MuSK transcript (Mm01346929_m1), the exon 8-9 junction of MuSK transcript (Mm00448008_m1), the exon 11-12 junction of MuSK transcript (Mm00448011_g1), and the exon 13-14 junction of MuSK transcript (Mm01346926_m1). The gene expression level was further analyzed with ΔCT, with β-2-microglobulin as a reference gene.

Immunofluorescence

NSCs were plated at 60,000 cells/coverslip on PDL-coated coverslips and incubated overnight at 37 °C and 5 % CO_2. NSCs were fixed with 1 % PFA and stained with DAPI, a human monoclonal anti-MuSK Ig2 antibody 189-1 generously provided by K. O'Connor at Yale

University [37], and a mouse monoclonal anti-MuSK Ig3 24C10 generously provided by Amy Payne at University of Pennsylvania [38]. Normal human IgG (Invitrogen) and PBS was used as a negative control. For secondary antibodies, donkey anti-human AlexaFluor 488 and goat anti-Mouse AlexaFluor 555 were used.

Chapter 3: Results

3.A. MuSK Expression in the Mouse Brain

First, to evaluate the transcription profile of MuSK in the brain, I conducted an RT-qPCR experiment on the mouse brain samples. Our lab previously observed a novel transcript variant of MuSK, T2, in qPCR analysis of mouse hippocampal RNA, analyzed based on the difference between the expression of the exons encoding the Ig1/Ig2 and Ig3 gene expression levels. Where T2 transcription starts and ends remains unknown, although the annotated MuSK short form transcription variant in the human database suggests that T2 extends to the tyrosine kinase domain-encoding exons (Supplementary Figure 1). In this chapter, I further investigated the RNA structure of T2 to confirm that the mouse T2 transcript also includes the exons encoding the tyrosine kinase domain. Furthermore, I compared the levels of the MuSK transcript variant in the hippocampus and 'Rest of the Brain (RoB),' which includes every brain region except hippocampus and olfactory bulb.

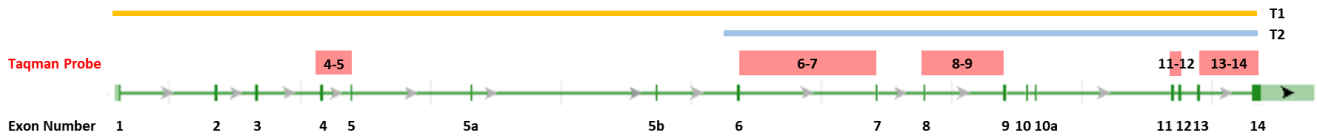

Figure 4. MuSK transcript map with Taqman probe spanning exon junction regions. The *Mus musculus* MuSK transcript in green line is from the NCBI gene browser, XM_006537659.4. Each vertical line represents the MuSK exon, which is enumerated in the black letter. T1 and T2 spanning regions are marked on top with the orange and blue lines, respectively. The exons with alphabet letters are alternatively spliced. The top red boxes represent Taqman probes spanning exon junction regions. All Taqman probes used in this study are shown in the figure. Also refer to Figure 3 for the MuSK protein structure and the corresponding exons.

To examine the transcript structure of T2, I conducted an RT-qPCR experiment with the Taqman probe sets (Figure 4). The exons upstream of exon 6 are expressed in T1 only, and both T1 and T2 express the rest of the exons. The differences in the gene expression levels of exon 4-5 and the rest of the exons allow the comparison of T1 and T2 expression. The previous T2 analysis only screened the MuSK cDNA up to exon 12, which is the transmembrane region of MuSK. I incorporated the Taqman probe for the MuSK exon 13-14 junction,which encodes for the cytosolic tyrosine kinase domain. We already confirmed that the gene expression of exon 1-2 (Ig1 domain) is comparable to that of exon 4-5 (Ig2 domain); thus, exon 1-2 probe is omitted from this study. Instead, I selected the probe sets targeting the exons downstream of Ig3 domain. The RNA samples were isolated from the hippocampus of 2 months old WT mice and reversely transcribed into cDNA.

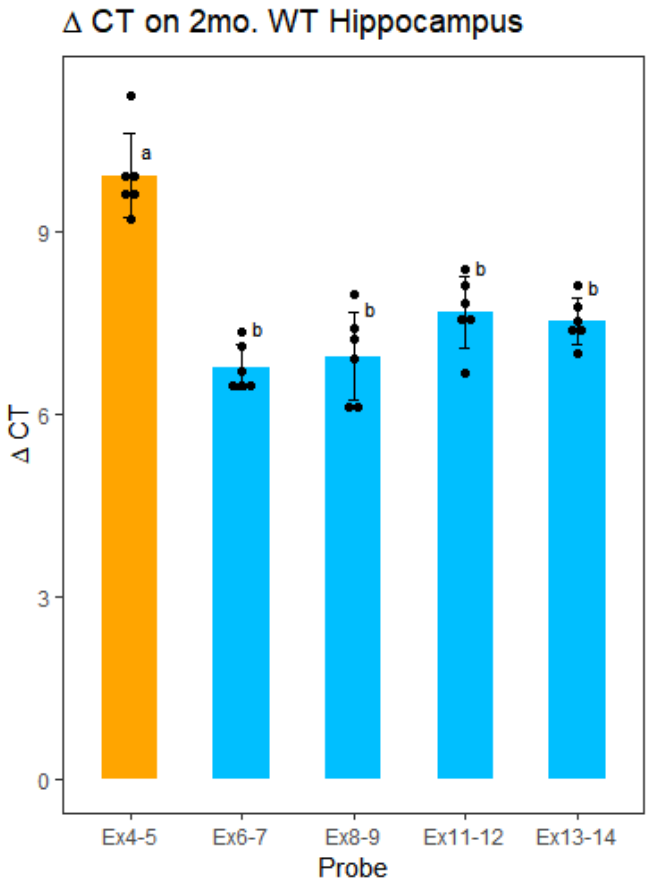

Figure 5. ΔCT between Housekeeping Gene Beta-2-Microglobulin (β2m) and MuSK on 2 months old WT Hippocampus (n=6). The ΔCT is inversely proportional to the gene expression. To yield the ΔCTs, the β2m CTs were subtracted from the corresponding MuSK CTs. T1-exclusive exon probes are marked in orange; T1 and T2 common exon probes are marked in blue. Significant differences among the probes were identified by one-way ANOVA with Tukey's multiple comparison post hoc test ($p<0.001$). Error bars indicate the standard error of the mean.

The differences between the β2m CTs and the MuSK CTs were calculated within each sample to yield the ΔCTs. This calculation minimizes the biological variability across the samples and increases the consistency. Similar to the raw CT, the ΔCT is inversely proportional

to the nucleic acid of interest quantity; the high ΔCTs indicate that the gene is lowly expressed, and vice versa. The data shows that the 2 month-old WT hippocampus expresses both T1 and T2 MuSK (Figure 5). There are significant differences between the ΔCTs of probe exon 4-5 (blue) and the rest of the probes (orange). More importantly, this study shows that the novel transcript variant of MuSK includes exons encoding the cytosolic tyrosine kinase domain (exon 13-14), which was first elucidated in this experiment.

The raw CT differences between the probe 4-5 and the rest of the probes is approximately 3. Considering that each cycle of the PCR doubles the target strand, these results indicate that there is an ~8-fold higher levels of T2 compared to T1. Intriguingly, this data establishes that T2 is the major MuSK transcript form in the hippocampus of the young animals. Further study is needed to examine the role of T2.

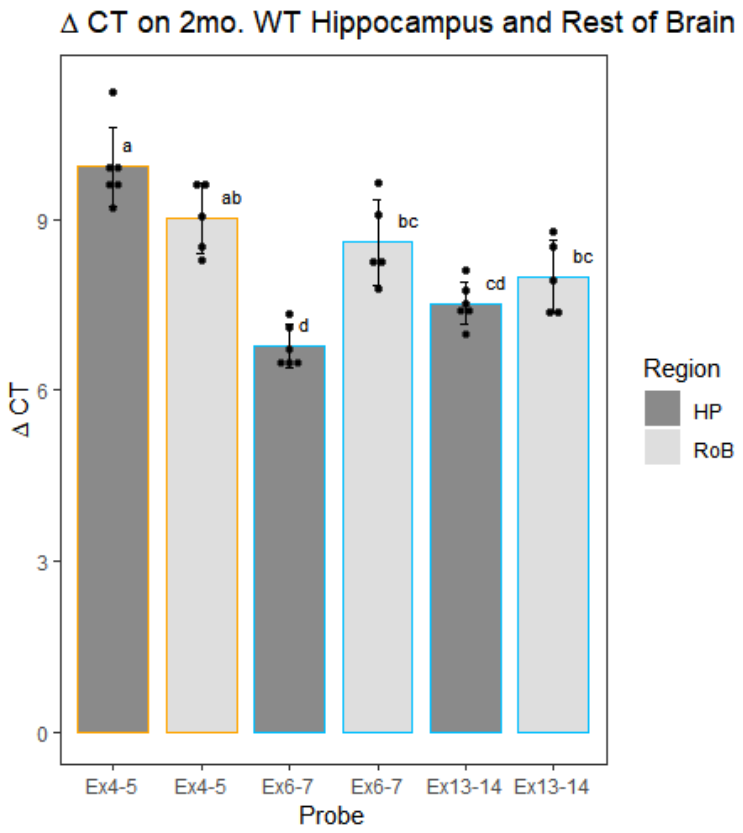

Figure 6. ΔCT between Housekeeping Gene Beta-2-Microglobulin (β2m) and MuSK on 2 months old WT Hippocampus (HP) (n=6) and Rest of Brain (RoB) (n=5). The ΔCT is inversely proportional to the gene expression. To yield the ΔCTs, the β2m CTs were subtracted from the corresponding MuSK CTs. T1-exclusive exon probes are marked with orange borders; T1 and T2 common exon probes are marked with blue borders. Significant differences among probes and regions were identified by two-way ANOVA with Tukey's multiple comparison post hoc test ($p<0.001$). Error bars indicate the standard error of the mean.

To examine whether T2 transcript variant is present in other brain areas, an RT-qPCR experiment was further conducted on the 2 months WT animals' hippocampus and RoB samples. Interestingly, the hippocampus and RoB have different MuSK transcript profiles (Figure 6).

Overall, the hippocampus expresses a higher level of MuSK compared to the RoB. The ΔCTs of the hippocampal ex 6-7 and 8-9 probes are significantly higher than those of the RoB, while the difference in the T1 (ex 4-5) MuSK is comparable between the hippocampus and the RoB. Within the RoB samples, there is no significant difference between the gene expression of T1 and T2 transcripts, indicating that T2 is not as prevalent in the RoB as in the hippocampus.

To conclude, the MuSK transcript variant T2 not only is present in the hippocampus but also is the predominant transcript. The hippocampus exhibits a significantly higher level of MuSK compared to the RoB. In the RoB samples, T2 was not the predominant transcript; however, this may be due to the lower number of samples. Further study with a larger sample size will reinforce the findings in this study. Taken together, this study confirms that our lead ASOs have a valid target in the region of interest. Nevertheless, because both T1 and T2 harbors Ig3 domain, our ASO may not be able to distinguish the T1 and T2 transcripts and bind without specificity. It is thus pivotal to analyze the presence of T2 in our target cell, NSCs.

3.B Establishment of primary NSC cultures

Our primary interest lies in the hippocampal neural stem cell population, which contributes to neurogenesis. All of the transcript analysis performed above was on RNA extracted from the whole hippocampus. Therefore, the NSC-specific transcript expression was unknown up to this point. For this reason, it was crucial to establish primary NSC culture to assess the efficiency of ASOs in the target cells. We first isolated NSCs from the mouse hippocampus.

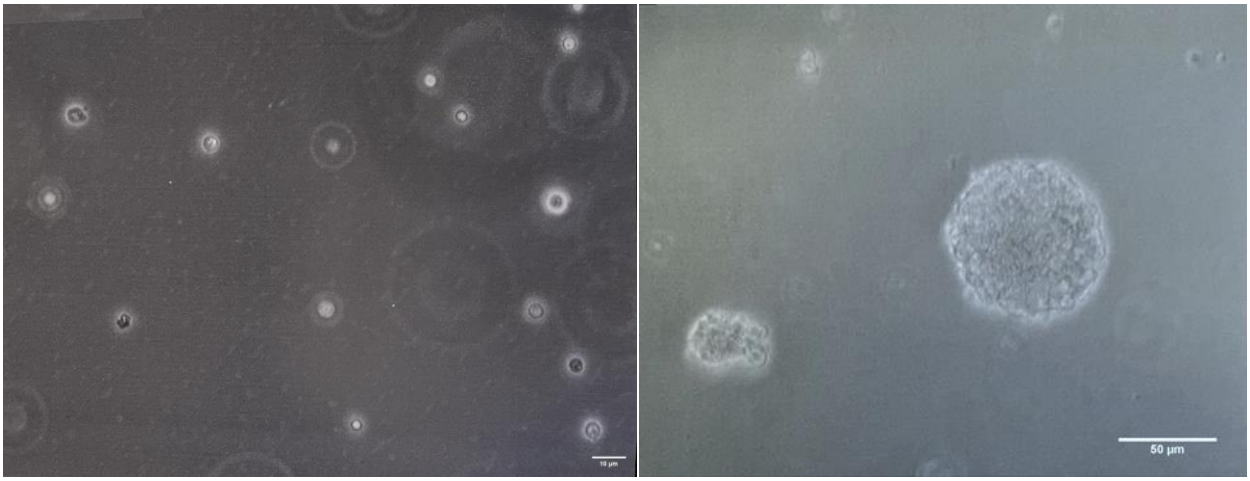

Figure 7. Representative Images of Non-Adherent, Isolated Neural Stem Cells and Neurospheres. Neural stem cells were isolated from the hippocampus of WT and ΔIg3-MuSK mice. Non-Adherent Single NSCs (Left) and neurospheres (Right) are cultured in the renewing media (see Methods). Typically, it takes 5-7 days for the dissociated NSCs to form neurospheres. Scale bars, 10 μm (Left) and 50 μm (Right).

Immediately after isolation, NSCs exist in the single cell state, floating in the renewing media (Figure 7). These NSCs expand to form the neurospheres within 5-7 days of culture; as the passage number increases, it takes longer for the neurospheres to form. NSCs are enzymatically dissociated regularly and passaged as floating single cells to prevent necrosis of the innermost cells in the sphere.

All experiments to be introduced in the next chapters are conducted on the adherent NSCs. After 24 or more hours of incubation, floating NSCs adhere to the PDL-coated plates or coverslips, confirmed by gently shaking the plate and observing no movement of the cells. The primary NSC culture was successfully established, and will be used for qPCR, immunostaining, and ASO transfection.

3.C MuSK Expression in NSCs

Although the *in vivo* hippocampal samples showed the MuSK expression (Figure 5 and 6), the characterization of the MuSK transcript was not yet evaluated in NSCs. I first investigated whether NSCs express T1 and T2 MuSK transcripts. Taqman probes that span MuSK exon borders were used to evaluate the MuSK gene expression in NSCs (Figure 4). Probes are chosen to represent the MuSK domains upstream and downstream of our region of interest, Ig3 domain. Ex4-5 probe was used to detect the gene expression of MuSK upstream of Ig3; ex6-7 probe was used to detect the gene expression of MuSK Ig3; ex8-9 probe was used to detect the gene expression of MuSK downstream of Ig3 (Figure 3). These probes can be used later to evaluate the alternative splicing efficiency of ASOs. The experiment was conducted on adherent WT mouse NSCs.

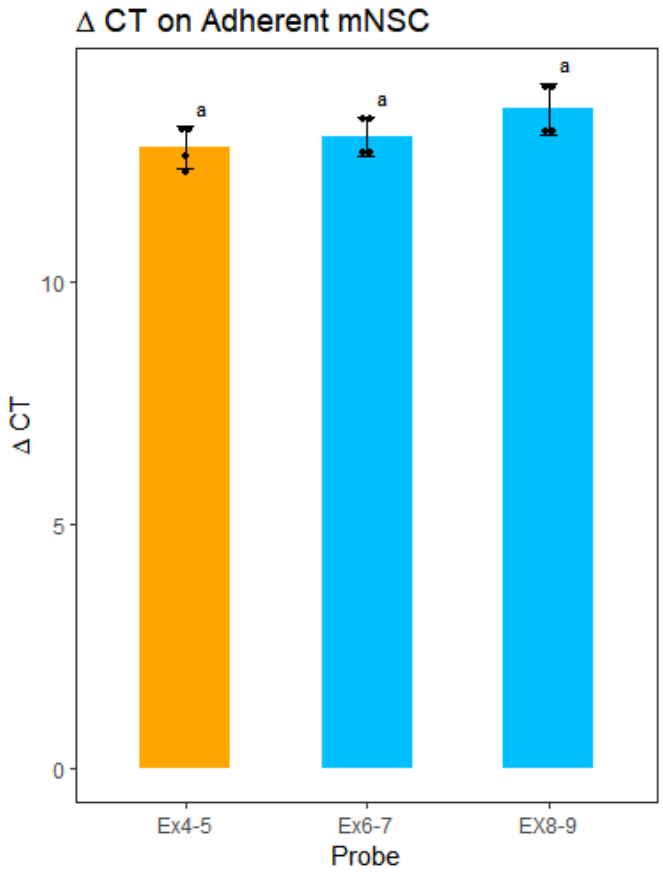

Figure 8. ΔCT between Housekeeping Gene Beta-2-Microglobulin (β2m) and MuSK in Adherent Mouse NSCs (n=4). The ΔCT is inversely proportional to the gene expression. To yield the ΔCTs, the β2m CTs were subtracted from the corresponding MuSK CTs. Significant differences among the probes were identified by one-way ANOVA with Tukey's multiple comparison post hoc test ($p = 0.0925$). Error bars indicate the standard error of the mean.

The qPCR result confirmed that MuSK is expressed at low levels in NSCs, with the raw CT at ~35 cycles while the B2M was ~22 cycles (Data not shown). Unlike the *in vivo* hippocampal samples (Figure 5), there are no significant differences among the probes, indicating that NSCs are likely to express only one transcript form of MuSK, T1 (Figure 8).

Although NSCs used for this study were isolated from the hippocampus of the young animals, there appears to be no apparent T2 in NSCs.

Next, I examined the expression of MuSK at the protein level by immunostaining NSCs with anti-MuSK antibodies. The experiment was conducted on adherent WT mouse NSCs. Antibodies specific for the MuSK Ig2 and Ig3 domains were used for MuSK protein detection, which can also be used in the future experiment to assess the efficiency of ASOs on alternative skipping.

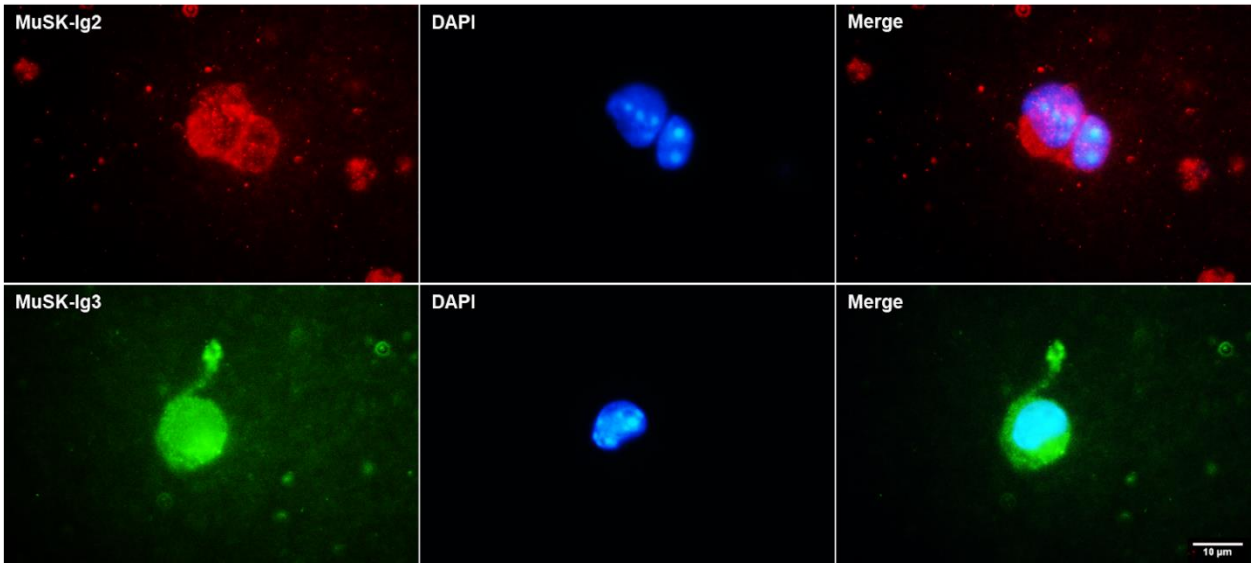

Figure 9. Representative Images of Immunostaining Adherent WT Mouse NSCs with MuSK Ig2 and Ig3 Antibodies. NSCs stained for MuSK-Ig2 (red), MuSK-Ig3 (green), and cell nucleus (blue). Scale bars, 10 µm.

WT NSCs are tagged with both MuSK Ig2 and Ig3 antibodies, confirming the translation of MuSK into the mature protein (Figure 9). Because MuSK is a transmembrane protein, with extracellular immunoglobulin domains and cytosolic tyrosine kinase domain, our MuSK

antibodies targeting Ig2 and Ig3 are stained outside of the cells. The levels of the signal from the antibodies to these domains was comparable, which is consistent with the result of the qPCR data, indicating that T2 is not present in NSCs; MuSK Ig3 staining is not significantly brighter than MuSK Ig2 staining. Taken together, our immunostaining images verify that MuSK protein is expressed in NSCs.

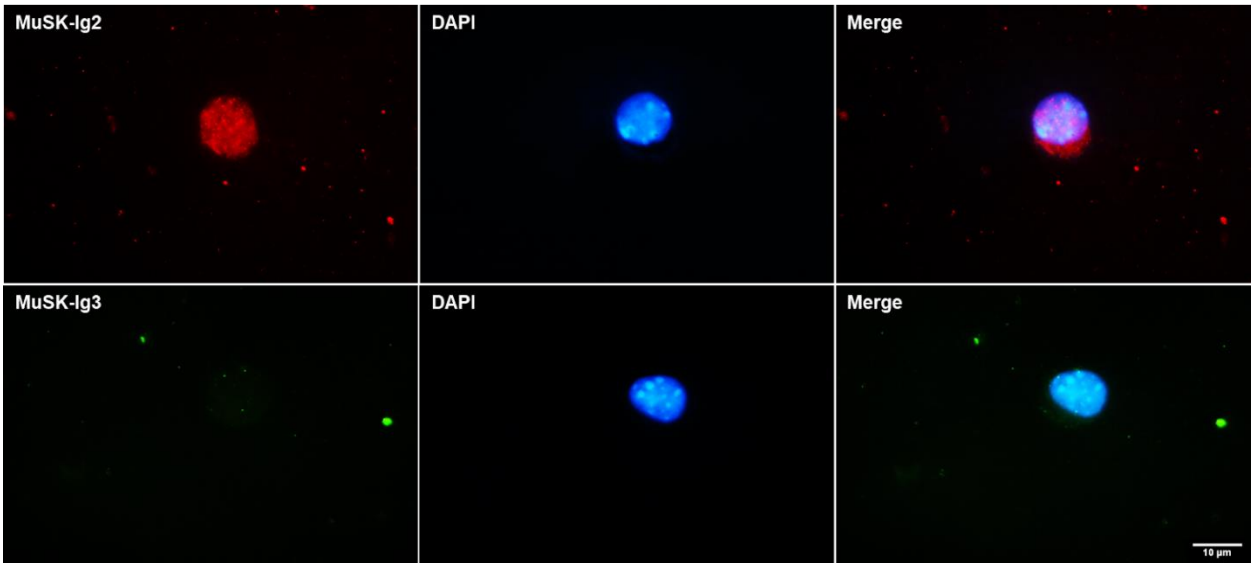

Figure 10. Representative Image of Immunostaining Adherent ΔIg3-MuSK Mouse NSCs with MuSK Ig2 and Ig3 Antibodies. NSCs stained for MuSK-Ig2 (red), MuSK-Ig3 (green), and cell nucleus (blue). Scale bars, 10 μm.

Similarly, MuSK is expressed in ΔIg3-MuSK mice-derived NSCs (Figure 10). The absence of Ig3 staining in ΔIg3-MuSK NSCs confirms genetic modification with CRISPR-Cas9 completely but selectively ablated Ig3 domain from the system. In our future NSC transfection study, ΔIg3-MuSK Mouse NSCs can be used as a positive control to evaluate the ASO efficiency of exon skipping.

In conclusion, NSCs express T1 MuSK in transcription and translation levels. Unlike the *in vivo* hippocampal samples, T1 is the predominant transcript form in NSCs, resolving the issue of non-specific targeting of MuSK Ig3 in T1 and T2 with our ASOs. In future studies, we will proceed to optimize the transfection of NSCs with ASOs using commercially available transfection reagents and gymnotic delivery and evaluate the efficiency of ASOs in these cells.

Chapter 4: Discussion and Conclusions

The primary goal of this thesis was to characterize MuSK transcript and protein expression in isolated NSCs. Understanding the structure of MuSK in NSCs is essential for the design and interpretation of our ASOs. We previously identified a novel MuSK transcript variant T2 in the hippocampus. Here, I investigated whether T2 is also expressed in NSCs. The RT-qPCR and immunocytochemistry experiments in the adherent NSC culture confirmed that NSCs transcribe and translate MuSK. Interestingly, NSCs did not show significant difference between T1 and T2 expression even though they are derived from the hippocampus (Figure 8). Instead, this aligns with the lab's previous characterization of MuSK structure in the muscle stem cells, where only T1 expression was detected. MuSK in the myoblast has been shown to play a role in the regulation of proliferation by interacting with BMP. Further study is warranted to examine whether MuSK in NSCs play the same role.

By profiling MuSK expression in NSCs, we confirmed that NSCs transcribe and translate MuSK Ig3 domain that will be targeted by ASOs. The issue of non-specific targeting of T1 and T2 by ASO is also resolved in this study. We already evaluated the efficiency of ASO in skipping Ig3 encoding exons in the myogenic cells, but we have not had an opportunity to verify its effect in the relevant cell type - NSCs. In the future experiment, we expect to develop the NSC transfection protocol and assess whether ASO induces exon 6-7 skipping and increase in proliferation in NSCs.

In this study, I also analyzed the T2 expression in the hippocampus and RoB. T2 was the predominant MuSK transcript in the hippocampus but not in the RoB (Figure 6). This raises the new question: what is the function of T2 in the hippocampus? Identification of the T2 transcription start site may allow the selective knockout and manipulation of T2, which may

uncover the role of T2 in the hippocampus. Based on our data, we speculate that there is a novel MuSK transcription start site between Ig2 domain encoding exon 5 and Ig3 domain encoding exon 6. I observed an open chromatin region in between exon 5 and 6 in the Ensembl genomic database (Supplementary Figure 2). Thoroughly screening for this region may help finding the transcription start site of T2.

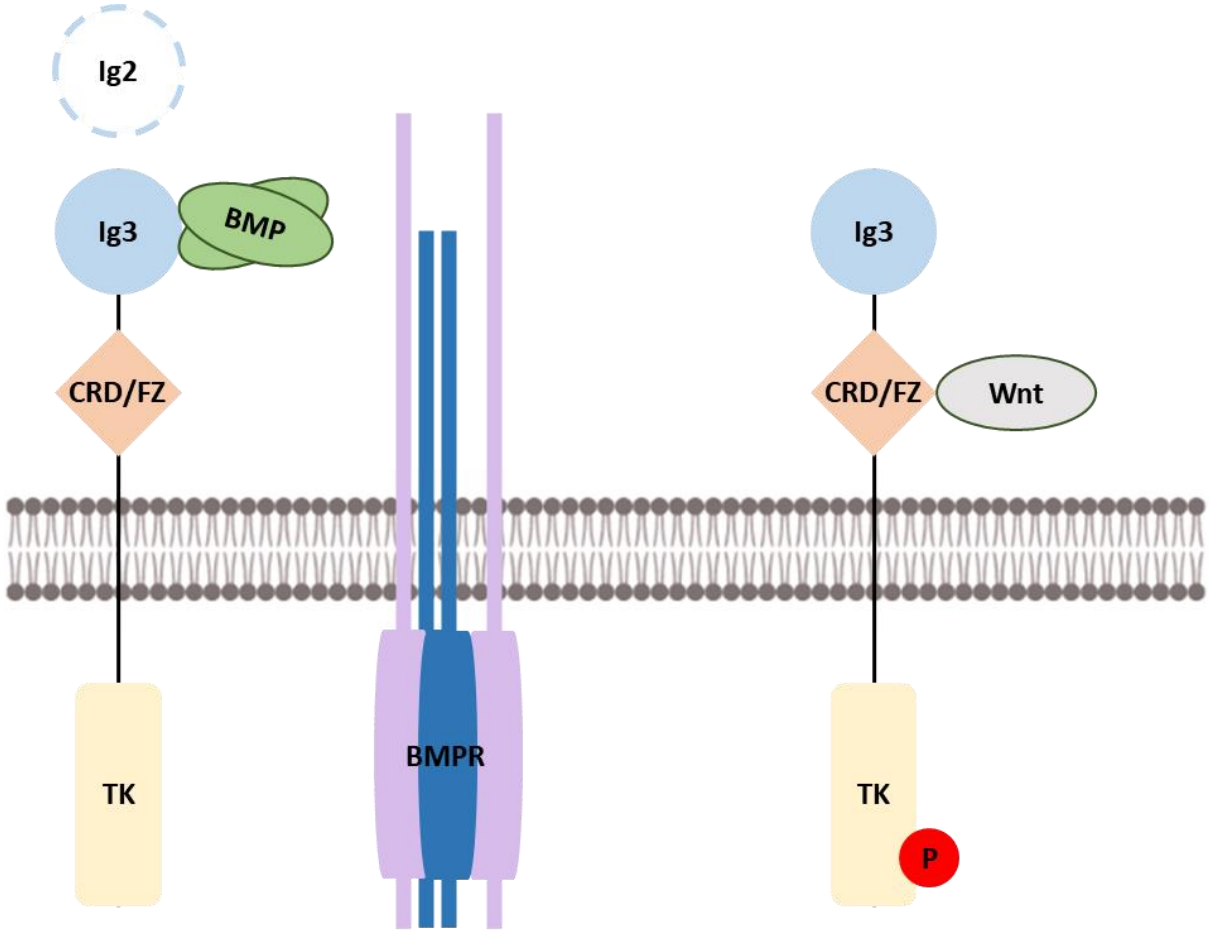

Figure 11. Hypothetical Structure of T2 Protein and Possible MuSK-BMP Pathways. The left pathway illustrates the inhibition of the MuSK-BMP pathway in T2 without the Ig2-BMPR binding. The right figure shows the MuSK-Wnt signaling. Wnt binds to the CRD/Fz domain to phosphorylate the tyrosine kinase domain.

We are still uncertain whether the T2 transcript will be translated into a functional protein. Although there is no apparent in-frame AUG start codon to initiate translation in intron 5-6, the recent study sheds light on non-AUG translation, a non-canonical translation initiation mechanism during development or stress [39]. Assuming that non-AUG translation allows T2 translation, the hypothetical signaling pathways of T2 protein are illustrated in Figure 11. Since the T2 is not encoding the Ig2 domain, the Type 1 BMP receptor cannot bind to T2 protein and induce a BMP signaling pathway. However, BMP will still bind to the Ig3 domain of T2 protein. This indicates that T2 protein may play a role as a BMP antagonist, regulating the availability of BMP to T1 MuSK.

T2 protein may also play a role in MuSK-Wnt signaling. This study is the first study to show that the tyrosine kinase domain encoding exons are included in T2 (Figure 5). With the CRD/Fz domain to bind to Wnt and the tyrosine kinase domain to be phosphorylated, T2 protein may transmit Wnt signaling and induce stem cell proliferation [40]. Again, further study is required to identify the function of T2 protein.

In conclusion, this study demonstrated that T1 MuSK is transcribed and translated by NSCs but not T2. This is the first study to identify MuSK transcript and protein in NSCs and sets the stage to evaluate the efficiency of our lead ASOs in the target cells, NSCs. In the next step, we plan to treat ASOs in NSCs to examine whether the ASOs can induce stem cell proliferation. Co-localizing Ig2 and Ig3 when immunostaining NSCs will also be helpful to assess whether ASOs induced alternative skipping *in vitro*. We look forward to ultimately developing the treatment for neurodegenerative diseases by enhancing AHN.

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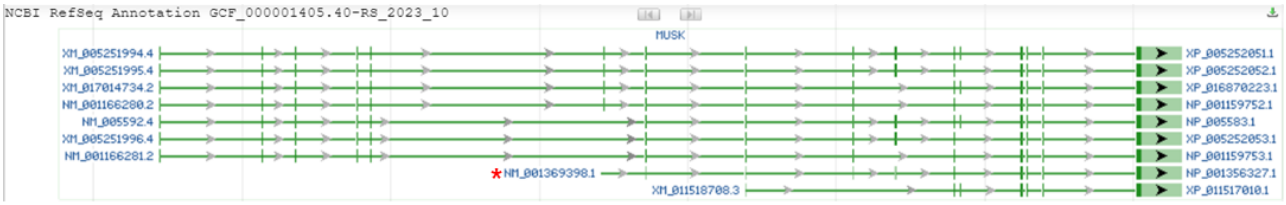
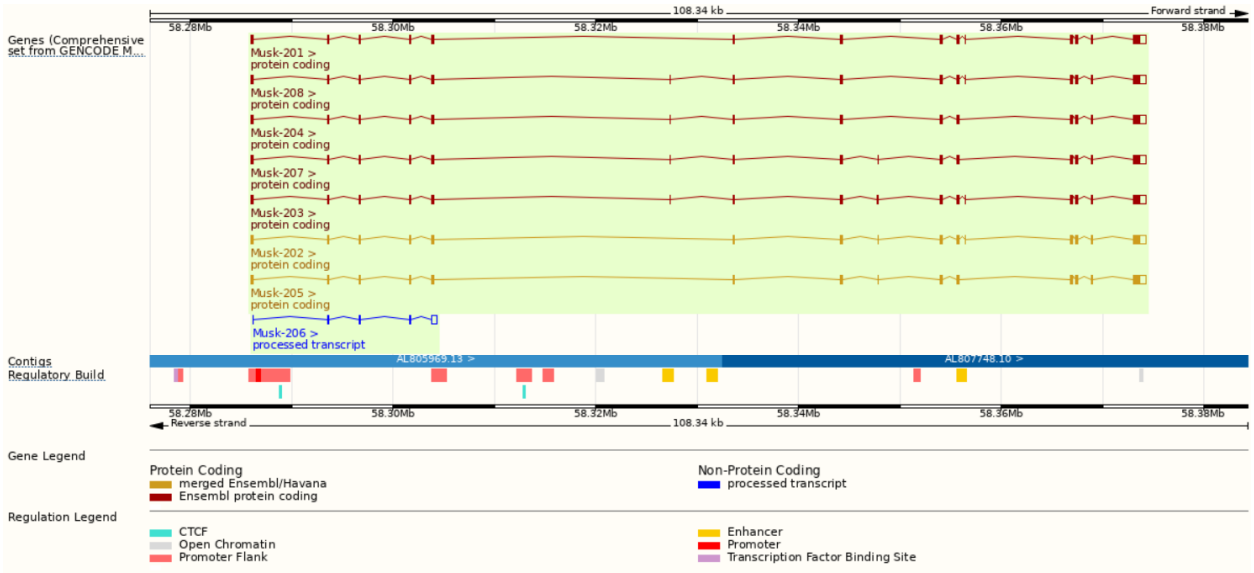
Appendix



Supplementary Figure 1. Human MuSK Transcript Variants from NCBI Genome Browser. NM_001369398.1 with red asterisk shows the short form of the MuSK variant is annotated in the human genome browser. The first exon transcribed in this MuSK variant is exon 6, which encodes the Ig3 domain.



Supplementary Figure 2. Mouse MuSK Transcript Variants from Ensembl Genome Browser. The regulatory build shows that there is an open chromatin region between the exon 5 and 6 (gray).