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Thesis

CRISPR/CAS13 BASED BIOSENSING OF MIRNA-21 AS A DIAGNOSTIC BIOMARKER FOR ALZHEIMER'S DISEASE

by

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B.S., Fairfield University, 2022

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CRISPR/CAS13 BASED BIOSENSING OF MIRNA-21 AS A DIAGNOSTIC BIOMARKER FOR ALZHEIMER'S DISEASE

ANNELISSE ZARA

ABSTRACT

Alzheimer's Disease is one of the most prevalent neurodegenerative diseases affecting the elderly population, with approximately 65 million individuals diagnosed worldwide. The hallmark pathological features include the accumulation of Aß senile plaques and the hyperphosphorylation of tau protein, ultimately leading to memory loss, cognitive deficits and loss of executive function. Due to its rapid progression and the inability to diagnose in early stages, mechanisms for detection and target biomarkers for AD have been well under study. In recent years, small extracellular vesicles, also known as exosomes, which contain a variety of macromolecules: protein, RNA, lipids and other metabolites, have been capable of acting as biomarkers for a wide variety of cancers and other diseases due to their ability to reflect the physiological status of the origin cell. This study will aim to characterize the presence of microRNA-21, a potential biomarker for AD, in SH-SY5Y neuroblastomal-derived exosomes using the CRISPR/Cas13 (Clustered Regularly Interspaced Short Palindromic Repeats) platform for diagnostic assay.

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LIST OF ABBREVIATIONS

Aß.....Amyloid Beta

AD.....Alzheimer's Disease

APP.....Amyloid Precursor Protein

BBB.....Blood Brain Barrier

crRNA.....CRISPR RNA

CSF.....Cerebrospinal Fluid

EV.....Extracellular Vesicles

iPSC-NPCs.....Induced Pluripotent Stem Cell-Neural Progenitor Cells

LOD.....Limit of Detection

MCI.....Mild Cognitive Impairment

miRNA.....MicroRNA

NFTs.....Neurofibrillary Tangles

OA.....Okadaic Acid

PBS.....Phosphate Buffer Saline

CHAPTER ONE

Introduction

AD as a Neurodegenerative Disease

One of the most prevalent multifactorial neurodegenerative diseases affecting the central nervous system (CNS), Alzheimer's Disease is a common illness in the aging population, ages 65 and older, manifesting as a decline in emotional, psychological and behavioral functioning. By 2050, one new case of AD is expected to develop every 33 seconds and total prevalence in the United States will reach 13.8 million (Kumar et al., 2015).

The most widely studied and accepted pathological basis of AD lies on the accumulation of senile amyloid beta (Aß) plaque production from the dysregulation of the Aß cascade along with the hyperphosphorylation of tau protein forming neurofibrillary tangle (NFT) aggregates – both of which contribute to brain atrophy and severe neurodengenerative changes within the neocortex and hippocampal regions (Mrdjen et al., 2019). From ongoing studies, it has been proposed that these accumulations of Aß and NFTs can be enhanced by a wide array of effects within neuronal cells including mitochondrial dysfunction, oxidative stress, neuroinflammation and the dysregulation of protein trafficking and regulatory pathways. Microglia and reactive astrocyte activation induce an adaptive stress response in neurons through the secretion of proinflammatory cytokines like tumor necrosis factor-alpha (TNF-a) and interleukin-beta (IL-ß) (P. K. Kamat et al., 2013; Mrdjen et al., 2019). This stress response further increases oxidative stress, reactive oxygen and nitrogen species within

these neuronal cells leading to further destruction of neuronal tissue exacerbating pathological inflammation. This neuroinflammatory stress is also seen to increase production of Aß via the amyloidogenic processing of amyloid precurcor protein (APP) (to be discussed in more detail below), enhancing progression of AD (Jeremic et al., 2021). Due to the multifactorial nature of AD, other risk factors may be of interest for studying population vulnerability. For example individuals diagnosed with diabetes, individuals seen to inherit genetic mutations in genes important for Aß regulation and those who are exposed to harmful environmental pollutants may be at increased risks for developing AD due to these simuli's ability to activate the adaptive stress response, triggering Aß production and further neuroinflammation, as discussed previously (Jeremic et al., 2021).

These pathological changes form well before the manifestation of prodrome symptoms, making it of great importance to find early biomarkers to diagnose AD. These symptoms include short-term memory loss, loss of visuospatial skills and executive functions, and cognitive decline affecting learning and emotional processes, all caused by atrophy to the cerebral cortex, hippocampus, temporal and parietal lobes (X. Yu et al., 2023).

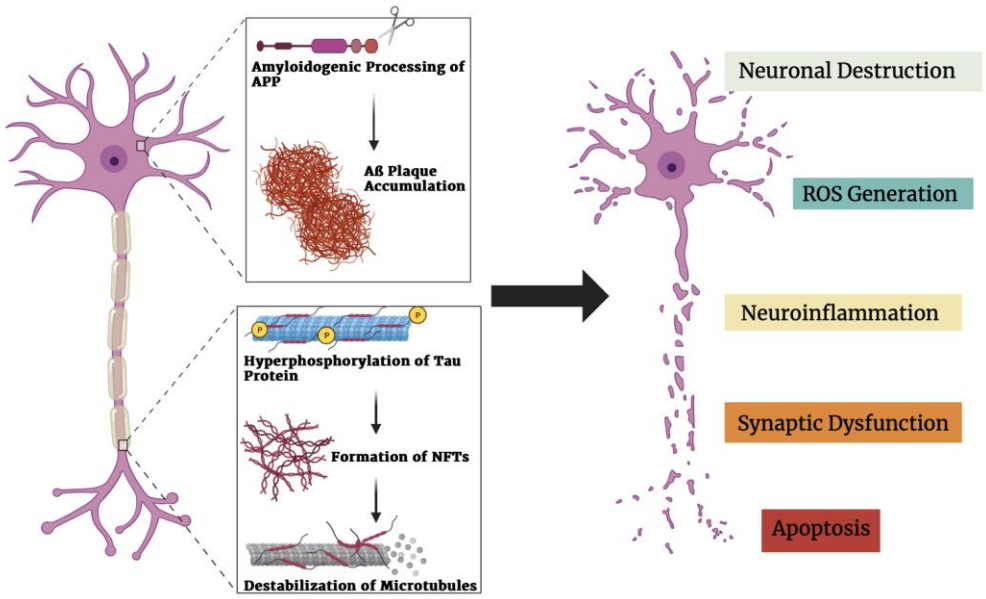

Figure 1: Alzheimer's Disease (AD) Pathology & Manifestations

The biological cascade of Aß formation in neuronal tissue occurs via the amyloidogenic processing and endoproteolytic cleavage of APP resulting in the formation of Aß plaque accumulations seen extracellularly within cortical and hippocampal tissue. In the non-amyloidogenic pathway, APP is cleaved by α-secretase followed by cleavage via γ-secretase leading to the generation of an extra soluble p3 peptide. On the other hand, during the amyloidogenic pathway APP is cleaved first by ß-secretase generating a soluble N-terminal fragment and membrane bound C-terminal component, then cleaved via γ-secretase leading to the generation of Aß protein which aggregates and leads to the formation of Aß plaques over time (Zhao et al., 2020). Aß40 and Aß42 are the two major isoforms produced following cleavage by γ-secretase, producing peptide lengths of 40 and 42 amino acids, respectively (Jeremic et al., 2021). Aß42 is cited to have more neurotoxic effects on synapses, can induce

hyperphosphorylation of tau protein leading to accumulation of NFTs, and is more susceptible to aggregation, in comparison to Aß40. For this reason Aß42 has become a critical component for studying AD (Jeremic et al., 2021; Kumar et al., 2015; Mrdjen et al., 2019).

There have been ongoing studies to elucidate the genetic predispositions for the inheritace of AD. It is estimated that the majority of AD cases occur in late onset, ages 65 and above (LOAD) and are seen to display heritability between 60% and 80% (Mrdjen et al., 2019). This heritability is seen to involve a wide array of risk genes, APOE ε4 being the main gene involved, accounting for 25% heritability. ApoE is known to regulate many homeostatic functions within hippocampal and cortical neurons. Released from microglia and astrocytes, ApoE regulates cholestereol levels and transports this cholesterol into neurons, stimulating synaptic generation and axonal growth (Mrdjen et al., 2019). Compared to the ε3 isoform, the ε4 isoform of the ApoE gene is known to trigger a loss of neuroprotective ability and stimulation of toxic function (NFT formation, oxidative stress and mitochondrial dysfunction) making neurons susceptible to pathological changes, specifically neurodegeneration (D'Argenio & Sarnataro, 2020).

While most diagnoses of AD are late-onset, some early onset cases are notable in presenting in individuals younger than 65 years old. Mutations in the APP gene and presenilin genes, PSEN1 and PSEN2 (present in the γ-secretase complex), all of which are involved in the amyloidogenic pathway, are linked to early onset AD (EOAD), also known as familial AD (D'Argenio & Sarnataro, 2020). Accounting for a range of 1%-10% of total AD cases, EOAD caused by these autosomal dominant mutations leads to

the upregulation of Aß, contributing to the toxic accumulation of Aß plaques within the brain (Mrdjen et al., 2019; Zhao et al., 2020). But overall, these mutations are seen to only account for ~10% of EOAD cases, thus eluding the involvement of recessively transmitted genes or those inherited via incomplete penetrance (D'Argenio & Sarnataro, 2020; Mrdjen et al., 2019). EOAD is seen to manifest more rapidly than LOAD and is known to include a few unconventional symptoms, like apraxia. It has also been questioned whether the differences between EOAD and LOAD can be attributed to the differing brain atrophic patterns as there is much debate over which brain areas display the most pronounced atrophic changes and the order neurodegeneration occurs in (Mrdjen et al., 2019).

Braak staging, first defined by Braak in 1991, classifies neurofibrillary tangle formation and progression as occurring in consecutive stages throughout regional areas of the brain. Stages 1-2 display NFTs in distinct regions of the transentorhinal portion of the brain, stages 3-4 with NFT formation spreading to multiple layers of the transentorhinal and entorhinal regions, and finally stages 5-6 embodying increased density of NFTs within the isocortex and hippocampus leading to destruction of these tissues (Braak & Braak, 1991).

On the otherhand, the formation of amyloid deposits occur in 3 stages, as per the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) methods (Boluda et al., 2014). Stage A embodies low density accumulations that are seen within the isocortex and the occipital, frontal and temporal lobes; stage B consists of medium density deposits found connecting all isocortical areas; and stage C exhibits all isocortical

areas affected along with regions of the subcortex (Boluda et al., 2014)(Braak & Braak, 1991). Overall, AD is seen to impact neuronal cells in a regional, stepwise manner, with the highest rates of synaptic loss due to neurodegeneration and Aß plaque formation in the later stages; first in the enthorhinal region followed by the temporal lobe spreading into the limbic system and finally to the neocortex (Jeremic et al., 2021). It is interesting to note that some areas of the brain, one being the cerebellum, is resistant to forming aggregates of NFTs, thus not involved in the progression of AD pathology (Mrdjen et al., 2019) (Walker, 2020). From a clinical standpoint, the stages and diagnosis of AD can be seen within a continuum ranging from stage 1-6, with 1 encompassing preclinical asymptomatic phase of the disease, followed by MCI at stage 3 and patients in stages 4-6 presenting with mild to severe dementia as the disease progresses (X. Yu et al., 2023).

Both NFTs and Aß deposits work in tandem to lead to the progression of AD. Under normal conditions, tau proteins form stabilization complexes with the axonal microtubules, promoting synaptic functioning via the transport of organelles and neurotransmitters. Thus dysfunction of tau protein result in its hyperphosphorylation leading to the loss of critical stabilizations and separation from microtubules, forming aggregates in the form of NFTs. These pathological changes manifest as a loss in cognitive decline and impaired neural functioning in the long term (Mrdjen et al., 2019). Aß, on the otherhand, is primarily generated extracellularly and released into the extracellular space of the brain tissue where its accumulation is directly linked to a decline in synaptic and neuronal activity (Walker, 2020).

As of today the treatment options available are limited. Certain drugs such as acetylcholinesterase inhibitors, functioning to increase cholinergic transmission, are the most widely known and may aid in temporary symptomatic relief but do not modify or hault the progression of the disease (Jeremic et al., 2021). With AD's irreversible pathology and rapid progression, ongoing studies are needed to conclusively determine the genetic and environmental risks, the etiology, along with mechanisms and target biomarkers for early detection and intervention before progression to later stages where treatment would be ineffective.

Exosomal miRNA as a Diagnostic Tool

With a diameter ranging from 30-130 nm, small extracellular vesicles, also known as exosomes, are synthesized within most cell types and contain unique protein markers on their surface: tetraspanin proteins CD63 and CD81 along with proteins responsible for cargo sorting and transport of exosomal content (ESCRT) (Gurung et al., 2021). Their endocytic-based biogenesis begins with the internalization of cargo molecules that are transported to the early endosome for sorting. Sorting is carried out through the use of these ESCRT proteins. After sorting into these early endosomes, intraluminal vesicle formation (ILV) occurs via the budding of late endosomal/multivesicular body (MVB) membrane, ultimately leading to the formation of hundreds of ILVs within the lumen of the MVB (Gurung et al., 2021; Urbanelli et al., 2013). If fusion occurs with the lysosomal membrane, the contents of the MVBs are directed towards a degradation pathway. If MVB fusion occurs with the plasma membrane of the cell, these ILVs are secreted as exosomes which can mediate cell-cell communication locally or systemically among

different cell types (Fig. 2) (Gurung et al., 2021) (D. Yang et al., 2020) (Mathew et al., 2021) (Kanninen et al., 2016). This intercellular communication is made possible through the uptake of exosomes into recipient target cells via surface receptors, endocytosis or direct internalization (fusion) with the plasma membrane (Fig. 2) (Gurung et al., 2021).

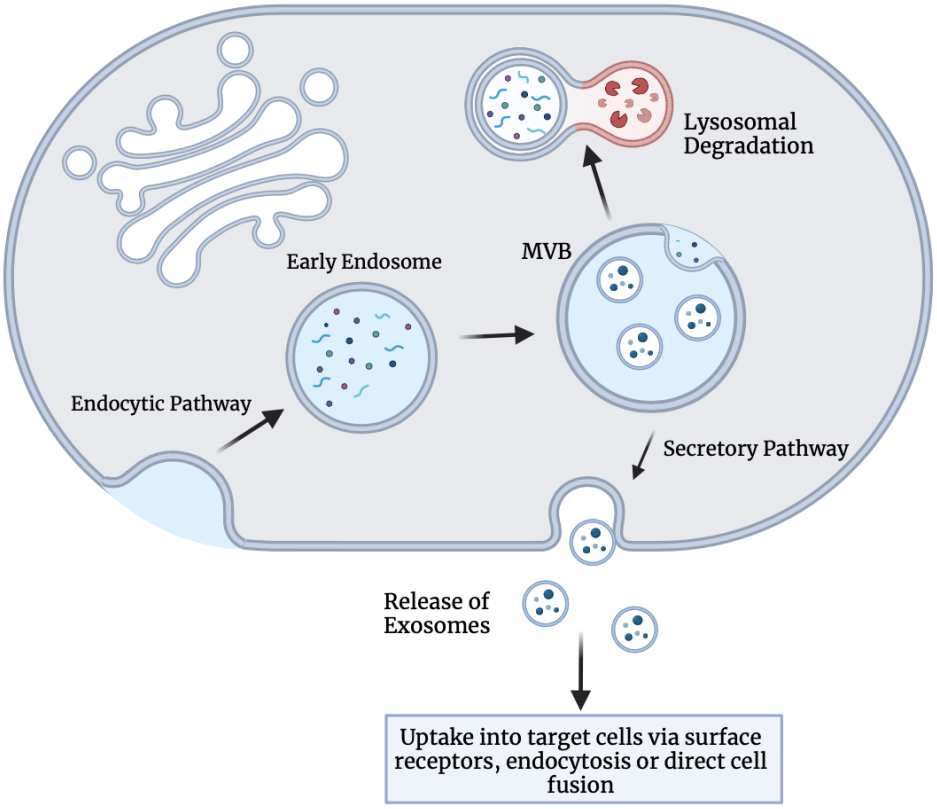

Figure 2: Exosome Biogenesis through the Endocytic Pathway & Release from Cell via the Secretory Pathway

Exosomes are typically found within the CSF, blood, saliva, urine and other biological fluids and their contents are known to reflect the physiological status of the origin cell due to the presence of certain metabolites, lipids, RNA and proteins. These exosomes have a primary ability of regulating inter- and intracellular communication through the trafficking of cargo molecules, cell signaling and transport of proteins and

nucleic acids, all of which play a role in the regulation of physiological function and pathological conditions of the cell (Kanninen et al., 2016) (Kawikova & Askenase, 2015) (Mathew et al., 2021) (D. Yu et al., 2022). For example, exosomes have been found in the synaptic cleft and may play a role in the physiology of synaptic transmission (Kawikova & Askenase, 2015).

Specifically for cells within the CNS (microglia, astrocytes, oligodendrocytes, Schwann cells, etc.), exosomes may have a role in synaptic plasticity (Hornung et al., 2020), intercellular communication (Kawikova & Askenase, 2015), myelination (Kanninen et al., 2016; Mathew et al., 2021), neuroprotection and neurogenesis (Fan et al., 2022). Due to their ability in reflecting cellular status, small extracellular vesicles have become of rising importance as CSF or blood-based biomarkers for diagnosis of diseases and cancer through analysis of their biomaterial content. Certain cell types are known to contain upregulated levels of certain cargo proteins which may be directly related to diseased states. For example, in exosomes derived from metastatic melanoma patients, upregulated levels of signature oncoproteins MET and TRYP2 were seen, thus pointing to the potential usage of exosomes as diagnostic biomarkers (Kawikova & Askenase, 2015).

In the case of AD, the signature hallmark proteins, Aß42 and tau were, as expected, upregulated within neuronal derived exosomes isolated from MCI and AD patient samples (Fiandaca et al., 2015). Since APP cleavage has been noted to occur in early endosomes, Aß42, normally confined to the cellular lumen,was seen co-localized with exosomal protein markers, CD63 and CD81, but the exact mechanim for this

presence of Aß in EVs is still to be determined (Mathew et al., 2021). Through exosome shuttling, Aß42 can be transported through the endosomal pathway, secreted into the CSF and promote further neurodengeneration and neuroinflammation as a result of astrocytic and microglial activation, leading to the release of proinflammatory cytokines like TNF-α and IL-ß (Jeremic et al., 2021) (Mathew et al., 2021) (L. Wang & Zhang, 2020) (Jiang et al., 2019). Hyperphosphorylated tau protein, also when shuttled through exosomes, contributes to further neurotoxicity and neurodegeneration (P. K. Kamat et al., 2013). Alongside Aß and tau, a previous study found exosome-mediated neuroinflammation to be linked to the transfer of inflammatory-miRNAs like miR-124, miR-21 and miR-155 from neuronal SH-SY5Y-ΔAPP neuroblastoma cells (capable of producing Aß at higher rates) origin to microglia (Fernandes et al., 2018). The transfer of these miRNAs caused acute pro-inflammatory changes in recipient microglia which was seen through the upregulation of their inflammatory markers TNF-α, S100B and HMGB1. This upregulation of inflammatory markers can be linked to miRNA's activation of various signaling pathways. For example, miR-21 has been shown to activate the PI3K/AKT pathway eventually leading to the activation of NF-kß pathway which releases proinflammatory markers like IL-ß and TNF-α (Lai et al., 2021). Thus it was shown how neuronal cell-cell communication through the exosome transfer of pro-inflammatory-miRNAs may be involved in the initiation of an inflammatory response and progression of AD pathology (Fernandes et al., 2018). For these reasons, exosome shuttling as a form of intercellular communication has been one ongoing area of study for understanding the mechanisms behind AD pathogenesis.

In the recent years, these small extracellular vesicles have become of rising importance due to their presence in a variety of biofluids and their intriguing composition. Along with acting as diagnostic biomarkers, they can also serve as a promising therapeutic vehicle for drug delivery. This was seen in a novel study delivering siRNA to neurons and oligiodendrocytes in a mouse-model brain. The siRNA was seen to successfully genetically silence BACE1, a protease responsonsible for the cleavage of APP at the ß-site (Alvarez-Erviti et al., 2011). So, as exosome-based studies expand, it is clear of their novel importance and contribution to revolutionary advancements in the field of medicine.

miR-21-5p as a Biomarker for AD

Due to AD's rapid progression and the challenges in effectively diagnosing patients in early stages of this disease, ongoing research has been of great importance in finding potential biomarkers that may be linked to its onset and progression. Using blood and CSF-based biomarkers like Aß and tau, along with MRI and PET scans, clinicians have used this technology as the foundation for AD diagnoses (Ausó et al., 2020). But due to these imaging technologies not being as readily available for routine visits and fairly expensive, researchers have been focusing on blood or CSF-based biomarkers that may contribute to Aß formation and tau pathology for the early diagnosis of AD. (Ausó et al., 2020). Biomolecules, like miRNAs, have emerged as potential biomarkers due to their involvement in many regulatory and disease processes along with their prescence in the CSF and blood. MiRNAs are single strands of endogenous RNA approximately 19-25 nucleotides in length with a primary function of regulation at the post transcriptional

level through the inhibition of translation or cleavage of RNA transcripts (Hong et al., 2023). They are also seen to impact cellular differentiation, metabolism and apoptosis via regulation of target genes (Bai & Bian, 2022) (Hong et al., 2023). A majority of known neuronal origin miRNAs have been studied for their involvement in the regulation of synaptic function and neurotransmitter release (Amakiri et al., 2019). In the realm of AD, miRNAs have also been said to play a role in cellular vulnerability, cell senescence and aging through their ability to disrupt regulation of Aß and tau protein as well as increase oxidative stress (Amakiri et al., 2019). Notably, previous studies cited the involvement of miR-1, miR-9, miR-16/17/138, among others in having the ability to enhance oxidative stress, induce tau pathology, and promote Aß deposition and aggregation, respectively (Hébert et al., 2009; Lu et al., 2019; Parsi et al., 2015; Smith et al., 2011; Wu et al., 2015). For example, miR-138 is seen to increase Aß production through the downregulation of the sirtuin-1 protein. On the other hand, miR-98 has the ability to decrease Aß production through the Notch signalling pathway, and in a diseased AD state, this Notch pathway is seen to be inhibited through the involvement of other miRNAs (Amakiri et al., 2019).

Specifically within AD, in addition to Aß42 and tau protein, the potential ability for miR-21-5p to act as a biomarker for this disease was shown in previous research (Bai & Bian, 2022; Fernandes et al., 2018; Garcia et al., 2022) . Since this particular miRNA has only recently become of rising importance for diagnosis of this neurodegenerative disease, ongoing studies are underway to discern both proinflammatory and anti-inflammatory properties miR-21 harnesses.

A majority of research has linked miR-21 to proinflammatory effects *in vivo*. One study revealed how, upon incubation of SH-SY5Y neuroblastomas with CHME3 microglia, SH-SY5Y-derived exosomes rich in miR-21 were uptaken by the recipient microglia, as these microglia displayed the same overexpressed levels of miR-21 as the neuroblastomas (Fernandes et al., 2018). MiR-21, along with other proinflammatory factors such as RAGE mRNA and TNF-α mRNA, were also upregulated within the SH-SY5Ys, the recipient CHME3 microglia and the microglia-derived exosomes following co-culture. This further contributes to the understanding of intercellular communication between neuron and microglia and the potential linkage between miR-21 and proinflammatory effects through the exosome-mediatied shuttling of RAGE mRNA and TNF-α. Thus it was concluded in this study how miR-21 harnesses the ability in acting as a potential biomarker for AD due to its involvement in a glial-related neuro inflammatory signaling pathway stemming from the SH-SY5Y based secretome (Fernandes et al., 2018). To further add to this point, a study conducted by Garcia et al. 2022, noted the upregulation of miR-21 isolated from exosomes in the CSF of MCI and AD patients.

Although the linkage to neuroinflammation along with the precise mechanism of inducing these effects is unclear, miR-21 is also seen to possess protective properties *in vivo*. In a study conducted by Feng, et al. in 2018, it was concluded that miR-21 has the ability to inhibit cellular apoptosis induced by Aß42 aggregations through the repression of the tumor suppressor gene, programmed cell death 4 (PDCD4). Using the SH-SY5Y cell model, this study also noted how the upregulation of miR-21 led to the activation of the PI3K/AKT/GSK-3ß signaling pathway, which is responsible for regulating cell

growth and survival (Feng et al., 2018). Taken together, miR-21 may serve an important role in protection against synaptic destruction and neuronal loss triggered by AD.

One study noted an upregulation of miR-21 in the CSF of MCI-AD patients compared to control, as mentioned previously (Garcia et al., 2022). These scientists also questioned whether a specific neuronal cell type was mainly associated with its upregulation, so using multiple cell line models isolated from AD patient samples: microglia, astrocytes, and iPSC derived NPCs, along with SH-SY5Y-Δ APP695 (swedish mutant for amyloid precursor protein), the researchers were able to successfully reproduce upregulated levels of miR-21 within all cell types and their derived exosomes. But since sample size was fairly small in this study, further research is necessary to confirm this.

While miR-21 induces both proinflammatory and anti-inflammatory effects, future studies should aim to investigate the precise mechanism as to how this upregulation of miR-21 controls both of these responses within the neuronal secretome, ultimately contributing to the progression of this disease.

Cellular Model of AD Neuropathological Feature Formation

In order to best study one of the hallmark features of AD, an AD cell model is used for *in vitro* conditions. A neuroblastoma SH-SY5Y cell line is typically used along with iPSC-NPC derived from fibroblasts. For the purpose of this study, SH-SY5Ys will be incubated with okadaic acid to mimic the pathological changes of tau protein, a microtubule-associated protein, that is hyperphosphorylated in AD pathology.

OA was orginally isolated from marine algae and known to be a Diarrheic shellfish poisoning toxin used in studying neurotoxicity. Later, it was discovered that OA is an inhibitor specific serine and threonine phosphatases'. One of its cellular effects is inhibition of protein synthesis through an increase in the phosphorylation of translation factor, E2F. In AD, tau protein is hyperphosphorylated and aggregates, forming neurofibrillary tangles (NFTs), although the exact pathway of this formation remain unclear (Boban et al., 2019). Tau protein is found within neuronal axons with a primary function of microtubule stabilization and phosphorylation decreases tau affinity to microtubules while increasing propensity to aggregate (Boban et al., 2019). The accumulation of NFTs contribute to neuronal destruction and loss of synaptic transmission due to hyperphosphorylated tau's inability to carry out microtubule assembly, thus leading to neurodegeneration.

As mentioned previously, an imbalance between tau phosphorylation and dephosphorylation is typically hypothesized to contribute to the dysregulation of tau. OA has the ability to inhibit the action of serine/threonine phosophatases, PP1 and PP2A, leading to tau hyperphosphorylation and NFT production (P. K. Kamat et al., 2013). Other targets may also be responsible for the dysregulation of tau, including kinases like glycogen synthase kinase 3ß (GSK-3ß) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), both of which contribute to tau hyperphosphorylation (Boban et al., 2019). Along with inducing tau pathology, OA has also been shown to increase Aß deposition and aggregation, further promoting cognitive deficiencies in vivo (P. Kamat & Nath, 2015).

From previous studies testing the reliability of OA as a model for AD, injection of OA into animal models led to oxidative stress, increasing the production of free radicals and lipid peroxidation, leading to neuronal stress and degeneration in hippocampal and cortical portions of the brain (P. K. Kamat et al., 2013). OA, along with inducing oxidative stress, also promotes DNA damage, a decline in mitochondria metabolic activity and, as mentioned, NFT formation by inducing tau hyperphosphorylation. These pathological changes in the brain lead to alterations in the neuronal cytoskeleton and ultimately, neuronal cell death. Phosphorylation is involved in many cellular processes such as protein signaling pathways, cell division and gene expression. As discussed before, the action of OA prevents the dephosphorylation of tau protein, promoting its hyperphosphorylation, thus contributing to NFT pathogenesis in AD. This process was examined in previous studies using SH-SY5Y neuroblastomas: upon incubation with OA, an increase in the 100kDa immunoreactive protein which reacted with phosphorylated tau antibodies -pS202 and pS396, was seen (Boban et al., 2019). Thus it was concluded that the hyperphosphorylation of tau protein within the SH-SY5Y neuroblastoma cell line represents a beneficial model for studying AD (P. Kamat & Nath, 2015)(Boban et al., 2019).

CRISPR/Cas13 Platform-Based Detection

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR associated (Cas) proteins were originally discovered in prokaryotic species as a means to avoid viral foreign invaders. Through cleavage and incorporation of the viral invader's genetic sequences into their own, this system prompts swift recognition and

destruction upon second invasion by the same pathogen, thus mirroring antibody function and memory cells of the human immune system (Tang et al., 2021). This molecular machinery consists of Cas effector proteins that are responsible for carrying out nuclease function and a non-coding CRISPR RNA (crRNA) with the primary role of guiding the Cas effector proteins to their appropriate nucleic acid targets sequence sites for cleavage (Tang et al., 2021). The crRNA contains two segments within it: the repeat sequence which makes up the hairpin loop structure and is homologous among all Cas effectors and the spacer sequence which binds to the nucleic acid sequence of interest (Tang et al., 2021). This system has become of extreme interest in the realm of genome editing which utilizes the Cas9 protein and has given scientists the ability to alter and edit genome sequences for many biomedical purposes. The Cas family of effector proteins is composed of a number of different proteins each with varying structure, function and mechanism of cleavage. Currently, there are two subclasses of the CRISPR/Cas system known: class I which includes a wide variery of several different Cas proteins (Cas5 & Cas7) and functions within the effector complex during interaction between crRNA and target DNA or RNA (Phan et al., 2022). This class differs from class II in that class II primarily contains 1 large effector protein which remains structurally similar among all class II cas proteins. Class II consists of Cas9, Cas12 and Cas13 effector proteins and although they have structural homologies to one another they differ each in their nucleic acid targets and degree of collateral activity (Phan et al., 2022). For example, Cas9 and Cas12's primary target is double-stranded DNA (dsDNA) while Cas13 is known to target single-stranded RNA (ssRNA) nucleic acids. The collateral activity, which is of the

greatest degree in Cas13, refers to its ability to cleave both target and non-target nucleic acid sequences and will be discussed further in the following paragraphs (Tang et al., 2021).

In the realm of biosensing for disease diagnostic purposes, a known nucleic acid target sequence that is related to disease or infection can be detected using this CRISPR/Cas13 platform. The crRNA can be designed complementary to the nucleic acid sequence of interest and upon this complementary binding, the activation of the Cas13 effector protein will carry out the collateral cleavage of both target ssRNA molecules containing a poly-U or poly-A tail along with neighboring non-target ssRNA molecules (Figure 3). Cas13's preference for cleavage at poly-U or poly-A tail can be incorporated in the design of signaling molecule sequences. These signaling molecules, upon cleavage, can produce a fluorescent or electrochemical signal, serving to provide a detectable readout for the presence or absence of the specific target sequence in patient samples (Phan et al., 2022). In previous literature, the specificity and diagnostic abilities of CRISPR/Cas-based assays have been demonstrated with the precise detection of infectious diseases (Dengue and Zika), early-stage lung cancer biomarkers along with SARS-CoV-2 infection (Gootenberg et al., 2017; Phan et al., 2022; Tang et al., 2021). To function as a diagnostic system, sensitivity at an attomomolar concentration is preferred for the detection of target molecules and it was shown that the CRISPR/Cas platform is capable of this attomolar and highly specific detection (Gootenberg et al., 2017).

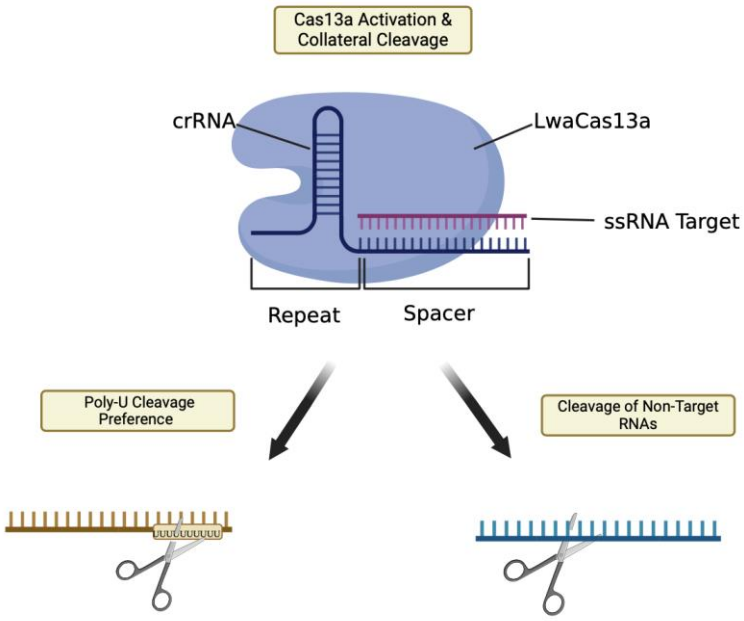

Figure 3: CRISPR/Cas13a Activation and Collateral Cleavage of both Target and Non-Target RNA

Specifically for Cas13, the potential of this system which has been termed SHERLOCK (Specific High Sensitivity Enzymatic Reporter unlocking) is known for its highly accurate and sensitive detection abilities through the recognition of desired target RNA molecules followed by the collateral cleavage of neighboring RNA signaling molecules. This yielding an accurate fluorescent readout, as mentioned before (Li et al., 2019; Phan et al., 2022; M. Wang et al., 2020). Under the SHERLOCKv2 platform, a further developed version of the SHERLOCK platform, it was discovered how this new system is capable of detection using instrument-free methods, like lateral-flow liquid biopsy (Gootenberg et al., 2018). SHERLOCK v2 also possesses the ability to identify genetically modified species within samples which further contributes to its diagnostic power in application and therapeutic development (Gootenberg et al., 2018). From a biomedical standpoint, this rapid and accurate detection can enable early disease

diagnoses through this recognition of specific target sequences known to be linked to disease or infection which can be isolated from patient samples. Unlike classic methods for nucleic acid detection, like reverse transcription-quantitative polymerase chain reaction (RT-qPCR), the CRISPR/Cas system uses less machinery, works in shorter detection cycles and is capable of highly specific detection (Yuan et al., 2021). Now, ongoing studies have been working towards optimizing this system and further enhancing the sensitivity, portability and efficiency of the CRISPR/Cas system for use in clinical settings.

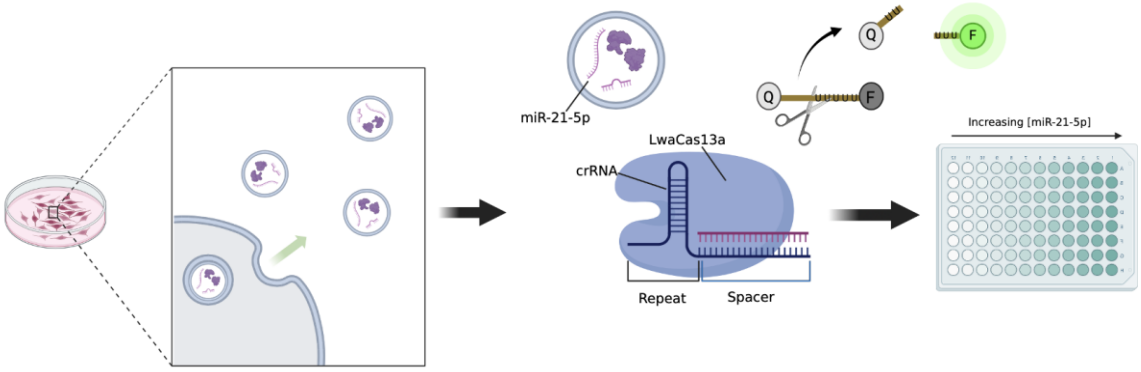

Figure 4: Schematic of CRISPR/Cas 13a-Based Detection of miR-21 Isolated from the EVs of OA-Induced SH-SY5Y Neuroblastomas

For the purpose of this study, the standard LwaCas13a ortholog derived from *Leptotrichia wadei* will be used on the SHERLOCKv2 platform to carry out the detection of miR-21 derived from isolated SH-SY5Y neuroblastomas following extraction of RNA from EV's (J. Yang et al., 2023). Upon complementary binding of miR-21 to the spacer region of the crRNA, Cas13 becomes activated. The ssRNA fluorophore-quencher reporter molecule containing a poly U tail, is then recognized for collateral cleavage following Cas13 activation (Figure 4). The cleavage of the RNA reporter molecule

allows for the separation of the fluorophore and quencher so that the fluorescent signal can no longer be quenched, leading to the production of a fluorescent signal that can be recorded through the use of fluorometer or plate reader. So the fluorescent signal measured increases as the concentration of miR-21 within a sample does. The linearity of the CRISPR/Cas13a system was shown in a study detecting potential breast cancer target, miR-17, with detected concentrations ranging from 10 aM to 100 fM and the quantity of miR-17 detected corresponding to the intensity of the fluorescent signal produced. This study, along with others, displays CRISPR/Cas13's ability in acting as a specific, sensitive and reliable diagnostic tool that can be used in clincal settings (Gootenberg et al., 2018; Granados-Riveron & Aquino-Jarquin, 2021; Shan et al., 2019).

Specific Aims

This study will examine the biosensing ability of the CRISPR/LwaCas13a platform for detection of miR-21-5p, a recently studied biomarker for Alzheimer's Disease. Through the induction of okadaic acid to the SH-SY5Y neuroblastoma cell line as a model for the induction of a neuropathological hallmark feature of AD, the presence of miR-21 within small extracellular vesicles released extracellularly from these neuroblastoma cells will be analyzed using the CRISPR/Cas13 system. This system is a novel platform that allows for the rapid and efficient detection of specific target sequences of interest, measuring their presence in biofluids ranging from CSF, blood, urine, etc., enabling early diagnosis and intervention. Early diagnosis of AD can enable prompt medical intervention before progression into later, irreversible stages, thus treatment, along with uncovering reliable target biomarkers are both of utmost importance for prevention, diagnosis and management of this detrimental neurodegenerative disorder. The aims of this study are two fold: first in utilizing the CRISPR/Cas13 platform with the sensitive and specific detection of potential biomarker, miR-21, and finally to discern the potential association between miR-21 and AD neuropathological features *in vitro.* Taken together, this can allow for the undertaking of future studies using CSF or blood plasma for the diagnosis of preclinical AD.

CHAPTER TWO

Methods

SH-SY5Y Cell Line Passaging

SH-SY5Y cells were originally isolated from the bone marrow of a female neuroblastoma patient and obtained from the American Type Culture Collection (ATCC). Using cell-specific conditions: incubation at 37 degrees with 5% CO_2 levels, cells were passaged using SH-SY5Y specific media (DMEM F12, 10% FBS (Fetal Bovine Serum), 1% P/S (Penicilin-streptomyocin)). Cells were thawed in a 37 degree water bath and diluted with 4 mL of media prior to centrifugation at 1.3 revolutions per minute (rpm) for 3.15 minutes. This centrifugation allowed for the removal of the cyroprotectant, dimethyl sulfoxide (DMSO) which was incorporated into cells during the storing process. Supernatant media was aspirated and cells were resuspended in fresh media, plated and incubated at 37 degrees for 3 days to allow the cells ample time to adhere to the plate.

Passaging began with washing the cells in Dulbecco's Phosphate Buffer Saline (PBS) followed by cellular detachment from the plate using 1x TrypLE Select Enzyme reagent. The plate was incubated at 37 degrees for 5 minutes, or until cells were completely detached from the plate. Media was added in a 2:1 ratio to the amount of TrypLE used for detachment. The detached cells were collected in a 15 mL tube and centrifuged at 1.3 rpm for 3.15 minutes. The supernatant media was aspirated off until only the cell pellet remained. The cells were re-suspended in 2 mL of media by pipetting up and down from the bottom to the top of the centrifuge tube. 10 uL of this cell-media solution was pipetted onto a hemocytometer and cell count was recorded using a light

microscope. A stock plate containing 500,000 cells along with 6-well experimental plate containing 200,000 cells/well were used for this experiment. Cells were passaged every 3-4 days or until space was limiting to growth and cells were confluent upon microscopic visualization. Prior to cell plating, in order to prevent detachment, cell culture plates were coated with a final concentration of 50 ug/mL solution of poly-D lysine prior to plating.

Okadaic Acid Induction

Cells were treated with a final concentration of 0.06 uM (from a 40 uM stock, diluted with PBS) of OA and incubated for 4 hours before media was changed. Cells were then allowed to proliferate for a 3-4 day period before media was collected for proceeding experiments.

Exosome Isolation

After collection of media, exosomes were isolated through differential centrifugation at 4° C. Culture supernatant was centrifuged in the following intervals: 500xg for 10 minutes to remove remaining cells in solution, 2,000xg for 10 minutes to discard dead cells and 10,000xg for 30 minutes to remove any cellular debris and apoptotic bodies. PEG-Dextran Buffer (75% Polyethylene Glycol 20,000, 200 nM NaCl, 10 mM EDTA, 1.65% Dextran) was added in a 1:1 ratio by volume and incubated at 4° C overnight to allow for phase separation.

After the overnight incubation two separate phases were visible: a hydrophilic PEG phase and a dense more hydrophilic Dextran phase below the PEG phase in which the exosomes preferentially accumulated (D. Yang et al., 2020). Solutions were

centrifuged at 1,000xg for 10 minutes followed by the removal of the PEG layer containing lipoprotein contaminants. The solutions were centrifuged again at 15,000xg for 20 minutes to allow for exosome pelleting. The supernantant was discarded. Exosomes were either resuspended in PBS for exosome characterization (DLS, NTA or TEM) or TriZol reagent for RNA extraction.

Dynamic Light Scattering (DLS) & Nanoparticle Tracking Analysis (NTA)

Following resuspension in 1 mL PBS, the sample was purified using a 0.22 μM syringe filter. The samples were transferred to a glass cuvette where exosome size and concentration was recorded using dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) instrumentation, respectively. Through analyzing the scattering intensity of particles within a sample, the average size of particles can be measured using an intensity distribution with DLS without the need to visualize individual particles. NTA, on the otherhand, captures the movement of particles using image analysis. This allows for increased resolution for both size and concentration of particles within a solution to be measured simultaneously (Malvern Panalytical, 2009).

Transmission Electron Microscopy (TEM)

Following resuspension in 400 uL PBS, samples were incubated overnight at 4° C. The copper coated carbon TEM grids were prepared by placing 10 uL of sample suspension on the surface and using a KimWipe to blot the sides of the grid to absorb excess sample. 4% uranyl acetate dye was then loaded onto the grids over the sample

suspension and the blotting technique was repeated. Grids were stored in a vacuum incubator overnight prior to imaging.

RNA Extraction

Immediately following exosome isolation procedure, 1 mL TriZol reagent was added to samples and mixed, pipetting up and down, to redisperse the pellet. Samples were then incubated at room temperature for 2-5 minutes, transferred to Eppendorf tubes and centrifuged at 12,000xg for 10 minutes at 4° C. The supernatant was transferred to a new Eppendorf tube following the addition of 200 uL chloroform. Samples were mixed and incubated at room temperature for 2-5 minutes followed by centrifugation at 12,000 xg for 15 minutes at 4° C. A precipitation buffer was prepared using 5 uL glycogen and 495 uL isopropanol. The aqueous phase of the samples, shown in Fig x, were transferred to the precipitation buffer and incubated overnight at 4° C.

The following day, the samples were centrifuged at 13,000xg for 10 minutes at 4° C. The supernatant was discarded. The pellet was resuspended in 75% ethanol (EtOH) to wash the RNA. Samples were vortexed and centrifuged at 7,500xg for 5 minutes. After this centrifugation, the EtOH was removed and RNA pellet air dried. The pellet was resuspended in 10 uL nuclease free water. Finally, the samples were incubated at 55° C for 10 minutes. The samples were then used for Nanodrop analysis or CRISPR/Cas13 detection of miR-21.

qRT-PCR for miR-21 Quantification & Amplification

Prior to qRT-PCR quantification of miR-21 RNA was extracted from exosomes using the standard RNA extraction protocal detailed above.

qRT-PCR was carried out using the Quiagen miRCURY LNA miRNA PCR assay kit. First cDNA was prepared using a total PCR reaction volume of 10 uL. The sample was reverse transcribed at 42°C for 60 minutes followed by incubation at 95°C for 5 minutes to inactivate DNA polymerase and finally, cooling to 4°C. Samples were stored at 4°C overnight followed by real-time PCR analysis using the standard protocal to prepare a reaction mix: a 1:60 dilution factor for cDNA, PCR primer, 2x miRCURY SYBR green master mix, and ROX reference dye for a 10 uL total reaction volume. Real-time PCR was carried out using Applied Biosystems StepOne Plus Real-Time PCR system and StepOne software.

CRISPR/Cas13 Based Detection of miR-21

The following reagents were used for CRISPR/Cas13 detection: 1uM commercial LwaCas13a ortholog protein from McLab, a 1 uM solution of crRNA from IDT, a 1 unit/mL RNase inhibitor solution from New England Biolabs, 1x Cas13 protein buffer from McLab, a 10 uM concentration of an RNA reporter solution from Thermo Fisher Scientific and finally, a 1 uM concentration of commercially produced miR-21-5p from IDT. Final concentrations of miR-21 were adjusted accordingly prior to experimentation.

For experimental purposes, a final volume of 100 uL was prepared for each experimental sample and contained the following: 5 uL Cas13a, 5 uL crRNA, 2.5 uL RNase inhibitor, 72.5 uL Cas13 reaction buffer, mixed and incubated at room

temperature for 15 minutes following the addition of 10 uL miR-21 target and incubated at 37° C for 1 hour. Samples were loaded onto a 396 well plate with the addition of 5 uL RNA reporter as fluorescent intensity was recorded on plate reader, with an excitation of 490 nm and emission at 520 nm.

CHAPTER THREE

Results

From microscopic analysis of cell culture samples, those neuroblastoma cells treated with okadaic acid (OA), as expected, exhibited cellular clusters and aggregated morphology due to its role in the hyperphosphorylation of tau protein, as mentioned previously(Boban et al., 2019; P. K. Kamat et al., 2013; P. Kamat & Nath, 2015). As shown in Figure 5b, the extracellular accumulations are, hypothesized to be a result of these physiological changes caused by the disruption of tau protein regulators, like PP2A (Boban et al., 2019) (P. Kamat & Nath, 2015). But in order to definitively link OA induction to tau pathology, future studies should investigate other *in vitro* effects OA is capable of inducing and how these changes might indirectly or directly alter tau pathological changes.

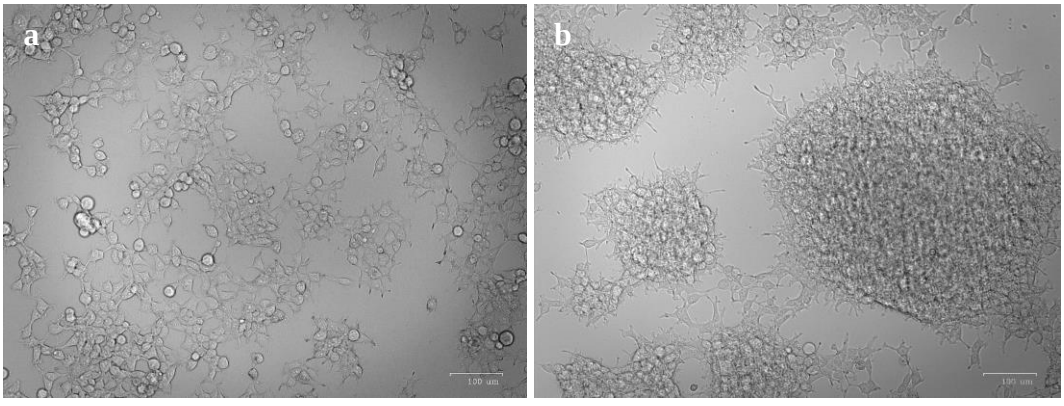

Figure 5: Neuroblastoma SH-SY5Y Cell Line (a) Non-Treated and (b) Treated with Okadaic Acid

After carrying out exosome isolation and RNA extraction from SH-SY5Y neuroblastoma cells, the presence of their derived exosomes, their composition and morphology was cofirmed using gold standard characterization techniques, with each

method providing beneficial data for analysis (Chernyshev et al., 2015; D. Yu et al., 2022). The most common characterization techniques include nanoparticle tracking analysis (NTA), dynamic light scattering (DLS) and transmission electron microscopy (TEM) to measure relative size and concentration, size distribution and intensity and visualization of individual exosomes, respectively.

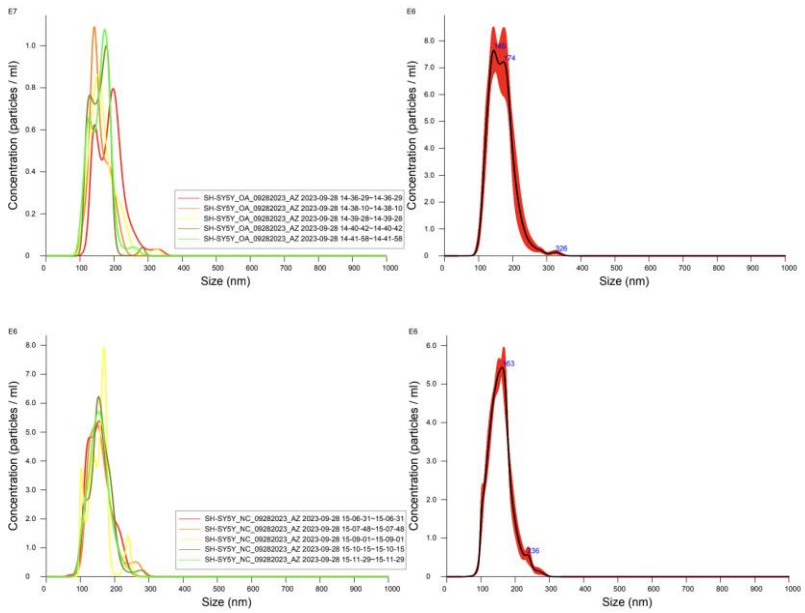

Figure 6: Nanoparticle Tracking Analysis (NTA) of SH-SY5Y Dervied Small EVs induced with OA (a) and NC (b) (Courtesey of Li Ling Goldston)

First, by carrying out 1 replicate using NTA machinery, which displays visual particle tracking measurements in 5 second intervals 60 seconds, producing replicates that are averaged for the final measurment, it was shown that the OA-treated SH-SY5Y neuroblatoma (SH-SY5Y-OA) derived exosomes displayed a concentration of ~5.5 x10^9 particles/mL with an exosome relative size of 163 nm. For the non treated, control SH-SY5Y (SH-SY5Y-NC) exosomes the concentration was ~7.5 x10^9 particles/mL with an exosome size of 150 nm (Figure 6). Since OA is not known to alter exosome size or

concentration in any way, it was expected that these values fall within the same range for SH-SY5Y-NC and SH-SY5Y-OA samples. From DLS analysis, exosome size was again confirmed and seen at approximately 100nm (Figure 7). On average, cell derived small extracellular vesicles are seen to be ~50-200 nm in size, along with exhibiting characteristic cup shape morphology that is recognizable through TEM (D. Yang et al., 2020). Within the single replicate TEM images shown in Figure 8, the prescence of exosomes was confirmed through the display of correct morphology and size distribution. The exosome sizes using TEM were seen to be 52.09 nm, 60.13 nm, 92.19 nm, 130nm, 144.7 nm and finally 181.3 nm, all of which are within range for the sizes expected. Thus it was concluded that neuroblastoma cell derived exosomes were successfully isolated and can be used for further experimentation using the CRISPR/Cas13 platform.

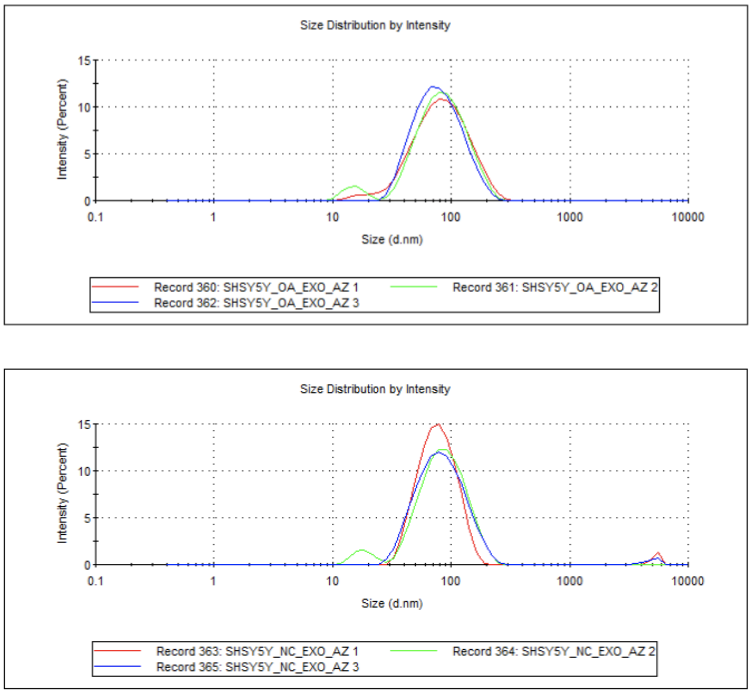

Figure 7: Isolated Exosome Characterization from (a) treated SH-SY5Ys and (b) non-treated SH-SY5Ys Using DLS (Dynamic Light Scattering) Analysis

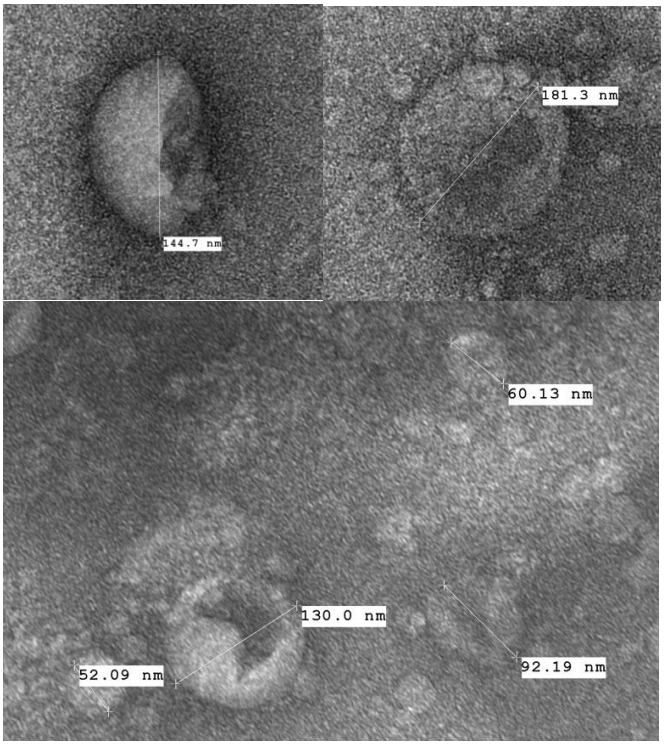

Figure 8: Transmission Electron Microscopy (TEM) of SH-SY5Y Derived Small EVs (Courtesey of Li Ling Goldston)

In order to quantify the concentration of RNA extracted from exosome samples for CRISPR/Cas13 detection, nanodrop measurements were recorded following the RNA extraction procedure. Table 1 displays a single replicate of measurements including the purity (A260/A280) and total RNA concentration of treated (SH-SY5Y-OA) and control (SH-SY5Y-NC) samples. A purity >1.80 is considered ideal in characterizing RNA-containing samples. Due to the SH-SY5Y-OA sample displaying a purity value of <1.80, this low purity ratio can be attributed to a potential protein contamination since nucleic acids are associated with absorbance values at 260 and proteins are associated at absorbance values of 280, thus lowering the purity ratio.

Table 1: Purity and Total RNA Concentration Isolated from SH-SY5Y EVs

	SH-SY5Y - OA	**SH-SY5Y - NC**
A260/A280	1.63	2.00
[Total RNA]	225.7 ng/uL	344.3 ng/uL

Prior to integrating the CRISPR/Cas13 platform for detection of miR-21-5p, optimizations were performed. First, by testing the range of detection using commercial miR-21-5p at varying concentrations, ideal points of fluorescence intensity (FI) were extracted and used to contruct a calibration curve to measure the limit of detection (LOD) of the CRISPR/Cas13a system (Figure 9). From this curve, the LOD of the system can be calculated by multiplying the standard deviation of the NC (negative control: no miRNA target added) by 3 and dividing by the mean of the NC. The LOD was seen to be ~1pM. Previous literature notes a LOD for the CRISPR/LwaCas13a platform ranging from 1 aM to ~50 fM (Gootenberg et al., 2017; Tang et al., 2021). There are several reasons that can be attributed to this discrepancy. First, the quality of CRISPR/Cas13 reagents may vary depending on the vendor these reagents are purchased from. Second, these studies citing low detection concentrations utilized CRISPR/Cas13 reagents that were designed and synthesized within their lab, so it was possible for the sensitivity of the system to be optimized and lowered (Shan et al., 2019). Finally, the concentrations of CRISPR/Cas13 reagents used for experimental studies may have varied between labs, so it is possible that this study utilized a smaller concentration of CRISPR reagents for the detection of RNA targets compared to the studies carried out in previous literature. For purchased

CRISPR/Cas13 reagents, a LOD at 1 pM is expected. Thus, these results lead to the confirmation of the sensitivity of the CRISPR/Cas system at 1 pM. Testing the specificity of the designed crRNAwithin the CRISPR/Cas13 system was the final measure for optimization parameters. Using the same protocal for CRISPR detection, FI was measured using the designed crRNA in samples containing either miR-21-5p or miR-23a. This specificity of the designed crRNA following CRISPR/Cas13 fluorescent assay can be seen in Fig. 10. These results are expected since the designed crRNA is complementary, it will bind to the target sequence of interest, miR-21, and will not bind to any off-target miRNAs, in this case miR-23a.

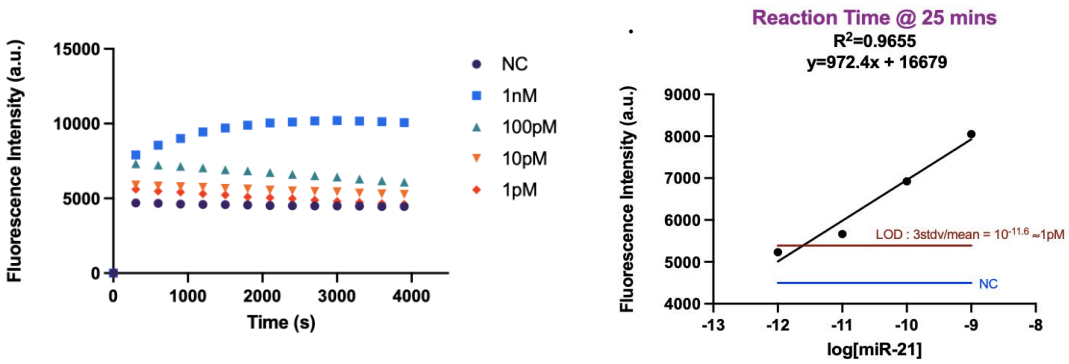

Figure 9: Sensitivity Optimization of the CRISPR/Cas13a Platform using (a) relative fluorescent intensity at varying concentrations of miR-21-5p and (b) calibration curve at reaction time 25 min for extraction of LOD; NC: negative control

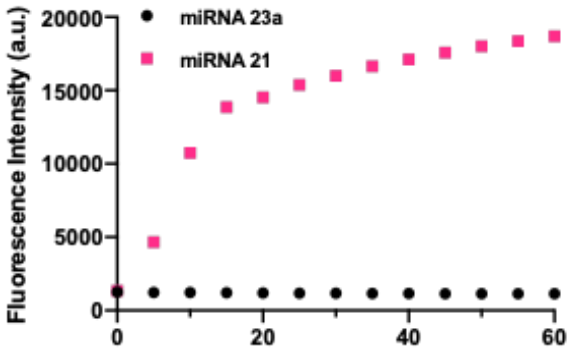

Figure 10: Selectivity of CRISPR/Cas13a Platform (Courtesy of Meizi Chen)

Following the CRISPR/Cas13 optimization parameters, detection of miR-21 isolated from small EV's derived from the AD SH-SY5Y cell model was recorded and can be seen in Figure 11. These experiments showed a trend for a higher fluorescence intensity in the SH-SY5Y-OA sample, confirming this study's hypothesis. But due to the variable and outlier data points among the sample. But the difference between amounts of miR-21 between the two samples was not significant. In order to determine the concentration of miR-21 available for CRISPR/Cas13 detectiont within the SH-SY5Y derived EVs, the average FI's from the SH-SY5Y-OA and SH-SY5Y-NC samples were plotted along the constructed calibration curve from figure 9b. These results can be seen in figure 12. Looking at this data, it is interesting to note how both samples were below the limit of detection threshold. From the values that were obtained, the concentration of miR-21 within SH-SY5Y derived EVs can be seen as ~$10^{-12.48}$ and ~$10^{-11.75}$ (~1pM) for the treated (SH-SY5Y-OA) and non-treated (SH-SY5Y-NC) samples, respectively. From this conclusion, it is clear that a larger sample volume of SH-SY5Y derived EVs are needed for the precise measurement of the concentration of miR-21 isolated from the EVs. If a larger volume of SH-SY5Y EVs cannot be obtained, the introduction of a nanomaterial, which will be discussed in detail within the concluding remarks section, would be of great use as it would allow for increased sensitivity at lower concentrations. This would fuction in lowering this platform's limit of detection <1pM.

Furthermore, upon analysis of the RT-qPCR in Figure 13, which measured the relative expression of miR-21, the same conclusion can be drawn: non statistically significant differences between those neuroblastoma SH-SY5Ys treated with OA and

those non treated. The LOD for RT-qPCR was not determined in this study, but based on previous literature, the approximate LOD range for RT-qPCR is 3 aM to 53 fM (J. Yang et al., 2023). Comparing this value to the LOD obtained in this study, RT-qPCR displays higher sensitivity and is able to detect RNA targets at lower concentrations. But since the CRISPR/Cas13 platform has the ability to be optimized, one example of this lies in the introduction of a nanomaterial as mentioned above, the platform's LOD could be drastically lowered in comparison to RT-qPCR. Furthermore, in a recent study, Yang, et al. engineered LwaCas13a variants with enhanced target RNA binding affinity and collateral cleavage activity through the introduction of RNA binding domains in the active site of the Cas13. This study was able to push the sensitivity to the lowest concentrations documented: 10 aM and 20 aM (J. Yang et al., 2023). So, although this study did not display the most sensitive detection abilities of CRISPR/Cas13 compared to RT-qPCR, LwaCas13a is capable of highly sensitive detection of target RNAs through its optimization.

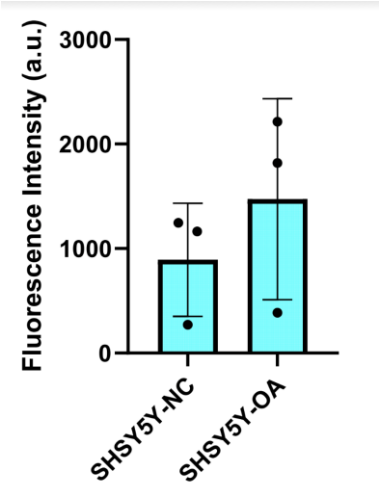

Figure 11: CRISPR/Cas13a Detection of miR-21 from isolated EV's derived from the SH-SY5Y cell line

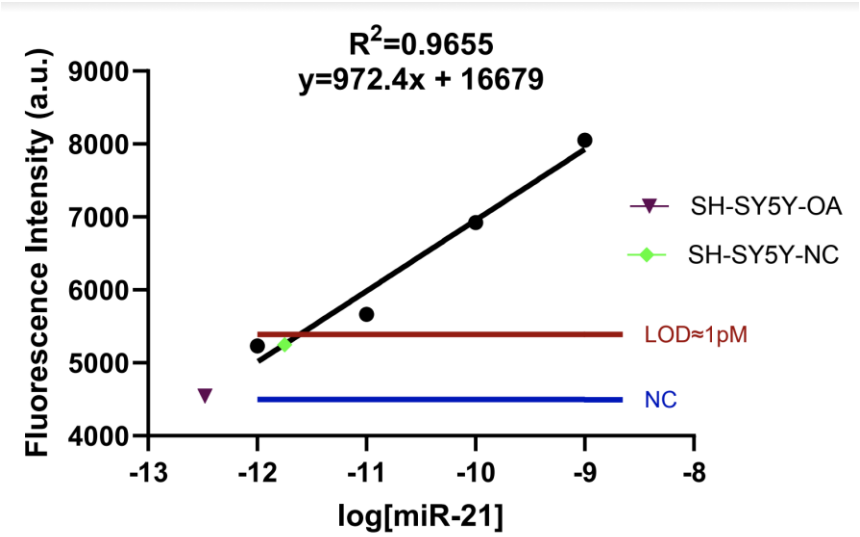

Figure 12: Concentration of miR-21 detected from SH-SY5Y derived EV's; NC: negative control

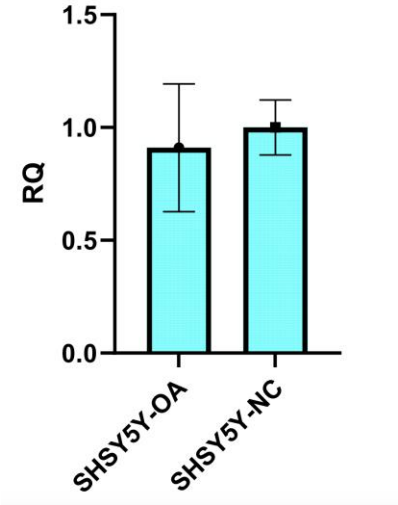

Figure 13: RT-qPCR Analysis of miR-21 Expression Isolated from SH-SY5Y derived exosomes

Overall, even though these results do not support the proposed hypothesis, this study has exemplified the novel ability that the CRISPR/Cas system holds within the field of biosensing and diagnostics. So, moving forward, as more target biomarkers are discovered, the flexibility of the CRISPR/Cas13 system and its optimization would allow

for the detection of any RNA-based biomarker that may be linked to the onset or progression of AD.

CHAPTER FOUR

Discussion & Concluding Remarks

Alzheimer's Disease, one of the most prevalent neurodenegerative diseases, negatively affects neuronal cellular function and impededs many physiological processes. This study, despite the highly sensitive and specific nature of CRISPR/Cas13 technology, it has not shown miR-21's presence within a cellular model of AD pathology. Some concerns surrounding the reliability and validity of this study are centered around the newness of the field and miR-21 as a reliable biomarker. Since there has been one main study in the recent years documenting the upregulated miR-21 levels found within AD patient CSF lumbar samples(Garcia et al., 2022), further research is necessary to discern miR-21's exact role within the pathology and progression of AD. It is also important to note that although many previously conducted studies cite miR-21's upregulation in AD neuronal cell lines along with the study documenting its presence in the CSF of AD patients, many articles have also noted its downregulation in AD patient CSF and serum samples through qRT-PCR (Burgos et al., 2014; Gámez-Valero et al., 2019; Sørensen et al., 2016). This reduces miR-21's significance in acting as a reliable biomarker for clinical trials and potential therapeutic measures (Gámez-Valero et al., 2019). Since most current knowledge regarding miR-21's upregulation stem from *in vitro* studies, little research in analyzing the miRNA profiles of AD patient samples has been carried out. In using miRNAs as blood or CSF-based biomarkers, there also lies a limitation in the reproducibility of results. Since miRNAs tend to be very unstable, variation within lab protocol, methodologies and handling most likely contributes to this variability seen

amongst these studies. So further research is necessary in order to extablish miR-21 as a reliable biomarker for AD as well as working towards standardization of methods for extraction, isolation and handling of miRNAs derived from patient samples which would both prove beneficial if moving forward in this field of research.

Although miR-21 has been cited for its involvement in AD pathogenesis, there have been other miRNA biomarkers that have been cited in directly contributing to the mechanisms behind AD pathology in vivo. For example, miR-346 has been noted to increase the production of Aß in HeLa cells hrough upregulating the translation of APP by targeting the 5'-UTR of APP mRNA (Sequeira & Godad, 2023). This study also found that levels of miR-346 were reduced in late-stage AD patient samples, concluding this miRNA's role in AD pathology. MiR-200c, along with the miR-200 family, have been shown to play a role in microtubule destabilization due to its interaction with the 14-3-3γ gene which can lead to tau pathology (Sequeira & Godad, 2023). MiR-101, on the otherhand, downregulates the amount of APP produced via interaction with the APP 3'-UTR in HeLa cells and SK-N-SH neuroblasmtomas (Long & Lahiri, 2011). This ultimately leading to a decrease in APP, and in turn, a decrease in the production of neurotoxic Aß present in the brain. This finding can facilitate the development of novel therapeutics through the integration of miR-101 or other miRNAs shown to attentuate AD pathology. In clinical studies, it was reported that miR-101 and -29a's downregulation in AD patient blood samples compared to non-AD controls can serve as a useful combination biomarker for non-invasive early diagnosis (Sequeira & Godad, 2023). MiRNAs like miR-124-3p, miR-128-3p and miR-146a-5p, which contribue to Aß

accumulation, oxidative stress and neuroinflammation may also be useful in early diagnosis (Ghafouri-Fard et al., 2021; Liu et al., 2019; Zhan-qiang et al., 2023)((Sequeira & Godad, 2023). But due to the modifiable nature of the CRISPR/Cas biosensing system, this poses no issue as virtually any miRNA could be targeted and detected by altering the crRNA sequence. So, as further research deduces miR-21's possible role among other miRNAs in AD pathogenesis, the CRISPR/Cas13 platform is reliable for its specificity and sensitivity and can be used in clinical settings for detection of precise nucleic acid sequences for diagnostic purposes.

In the limited number of studies that have examined miR-21 levels in AD patient samples, clinicians carried out CSF lumbar punctures to extract samples for analysis, which is a more invasive procedure that may not be accessible to patients in clincal settings as compared to a fingerstick or blood draw. But although a blood sample may serve as a less invasive measure for patients, it has the potential drawback of not encompassing the full profile of biolecules present in the CNS due to the presence of the BBB which functions as a filter and can alter the RNA, protein and nucleic acid profiles within circulation. It is also important to note the potential degradation and processing/cleavage of proteins and other molecules within the circulation as this can also alter the quantification of biomolecules within the blood compared to the CSF (X. Yu et al., 2023).

While both CRISPR assay and PCR data conclude the same results: no significant difference between control sample and treated sample with OA, this can be explained partly due to the nature of the neuroblastoma cells themselves. Since all cancer cells are

known to exhibit high levels of this miRNA due to its involvement in oncogenic pathways that inhibit tumor suppressor genes, thus enhancing tumor progression, this can be a reasonable explanation as to why the data yielded this result (Hashemi et al., 2023). If this study were to be replicated, it would be beneficial to instead utilize iPSC-NPCs to address this challenge and in doing so, would serve as a more representative cell model for AD. Furthermore, following treatment of iPSC-NPCs with OA or Aß and CRISPR/Cas13 detection, this model would provide more encompassing miRNA profiles *in vitro* and *in vivo*.

In optimizing biological platforms like CRISPR/Cas, novel measures are currently being undertaken to improve the sensitivity of fluorescent assay readouts through the phemonenon of metal-enhanced fluorescence (MEF) and surface plasmon resonance using a distance-dependent quenching mechanism and energy transfer within an electromagnetic field (S. Lee & Kang, 2023). The field of nanotechnologies has revolutionalized science and medicine by allowing scientists to detect, diagnose, monitor and treat a wide variety of disease through the introduction of nanomaterials to increase the sensitivity of detection at lower patient sample concentrations. Currently, a wide variety of nanomaterials exist in this field, with those capable of inducing a MEF effect containing a noble metal component such as Au, Cu, Al, and Ag. By introducing a metal-based nanoparticle or nanorod, this would allow for the enhancement of the readout signal of the CRISPR/Cas13 platform, producing efficient and highly sensitive detection of target biomolecules, making it a novel asset to the field of biosensing (J.-H. Lee et al., 2019). In one study conducted by Lee, et al., the novel mechanism of immune-magnetic

exosome capture to monitor stem cell differentiation using a miRNA biomarker was carried out using a Au-Ni-Au nanorod, which exploits the function of both localized surface plasmon resonance and distance-dependent metal enhanced fluorescence. Through the use of a functionalized CD63 Ig, exosomes could be successfully captured and miRNA targets isolated using a complementary molecular beacon functionalized to the Au portion of the nanorod, providing both a specific and sensitive readout confirming the presence of miRNA targets.

If this study were to be continued, it would be a novel measure to introduce a nanomaterial or nanorod for increasing the selectivity and sensitivity of the CRISPR/Cas13 platform. For biosensing purposes, introducing a nanomaterial may push the sensitivity as low as possible which is beneficial in the field of nanotechnology. In utilizing the Au-Ni-Au nanorod, as described above, exosomes specific to certain cell types may be selectively captured using an aptamer or antibody functionalized to the nanorod. For example, it has been shown that neural cell-derived exosomes display the L1 cell adhesion exosome surface marker (L1CAM) which would be beneficial to select for using the Au-Ni-Au nanorod (Huo et al., 2021). As mentioned before, it would also be beneficial to carry out this protocal using iPSC-NPCs in order to replicate a more accurate AD model to deduce miR-21's ability in acting as a potential biomarker for this neurodegenerative disease. From past literature, it has been shown that the mutant iPSC-NPCs isolated from AD patients, when differentiated, are capable of exhibiting hallmark features of AD: Aß production and hyperphosphorylation of tau, and were also seen to display notably upregulated levels of miR-21, miR-124 and miR-125b (Garcia et al.,

2022). So by using the iPSC-NPC cell model as opposed to a neuroblastoma cancer cell line (SH-SY5Y) model, it would potentially provide a more encompassing cell model for analyzing a potential upregulation of miR-21 attributed to AD pathology.

When it comes to the analysis and characterization of exosomes, alongside DLS, NTA and TEM, Western Blot is another gold standard measure to accurately confirm presence and characterization of exosomes using Ig-CD63 (an exosome-specific tetraspanin protein), which would also be beneficial to carry out for the nature of this project (Khushman et al., 2017). This study aimed to correctly isolate exosomes derived from the neuroblastoma, SH-SY5Y cell line. Due to the SH-SY5Y specific media containing FBS which contains exosomes of its own, another limitation lies in the inability of passaging the cells using exosome-depleted media. These exosomes derived from the FBS may also contain miR-21 and this could potentially skew the FI value recorded, leading to an overestimated value of miR-21 in SH-SY5Y samples.

Due to current studies examining the diagnostic ability of exosomes being in the early stages of development, some concerns have arisen due to the newness of the field and the complex pathological disease stages AD and other neurodegenerative diseases exhibit, ultimately affecting biomarker quantification (L. Wang & Zhang, 2020). Furthermore, within exosome-based diagnostic research, some concerns arise around the cost of materials and equipment needed for exosome isolation, extraction and analysis of the target biomarker within exosomes- making it a time consuming procedure, leading to samples not as readily available for diagnostic assay. Another limitation lies in the feasibility of integrating this exosomal-based technology into clinics, which may pose a

challenge due to the time consuming and costly nature of the current extraction and isolation procedures. All of which present challenges when it comes to the practicability of exosome-based detection measures.

Exosome-based research, along with being a beneficial origin for diagnostic biomarkers, can also serve as a promising vehicle for drug delivery through due to their ability in crossing the BBB. For example, as mentioned previously, a novel study noted how the delivery of exosomes containing siRNA targeting BACE1 mRNA decreased the amount of cytotoxic Aß production upon delivery to a mouse brain model, making exosome-related studies of great interest for future diagnostic and therapeutic purposes (Alvarez-Erviti et al., 2011; Fan et al., 2022; Hornung et al., 2020).

Overall, as AD research progresses, the CRISPR/Cas systems can be of great use in the field of diagnostics. With its specific and sensitive nature, it allows for the rapid and efficient detection of virtually any miRNA biomarker. Due to AD's quick progression, future studies should focus on standardization and reproducibility of target biomarkers from study to study and work towards incorporating AD patient samples for precise analysis. In the recent years, exosomes-based biomarkers have become of rising research interest for the diagnoses of neurodegenerative diseases due to EV's ability in crossing the BBB. But further research is necessary to standardize methodologies, like the isolation and extraction procedures, in order to be used in clincal settings. In conclusion, although it is unlikely that one molecule alone can act as a biomarker or is directly responsible for a diseased state due to the complex nature of AD pathology and interplay between biolmolecules within organ systems, ongoing studies should have aims

of evaluating the complex interplay between newly discovered miRNA, proteins and other molecules that may play a role in the onset and progression of AD.

As research within the field of diagnostics expands, this will open up avenues towards early detection and hopefully, in combination with other research efforts, a cure for this fatal neurodegenerative disorder.

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CURRICULUM VITAE