I. Introduction

A defining characteristic in individuals with dementia, particularly those with Alzheimer's disease (AD) and primary progressive aphasia (PPA), is the difficulty to transmit words in written and spoken communication (Burke; Premi).[1,2] Projections in prevalence rates indicate that 4.6 million new cases of dementia are diagnosed per year (one case per 7 seconds), whereby this number is expected to result in 81.1 million people with dementia by the year 2040 (Ferri 2005).[3] As a result of this alarming incidence rate, questions have risen regarding how to better understand the biomarkers that give light to the disease and its diagnostic criteria.

MicroRNAs (also referred to as miRNA) are small, non-coding regions of RNA that have been of particular interest in the AD and PPA arena for their influence in cognitive pathways. Such pathways, like the commonly cited amyloid beta and protein tau pathways of neuronal degradation, have long been studied as underlying agents of dementia-related symptoms.[4,5,6] Language network disruption pathways, however, have only sparsely been considered because of their lesser understood vulnerabilities in language-associated brain regions. These brain regions heavily involved in language acquisition, production, and impairment include those found in the left hemisphere, primarily in the frontal and temporal regions (Premi).[2]

Extant evidence has found that mutations in the foxhead box P2 gene (FOXP2) directly results in speech and language impairments and corresponds with the frontotemporal degeneration typical of some dementia types (specifically, PPA; Premi; Fu).[2,7] What is still unknown, however, is whether this FOXP2 gene actually gives rise to language impediments through miRNA regulation. MiRNAs have been repeatedly found to dictate the normal functioning of brain networks, whereby specific alterations in these miRNA profiles (ie. their genes) can contribute to AD pathways and thus neurodegeneration (sources 2,6).[5,8]

MiRNAs maintain a complex role as moderators of transcriptome processes, and are comprised of multi-target networks. That is to say, miRNAs not only target multiple genes, but multiple genes can be affected by more than one miRNA. MiRNAs are post-transcriptional in level, meaning that any regulation caused by these molecules occurs between the transcription and translation processes. As demonstrated in Figure 1, miRNA is processed outside of the nucleus where it then

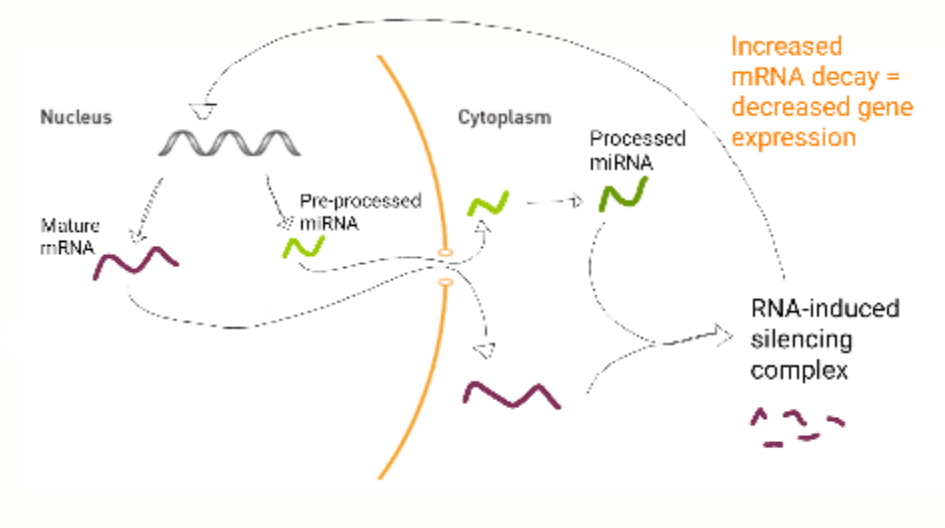

Figure 1. (Down-)Regulation of mRNA by miRNA in neuronal cells. mRNA and precursor-miRNA (pre-miRNA) is transcribed from DNA within the nucleus. The mature mRNA exits the nucleus to begin the translation process while pre-miRNA is processed by enzymes into mature miRNA. This miRNA is incorporated into a ribonuclear particle to form the RNA-induced silencing complex, RISC. RISC targets sites on mRNA and initiates degradation. Picture adapted from Ref 9.

mediates gene silencing through mRNA degradation.[9]

As a result of AD's poligenetic nature, it is important to study miRNA profiles of not only the most popular genes associated with AD, like APP and BACE1, but also those like FOXP2 whose connection with dementia is still unclear. Schonrock et al. (2010) suggest that not only do miRNAs influence the pathways of these common AD-associated genes, but that the genes themselves lend a hand towards the regulation of miRNA systems. In an effort to identify what regulation (if any) is affected by genetic mutations, Schonrock and her colleagues explored miRNA expression of the amyloid precursor protein (referenced above as APP) gene, a gene that typically codes for a 40 amino acid long polypeptide. Mutations to this gene can lead to production of a longer, 42 amino acid long unit (Aβ42) that is known to form destructive, signal-interrupting plaques in the brain.

To test their hypothesis that Aβ42-treated neurons cause neuronal miRNA deregulation, the group used RNA from the hippocampal neurons of mice that expressed the human APP gene. The study found 230 miRNAs (60%) that were able to have expression levels analyzed, and that of these 230, 47% of the miRNA yielded a down-regulating effect. Moreover, 20 miRNAs were found to significantly down-regulate as a result of Aβ42 exposure (Figure 2). As a final step, Schonrock et al. analyzed predicted gene targets, finding that the strongest significantly down-regulated miRNAs functioned primarily in axon guidance pathways (proper brain functioning pathways).[10]

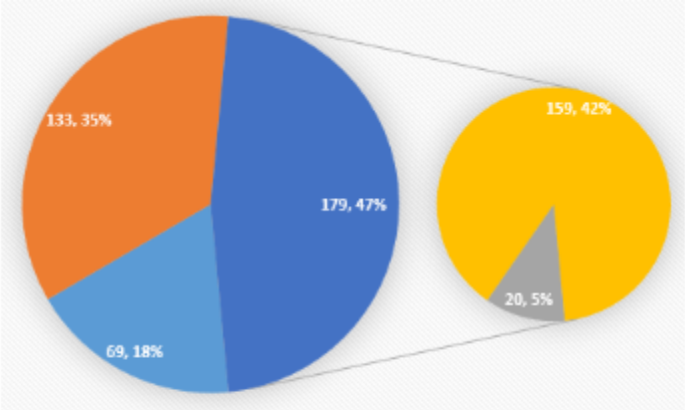

Figure 2. Neuronal miRNA response to Aβ treatment. Of the original 381 miRNA tested, 230 (60%) miRNA were reliably detected. 47% of these 230 down-regulated miRNAs, 18% up-regulated miRNAs, and 35% were considered unchanged (expression levels only varied 15%). Twenty miRNA showed significant down-regulation at a $p<0.05$ level. (Ref. 10)

What would be interesting to report, however, is a similar occurrence with the FOXP2 gene. The present study aims to elucidate whether miRNAs are in fact down-regulated as a result of mutated FOXP2.

II. Experiment

The primary goal of this experiment is to determine the regulatory influence of mutated FOXP2 on miRNA found in the human brain. To accomplish this task, the present proposal will utilize components of the Schonrock et al. (2010) study, particularly those involving cellular preparation and gene expression profiling.

II.A. Murine Cell Preparation

Mouse frontotemporal neurons will be plated and cultivated with a supplemental medium that will prevent growth of astrocytes and microglia. Synthetic FOXP2 peptides will be dissolved and introduced to the cells at 23 days after initial incubation (per Ref. 10). Half of the sample dishes will be treated with

undamaged FOXP2, while the other half will be treated with the point mutation affected FOXP2 (denoted here as pma-FOXP2) that is associated with language defects.[11]

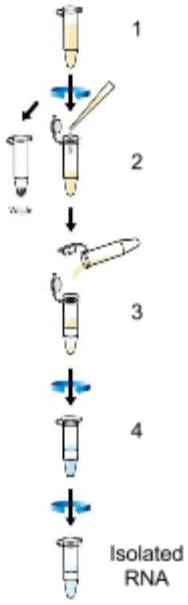

Figure 3. InviTrap RNA Kit use to isolate RNA. 1) Buffer designed to lyse tissue is added to cell culture of FOXP2 and control. 2) DNA is fixed to the surface of carrier particles, and the matrix loaded with this DNA is removed by centrifugation. 3) The RNA remains in the supernatant and is then introduced to an RNA binding membrane. 4) The RNA binds, contaminants are washed away, and the pure RNA is left behind. Picture adapted from Ref. 12.

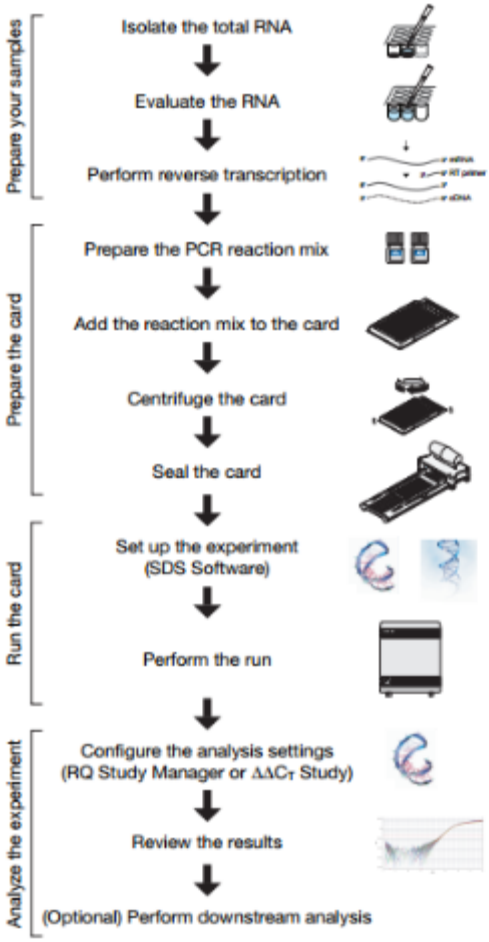

Figure 4. TaqMan Low-Density Microarray analysis of samples. 1) Reverse transcription (RT) is performed on prepared RNA. 2) PCR card prepared with the RT-samples. 3) Run PCR card through TaqMan Array software. 4) Perform analyses on amplification plots. miRNAs will be discarded if resultant values indicate miRNA expression is too low for accurate detection. Picture adapted from Ref. 13.

II.B. RNA Extraction and Microarray Analysis

Following treatment with either FOXP2 or pma-FOXP2, the RNA of each sample type will be isolated and extracted using the InviTrap RNA Kit (note: differs from the miRNeasy Kit used by Schonrock et al.) according to the method illustrated in Figure 3.[12] Following RNA extraction, microarray analysis will be performed according to the methods of Figure 4 in order to determine miRNA expression levels.[13] In the case where pma-FOXP2 indeed affects regulation of miRNA, I would expect to see increased levels of down-regulating miRNAs between the pma-FOXP2 and the controls. Specifically, these down-regulating miRNAs might include miR-9 and let-7, miRNAs that have been associated with gene regulation in the anterior temporal cortex.

III. Discussion

If my above experiments produce expected results, the pma-FOXP2 samples of miRNA will show considerable (significant) down-regulation, especially for miRNA groups that are associated with the temporal and frontal lobes of the brain. Moreover, an interesting finding would be the down-regulation of most or all of the twenty miRNAs whose expression was significantly changed as a result of Aβ42 treatment. Other studies have suggest evidence that confirm these twenty miRNAs show significant differences in AD status versus a control.[14,15] This finding could potentially elude to miRNA networks of

AD and PPA that are still unclear. For instance, while it is known that miRNAs target multiple genes and that each gene can be targeted by multiple miRNAs (Figure 4), it would be fascinating to produce results demonstrating that dementia-related diseases exhibit the same miRNA network despite differences in the biological pathways (memory decay versus language production, for example).

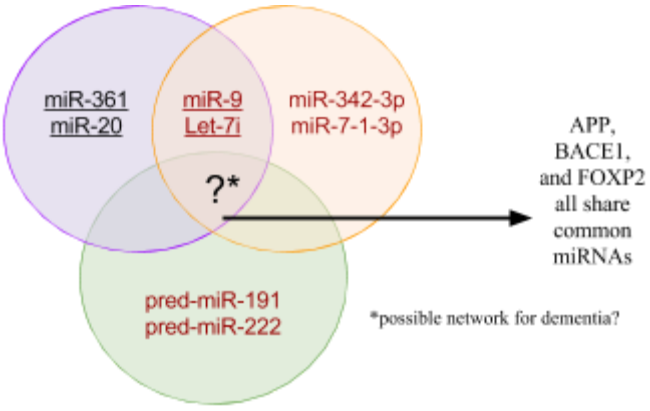

Figure 4. Possible miRNA Network for Dementia. Each circle displays samples of previously studied miRNAs to reflect connectivity: purple for Ref. 10, orange for Ref. 16, and green for Ref. 17. Underlined values indicate that miRNA expression was affected by gene expression while red values indicate the reverse (miRNA expression affects gene expression).

While these findings would prove to be the ideal outcome, it might also be the case that 1) no previously studied miRNAs are affected by FOXP2 or 2) FOXP2 displays no regulatory function in miRNA expression. The former of these arguments would suggest that perhaps language transmission deficiencies are a completely separate pathway than the amyloid beta and protein tau pathways--a finding that would dispute prior work indicating this is not the case.[2] The latter of these scenarios, however, would instead provide support for a unilateral model of miRNA functionality. Though it has not been heavily investigated by others, there has been prior work that suggests miRNAs readily target the FOXP2 gene. This characteristic does not imply by any means that FOXP2 must then affect miRNA expression, but it would be rather interesting to reveal a one-sided pathway such as this.

Unfortunately, the breadth of this proposal does not expand to analyzing potential gene targets of deregulated miRNAs as was done in Schonrock et al. (2010). Schonrock et al. were successfully able to identify which pathways were most affected by their twenty selected down-regulated miRNA. These pathways were primarily involved in axon guidance pathways, confirming the notion that brain function heavily relies on these particular miRNA.[10] My study, in an ideal situation, would perform this predictive target gene analysis to further identify whether links can be made between language pathways and the miRNA that display higher levels of expression. Despite these constraints, however, the potential for novel understanding permeates throughout the exploration of pma-FOXP2 and its effect on miRNA.

References

[1] Burke, D. and Shafto, M. (2004). Aging and Language Production. Current Directions in Psychological Science 13(1): 21-24.
[2] Premi, E., et al. (2012). FOXP2, APOE, and PRNP: new Modulators in Primary Progressive Aphasia. Journal of Alzheimer's Disease 28(4):941-950.
[3] Ferri, C., et al. (2005). Global Prevalence of Dementia: a Delphi Consensus Study. Lancet 366(9503):2112-2117.
[4] Panigrahi, P. and Singh, T. (2013). Computational Studies on Alzheimer's Disease Associated Pathways and Regulatory Patterns Using Microarray Gene Expression and Network Data: Revealed Association with Aging and Other Diseases. Journal of Theoretical Biology 334:109-121.
[5] Kumar, P., et al. (2013). Circulating miRNA Biomarkers for Alzheimer's Disease. PLoS One 8(7).
[6] Luo, H., et al. (2014). Genome-Wide Analysis of miRNA Signature in the APPswe/PS1(delta)E9 Mouse Model of Alzheimer's Disease. PLoS One 9(8).
[7] Fu, L., et al. (2014). Multiple microRNAs regulate human FOXP2 gene expression by targeting sequences in its 3' untranslated region. Molecular Biology 7(71).
[8] Li, Y., et al. (2011). Increased Expression of miRNA-146a in Alzheimer's Disease Transgenic Mouse Models. Neuroscience Letters 487(1):94-98.
[9] (Picture reference) Exiqon: Seek and Verify. What are microRNAs? http://www.exiqon.com/what-are-microRNAs
[10] Shonrock, N., et al. (2010). Neuronal MicroRNA Deregulation in Response to Alzheimer's Disease Amyloid-β
[11] Liégeois, F., et al. (2003). Language fMRI abnormalities associated with FOXP2 Gene Mutation. Nature Neuroscience 6:1230-1237.
[12] (Picture reference) Stratec Biomedical. Invitrap Spin Tissue RNA Mini Kit. http://www.stratec.com/en/molecular/Products_Molecular/Total_RNA/invitrap_spin_tissue_rna_mini_kit/invitrap_spin_tissue_rna_mini_kit.php
[13] (Picture reference) Applied Biosystems: TaqMan Array Micro Fluid Cards. https://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_062836.pdf
[14] Lugli, G., et al. (2015). Plasma Exosomal miRNAs in Persons with and without Alzheimer Disease: Altered Expression and Prospects for Biomarkers. PLoS One 10(10).
[15] Ravetti, M., et al. (2010). Uncovering Molecular Biomarkers that Correlate Cognitive Decline with the Changes of Hippocampus' Gene Expression Profiles in Alzheimer's Disease. PLoS One 5(4).
[16] Sebastien, H., et al. (2008). Loss of MicroRNA Cluster miR-29a/b-1 in Sporadic Alzheimer's Disease Correlates with Increased BACE1/Beta Secretase Expression. Proceedings of the National Academy of Sciences of the USA 105(17):6415-6420.
[17] Smith, B., et al. (2010). Large-Scale Expression Analysis Reveals Distinct MicroRNA Profiles at Different Stages of Human Neurodevelopment. PLoS One 5(6).