The role of matrin 3 in the pathogenesis of amyotrophic lateral sclerosis

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Boston University
THE ROLE OF MATRIN 3 IN THE PATHOGENESIS OF
AMYOTROPHIC LATERAL SCLEROSIS

by

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HAO WANG

ABSTRACT

The cause of amyotrophic lateral sclerosis (ALS), a cruel neurodegenerative disease, remains unclear. Trans-activating response region (TAR) DNA-binding protein of 43 kDa (TDP-43) has been suggested to have an important role in ALS pathogenesis. In this thesis, we show that a disease linked mutation in matrin 3 (MATR3), a DNA/RNA-binding protein, corresponds to an increased tendency for TDP-43 to aggregate into large and more numerous cytoplasmic inclusions that are the hallmark of ALS.

Immunocytochemistry experiments show that MATR3 colocalizes with TDP-43 in vitro. These experiments also show TDP-43 is a component of both MATR3 granules and stress granules, and that MATR3 inclusions are directly adjacent to stress granules or eIF3α inclusions. We hypothesize that, while not being a part of stress granule complex, MATR3 granules are involved in RNA processing via the stress granule pathway by relaying crucial components such as TDP-43. We have also found that compound 8J is able to disaggregate and relocate TDP-43 and MATR3 positive inclusions in vitro. While the mechanism of action of compound 8J remains unclear, fluorescence activated cell sorting (FACS) experiment showed that there was a significant increase in viability in double wild type (matrin 3 and TDP-43) cells when treated with C8J (p-value <.001), which suggests that the TDP-43 and MATR3 cytoplasmic inclusions that were previously observed have a net cytotoxic effect. Together with the in vitro result on C8J, this result
also suggests that C8J enhances the survivability of cells by restoring TDP-43 back to the nucleus. MATR3 biochemistry seems to connect to neurodegenerative diseases in several ways. Identifying the pathological connections between MATR3 and TDP-43 physiology will provide us with a greater understanding of ALS pathology.
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LIST OF ABBREVIATIONS

BU .......................................................................................... Boston University
ALS .......................................................................................... Amyotrophic lateral sclerosis
FDA ......................................................................................... Food and Drug Administration
SOD1 ...................................................................................... Superoxide dismutase 1
TDP-43 .................................................................................... T binding protein 43
MATR3 ..................................................................................... Matrin 3
SG ............................................................................................... Stress Granule
INTRODUCTION

Amyotrophic lateral sclerosis is a human neurodegenerative disease that affects the motor neurons in the brain, spinal cord, and peripheral nervous system\(^1\). Clinical features are characterized by weakness of arms, legs, and face and difficulties with speech, swallowing and respiratory functions\(^2\). Current clinical observations suggest that it affects women and men, regardless of ethnicity, and risk increases substantially with age\(^2\). The clinical progression of ALS is one of the fastest among neurodegenerative diseases and the patients typically die from respiratory failure within 3 to 5 years after onset\(^1\)\(^{-2}\). Currently, there is no cure for ALS. The only FDA approved treatment is Riluzole, which prolongs survival by three months\(^1\). The cause of the ALS is currently unknown. Approximately 10% of ALS is familial, and the remaining 90% of cases appear to be idiopathic\(^1\). Missense mutations in superoxide dismutase 1 (SOD1) have been identified more than 20 years ago in ALS patients, and population studies have suggested that mutation in SOD1 accounts for ~20% of the familial cases and ~2% of all cases\(^3\)\(^{-4}\). This discovery has led to the creation of SOD1 transgenic mice. The usefulness of this model to screen for therapeutic agents in human trials has been repeatedly called into question because treatment that suppressed symptoms in SOD1 mutant mice have consistently failed in human trials\(^5\). In addition, the pathology of SOD ALS is distinctive in that it lacks the TDP-43 positive cytoplasmic inclusions or FUS pathology present in other ALS pathologies\(^6\). With this discovery, many researchers have begun to explore new mechanisms of ALS pathogenesis.
More recently, TAR DNA-binding protein (TARDBP) has been identified as a major component of the ubiquitin-positive neuronal inclusions that are the hallmarks of ALS and fronto-temporal dementia (FTD)\(^7\). TDP-43 normally resides inside the nucleus, but pathological inclusions containing TDP-43 tend to aggregate in the cytosol, which suggests that subcellular protein disposition is a crucial step in ALS pathogenesis\(^7\). Although TDP-43 has been subjected to great investigation, little is known about whether the pathology is due to a gain of toxic function or loss of important biological functions. Moreover, very little is known about the pathways of TDP-43 pathogenesis or how other key players interact with TDP-43 that contributes to the pathogenesis of the disease. The discovery of TDP-43 in ALS pathology has highlighted the importance for RNA processing in neurodegenerative diseases. Subsequently, FUS and other RNA-binding proteins have been discovered\(^8,9\). We propose that the pathological inclusions of many neurodegenerative diseases proceed through the stress granule pathway, and mutations in key stress granule proteins or prolonged stress lead to increased stress granule formation and pathological inclusion in neurodegenerative diseases\(^10\). Recently, Johnson et al. found that mutations in MATR3 genes are associated with familial ALS\(^11\). Thus, we shift attention to MATR3—an important RNA- and DNA-binding protein—that interacts with TDP-43, in effort to understand how its mutations might play a role in ALS pathology. Although the biochemistry of MATR3 has been studied extensively, very little attention has been given to its relevance in neurology. It is known to bind and stabilizes mRNA\(^12\), retain hyper-edited RNA\(^13\), regulate formation of RNAi\(^14\), chaperone stem cell
differentiation\textsuperscript{15}, and more. Thus, we set out to explore the interactions of MATR3 with TDP-43, in effort to clarify its role in ALS pathogenesis.
METHODS

Cell Lines

HT-22 (Murine hippocampal cell line) cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) plus 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% nonessential amino acid (Fisher) at 37 C and 5% CO₂ atmosphere.

Plasmids

The TDP-43 A315T and wild type cDNAs were inserted into a pEGFP-C1 vector at BamH1/Xbal sites. The MATR3 S85C and wild type cDNAs were inserted into a pmCherry vector at BamH1/Xho1 sites modified to express monomeric mCherry fluorophore. Cloning was performed by Shamol Saha (Boston University School of Medicine).

Cell Culture and Transfection for Fluorescence Microscopy

12mm coverslips (Fisher Scientific) were washed with concentrated nitric acid overnight to remove any organic contaminants. The coverslips were then washed with distilled water until pH returns to 7. When coverslips were completely dry, the cleaned coverslips were coated with 1% poly-L-lysine solution overnight in 12 wells plates (Falcon). The poly-L-lysine coated slips were washed with deionized water three times under sterile conditions before culture. HT-22 cells were plated on to poly-L-lysine coated slips (60,000 cells/coverslip) and cultured overnight in DMEM plus 10% fetal bovine serum, 1% penicillin/streptomycin,
and 1% nonessential amino acid. After 20 hours, serum free media was replaced in preparation for transfection. Lipofectamine 2000 (Invitogen) solution was prepared separately with Opti-MEM Reduced Serum Medium, mixed well and incubated at room temperature for 15min. TDP-43 and MATR DNA samples were prepared and mixed with lipofectamine solution at a ratio of 1µg/2µL and incubated at room temperature for 20 min. For each sample, 600ng of MATR3 and TDP-43 DNA was added for a total of 1.2µg DNA per well. After 12 hours of transfection, culturing medium was replaced and experiments were initiated 24 hours after transfection.

**Stress Induction**

1mM of Sodium Arsenite was prepared in deionized water and filtered with Corning vacuum filters. The desire concentration of 7µM NaAsO₂ solution was prepared by titrating the proper amount of 1mM NaAsO₂ with culture medium. Cells were then treated with the 7µM NaAsO₂ medium for 24 hours. All stress experiments were initiated 24 hours after transfection.

**Immunocytochemistry**

HT-22 cells were grown on poly-L-lysine-coated slips and were treated 7µM NaAsO₂ for 24 hours. Following treatment, the cells were rinsed with PBS, fixed with 4% paraformaldehyde for 15 min, permeablized with 0.2% Triton-X100/PBS for 10 min, and blocked for 1 hour in PBS containing 10% donkey serum. The primary antibody used were: goat anti-eIF3α (1:200, Santa Cruz Biotechnology, Inc, sc-16377), rabbit anti-
DDX6 (1:400, Berthyl Laboratory, Inc), and goat anti-TIA-1 (1:200, Santa Cruz Biotechnology, Inc, sc-1751). Secondary antibodies used were: Dylight 405 conjugated donkey anti-rabbit IgG and Dylight 405 conjugated donkey anti-mouse IgG (1:400, Jackson ImmunoResearch). Samples were incubated with primary antibodies in 5% donkey serum/PBS, overnight at 4°C. After, cells were washed three times with PBS, and were subsequently incubated with secondary antibodies diluted in PBS for 1 hour at room temperature, covered from light. Cells were then washed three times with PBS, one time with 0.2% Triton-X100/PBS, and then one last time with PBS. The coverslips were then mounted on glass microscope slides (Fisher Scientific) using 8 μL of Prolong-Gold Antifade reagent (Invitrogen). Slides were then left to dry overnight in fume hood and sealed using clear nail polish.

C8J experiments

C8J dissolved in dimethyl sulfoxide was obtained as 30mM solution from Dr. Benjamin Wolozin. In previous high content screening, the EC50 was determined to be 200nM. The desired concentration of 5μM was prepared from titrating with the proper volume of 30mM C8J. Cells were then treated with 5μM C8J for 24 hours against stress and MATR3 pathology to determine its possible therapeutic effect.

Microscopy

Fluorescence microscopy was performed using a Carl Zeiss Axio Observer.Z1 carrying lasers at 405, 488, 543 and 633nm. All images were captured using a 40 or 63X len. Zen
Pro was used for digital image analysis. Imaris Bitplane was used for analysis of subcellular inclusions. Images were prepared and combined into figures for the manuscript using Adobe Illustrator and Adobe Photoshop software.

*Fluorescence Activated Cell Sorting (FACS)*

All FACS experiments were performed using FacsCalibur (BD Biosciences) carrying 488nm Argon laser with three fluorescence channels at 515-545nm, 564-606nm and >650nm. Cells were grown on 12-well plate (Falcon), harvested using 0.25% trypsin-EDTA solution (Sigma), and subsequently washed with PBS and Binding Buffer (eBioscience, cat. 00-0055). Each cell sample was resuspended in 75µL of Binding Buffer, and 2.5µL of Annexin V PE (eBioscience, cat. 12-8102) was added to the cell suspension and mixed well. The cells were incubated at room temperature for 20 min, and subsequently washed, transferred to 5mL polystyrene round-bottom tubes (BD Falcon) and resuspended in 250µL of Binding Buffer. Then, 2.5µL of 7AAD Viability Staining solution (eBioscience, cat. 00-6993) was added to each sample, mixed well, and readied for FACS analysis. For proper compensation, Negative control was unstained cells, and positive control was prepared by 90°C heat shock with Annexin V PE and 7AAD Viability Staining solution.

*Statistical Analysis*

All statistical analysis was done using one-way ANOVA followed by t-test. Statistical
calculations were performed using R-statistics program. Error bars in figures are shown as standard error of the mean.
RESULTS

Figure 1. MATR3 colocalizes with TDP-43. HT22 cells were transfected with plasmids encoding eGFP-WT TDP-43, eGFP-TDP-43 A315T and/or mCherry-WT MATR3, mCherry-MATR3 S85C. MATR3 S85C. MATR3 colocalizes with TDP-43. Normally MATR3 is localized to the nucleus. Under basal conditions, HT22 cells transfected with WT TDP-43 and WT MATR3 shows mainly localization TDP-43/MATR3 in the nucleus with occasional cytoplasmic inclusions. S85C mutation of MATR3 greatly increases the formation of both TDP-43 and MATR3 cytoplasmic inclusions. The translocation effect is observed most dramatically in mutant TDP-43 and MATR3 condition, where TDP-43 and MATR3 is almost completely absent from the nucleus. It is interesting to note that HT22 cells that are only transfected with MATR3 exhibit a “stretched out” morphology.
Matrin 3 colocalizes with TDP-43

The mislocalization of TDP-43 is a hallmark of ALS pathology. TDP-43 normally resides in the nucleus, but the pathogenesis of ALS involves abnormal localization of TDP-43 in the cytoplasm, forming insoluble aggregates. Many investigators, including our lab, have reported that TDP-43 regulates RNA processing through the stress granule pathway. The discovery of TDP-43 proteopathy in ALS has prompted the discovery of other key factors that regulate RNA metabolism. Johnson et al. reported that a series of mutations in MATR3 cause familial ALS. A Ser85Cys missense mutation was
previously reported to cause autosomal dominant, distal, asymmetric myopathy with vocal cord paralysis. Further clinical findings reclassify these symptoms as slow progressive forms of ALS. To test whether Matrin 3 interacts with crucial disease proteins of ALS, murine hippocampal cell lines HT22 were transfected with WT TDP-43 or TDP-43 A315T constructs N-terminally tagged with EGFP, and WT MATR3 or MATR3 S85C constructs N-terminally tagged with mCherry. Our result shows that cells that were transfected with WT TDP-43 had few cytoplasmic and total TDP-43 inclusions, which concurs with previous reports that full length TDP-43 predominantly resides in the nucleus under basal conditions. WT TDP-43 has ~2.5 inclusions/cell and ~11 total. Cells that were transfected with TDP-43 A315T (a known mutation that causes ALS) exhibit a significant increase in total number of cytoplasmic inclusions, which conforms with the report by Liu-Yesucevitz et al. that mutations in TDP-43 cause aggregation of TDP-43 and stress granule associated proteins. TDP-43 A315T has ~1/cell and ~33 inclusions total. The WT MATR3 and WT TDP-43 are predominantly
nuclear under basal conditions in vitro (Figure 2). No granule formation is observed for WT MATR3, however, an increase in cytoplasmic and total granule formation is observed with the S85C mutation, suggesting that mutation in MATR3 correlates with the pathological phenotype. We show that an average cell with WT MATR3 has 0 matrin inclusions per cell, while a cell with MATR3 S85C has 4 inclusions on average (Figure 3). Cells expressing both TDP-43 and MATR3 show that the majority of TDP-43 colocalizes with MATR3, which demonstrates that MATR3 physically interacts with TDP-43. In all conditions, more than 90% of TDP-43 colocalized with MATR3(Figure 2C). In addition, our colocalization data show that MATR3 S85C has a greater interaction with TDP43 than WT MATR3. The S85C mutation correlates with a 1.2 fold increase in interaction compared with WT MATR3, which concurs with previous reports from Johnson et al. that the S85C mutation has the strongest effect on TDP-43 structure.
and affinity\textsuperscript{11} (Figure 1, 2C). Most importantly, this result shows that MATR3 modifies TDP-43 subcellular location. Under the WT TDP-43 and WT MATR3 condition, there are very few total and cytoplasmic inclusions (Figure 2A). Introduction of the S85C mutation greatly increased the number of cytoplasmic and total inclusions, which indicates that MATR3 substantially modifies TDP-43 pathology. When the condition of WT TDP43 and WT MATR3 was compared with WT TDP43 and S85C MATR3, we observed about a 6-fold increase in total inclusions (Figure 2B). The greatest number of inclusions was observed in the double mutant condition, with TDP-43 A315T and MATR3 S85C. In that condition both TDP-43 and MATR3 are almost completely absent in the nucleus and instead are observed as cytoplasmic inclusions (Figure 2B). This represents about a 25-fold increase from the pure wild type condition (Figure 2B). It is also interesting to note that when WT TDP-43 is expressed together with WT MATR3, less inclusions were observed compared with TDP-43 alone, which suggests a potential protective function of WT MATR3 from aberrant TDP-43 localization (Figure 2B). WT TDP-43 alone has about cytoplasmic inclusions per cell, while WT TDP-43 co-expressed with WT MATR3 has ~0.5 inclusion/cell (Figure 2B). Large, insoluble, cytoplasmic inclusions in neuronal cells are defining characteristics of most neurodegenerative disorders\textsuperscript{7}. This prompted us to investigate how MATR3 might modify the size of TDP-43 positive inclusions. We developed an algorithm to detect granule sizes greater than 0.5\textmu m using Imaris Bitplane, and ran this algorithm on our images to assess the average size of granules in each condition. We found that the S85C mutation causes a significant increase in the size of both TDP-43 and MATR3 granules, while the WT TDP-43 & WT
MATR3 condition shows no large inclusions, which may suggest that WT MATR3 has a protective effect while mutations such as S85C have a role in pathological phenotypes (Figure 4). Cells with WT MATR3 had no matrin 3 inclusions greater than 0.5µm, while cells with MATR3 S85C had inclusions with an average size of 1.2µm. Double wild type cells had no inclusions greater than 0.5µm, while cells with WT TDP43 alone showed inclusions with average size of 0.9µm. Observations of either MATR3 S85C or TDP43 A315T showed larger inclusions around 1µm in size, while cells with the double mutation condition had very large inclusions around 1.3µm (Figure 4).
Figure 5. The effect of MATR3 on TDP-43 is greatly enhanced by stress. HT22 was expressed with wild type and mutation of TDP-43 and MATR3. Cells were exposed to simulated stress (7µM NaAsO₂, 24 hrs) WT TDP-43 shows little inclusion formation, while MATR3 mutation greatly increase the number and the size of TDP43 inclusions.
The effect of MATR3 on pathology is greatly enhanced by stress

Many investigators have reported than protein aggregation increases under stressful conditions. To investigate how MATR3 aggregations might be different in a stressful environment, HT22 cells were exposed to 7µM sodium arsenite for 24 hours. Arsenite causes stress through multiple pathways and is commonly used as stress inducer for neurological studies\(^\text{19}\). Arsenite can directly cause oxidative stress by oxidizing oxygen and producing superoxides in a process analogous to the well-known Fenton reaction\(^\text{19}\). Arsenite also oxidizes glutathione, which is further conducive to oxidative stress\(^\text{19}\).

Metabolic responses to arsenite include synthesis of heat shock proteins, stimulation of NFkB and increased synthesis of glucose regulated proteins and transporters\(^\text{19}\). Many of the stress responses to arsenite are known to inhibit protein translation and induce stress granule formation, which makes arsenite an excellent stress inducer for stress granule-related studies\(^\text{19}\). Many studies previously have used various concentrations and exposure times of arsenite. 7µM sodium arsenite for 24 hours was chosen to more closely mimic the slow and prolonged stress involved in neurodegeneration. Upon exposure to arsenite WT TDP-43 remained largely nuclear with very little presence of inclusions and translocation to the cytoplasm. The expression of MATR3 S85C causes the formation of very large 3-5µm inclusions, while WT TDP-43 with WT MATR3 forms more smaller 1-2µm inclusions (Figure 5).
MATR3 inclusions are TDP-43 positive but are not stress granules.

In our previous vitro experiment, we were able to show that MATR3 colocalizes with TDP43, and mutation in MATR3 causes mislocation and formation of large cytoplasmic TDP-43 positive inclusions that are characteristic of ALS pathology. To gain further insight into MATR3 pathway and functions, immunocytochemistry was performed to characterize MATR3 inclusions and identify its components. HT22 cells were transfected with plasmids encoding eGFP–TDP-43 A315T and mCherry–MATR3 S85C, were exposed to stress (7 μM NaAsO₂, 24 hrs). Subsequently, cells were immunostained for elf3α. MATR3 colocalizes with TDP-43 but not elf3α (indicated by white arrows).
with eGFP-TDP-43 A315T + mCherry-MATR3 S85C and exposed to arsenite (7µM, 24hr). The cells were fixed and then immuno-labeled with antibodies to eIF3α, a stress granule marker. A notable feature of stress granules compared to other RNA granules is that they contain translation initiating factors and 40S ribosomal subunits. Therefore eIF3 is an ideal marker for stress granules. Similar to our previous result, most of the TDP-43 and MATR3 translocated to the cytoplasm and formed large inclusions, and a majority of the TDP-43 inclusions were colocalized with MATR3 (Figure 6. Yellow indicates colocalization of TDP-43 and MATR3). A proportion of TDP-43 inclusions were found to be eIF3α positive, which agrees with many previous findings that TDP-43 is a part of the stress granule, (purple indicates eIF3α positive TDP-43 granules). However, interestingly, MATR3 inclusions were not eIF3α positive, suggesting that MATR3 is not a part of the stress granule complex. Our result indicates that TDP-43 is a component of both MATR3 granules and stress granules, and that MATR3 inclusions are directly adjacent to stress granules or eIF3α inclusions, suggesting that, while not being a part of stress granule complex, MATR3 granules are involved in RNA processing via the stress granule pathway by relaying crucial components such as TDP-43.
Several key features of MATR3 granules include forming inclusions with TDP-43, not being stress granules, and residing physically next to stress granules. All these features led us to investigate the possibility that MATR3 granules might be processing bodies (p-bodies). P-bodies are discrete cytoplasmic domains and known to be involved in mRNA degradation, translational repression, and RNA-mediated gene silencing. Under stress conditions, they are found closely associated to stress granules and have been observed to exchange components with stress granules. To test whether MATR3 granules are p-bodies, immunocytochemistry was performed to stain for DDX6. MATR3 colocalizes with TDP-43 but not DDX6.

**MATR3 inclusions are not P-bodies**

Several key features of MATR3 granules include forming inclusions with TDP-43, not being stress granules, and residing physically next to stress granules. All these features led us to investigate the possibility that MATR3 granules might be processing bodies (p-bodies). P-bodies are discrete cytoplasmic domains and known to be involved in mRNA degradation, translational repression, and RNA-mediated gene silencing. Under stress conditions, they are found closely associated to stress granules and have been observed to exchange components with stress granules. To test whether MATR3 granules are p-bodies, immunocytochemistry was performed to stain for DDX6. MATR3 colocalizes with TDP-43 but not DDX6.

**Figure 7. MATR3 inclusions are not p-bodies.** HT22 cells transfected with plasmids encoding eGFP-WT/mut TDP-43 and mCherry-WT/mut MATR3, were exposed to stress (7 µM NaAsO₂, 24 hrs). Subsequently, cells were immunostained for DDX6. MATR3 colocalizes with TDP-43 but not DDX6.

DDX6 is a major component of p-bodies and its functions include translational suppression and mRNA degradation, and microRNA-induced gene silencing. HT22 cells were transfected with eGFP-TDP-43 + mCherry-MATR3, and exposed to arsenite.
The cells were fixed and then immuno-labeled with antibodies to DDX6. DDX6 was stained blue, and our result shows that cherry MATR3 granules were not stained with DDX6 (Figure 7), which suggests that the MATR3 granules did not contain any DDX6 and therefore are not p-bodies.

**C8J alleviates TDP-43 and MATR3 pathological inclusions**

![Image](image_url)

**Figure 8. Compound 8J reverses MATR3 induced cytoplasmic inclusion with no significant cytotoxicity in vitro.** HT22 cells transfected with plasmids encoding eGFP- TDP-43 A315T and mCherry-MATR3 S85C, were exposed to stress (7µM NaAsO2, 24 hrs) and C8J (5µM 24hrs). No difference in cell viability and morphology is observed under basal conditions. Under stress condition, C8J inhibits and reverses TDP-43 and MATR3 cytoplasmic inclusions comparing to the untreated.
As we mentioned previously, there is currently no cure for ALS, and the only FDA approved treatment for ALS is Riluzole, which prolongs patients’ survival for an average of three months\textsuperscript{1}. Riluzole blocks TTX sensitive sodium channels, which associate with neuronal damage by excitotoxicity\textsuperscript{22}. This method alleviates some symptoms of ALS with detrimental side effects and does not target the central cause. With greater understanding of ALS pathology and new technologies such as high throughput screening, we are more hopeful to find treatments that target the core pathology of ALS.

In a recent high-content screen for compounds that inhibit stress-induced TDP43 aggregation, C8J was found to be effective at inhibiting TDP43 aggregation. Our results have shown MATR3 as an important modifier of TDP-43 pathology, as the S85C mutation of MATR3 greatly increased TDP-43 aggregation. To determine if C8J is effective at inhibiting MATR3 induced TDP-43 aggregation, HT22 cells were plated and transfected with eGFP-TDP-43 A315T + mCherry-MATR3 S85C, and treated with 5µM C8J. The cells were divided into two groups: a basal condition group and a stress condition group that was treated with 7µM arsenite for 24 hours (Figure 8). Under no stress, we found that there was no difference between cells treated with C8J and cells that were not. This suggests that C8J has no innate toxicity. Under stress conditions, the untreated cells show striking TDP-43 pathology, in that most of all the TDP-43 was absent in the nucleus and translocated to the cytoplasm where it formed inclusions. Stress condition cells that were treated with C8J, however, showed significantly less cytoplasmic TDP-43 inclusions. Most interestingly, the mostly nuclear TDP-43 indicates
that C8J not only inhibits TDP-43 aggregation, but also reverts TDP-43 back to its nuclear state.

Fluorescence activated cell sorting (FACS) experiment shows important MATR3 physiology

A.
One of many important unanswered questions in RNA processing is the potential consequences of stress granule (SG) persistence\textsuperscript{10}. On one hand, SGs protects neurons from toxic proteins and oligomers. On the other hand, as we hypothesize, the persistence and accumulation of SGs in neurons might lead to a loss of normal function and produce toxic pathological SGs\textsuperscript{10}. Thus, it is important to perform cytotoxicity experiments to confirm whether disease-linked mutations in MATR3 that increase SG formation are responsible for low cell survivability. In preparation for FACS, HT22 cells were transfected with 8 different conditions shown above (Figure 9) plus 4 control conditions (unstained, unstained eGFP + βgal, heat shocked cell + Annexin V PE, heat shocked cells +7AAD). The controls were necessary for FacsCalibur instrument settings and fine-tuning of detector amps and compensation. After 24 hours of transfection, cells were washed and stained with both annexin V PE (apoptosis marker) and 7AAD (cell viability marker). Annexin V is a phosphatidylserine marker, and when cells undergo apoptosis,
phosphatidylserine is expressed on the surface and therefore marked by annexin V\textsuperscript{23}. In order to stain cells that die and lose integrity, the DNA intercalating agent 7AAD was used. The combination of annexin V PE and 7AAD assay can distinguish viable cells from apoptotic and necrotic cells. Viable cells would not be stained by either annexin or 7AAD. Cells undergoing early apoptosis would be stained by annexin V only. Cells undergoing late apoptosis would be stained by both annexin V and 7AAD, and necrotic cells would be stained only by 7AAD. Next, stained cells were analyzed by FacsCalibur. Detector amps and other parameters were set based on controls, and FACS data were obtained for each condition based on a sample of 20,000 cells/condition. Our results indicated that over-expression of MATR3 is toxic for the HT22 cells. Less than 10\% of cells were viable for the WT MATR3 condition (Figure 9). TDP-43, however, seems to reduce cytotoxicity, with the expression of TDP-43 bringing viability back to 30\%+ compared with MATR3 alone. Previous reports have suggested that MATR3 is an important factor for neuronal cell death in response to NMDA-type glutamate receptor activation\textsuperscript{24}. However, the explanation for this is not straightforward since reports have also confirmed that HT22 cells lack glutamate receptors\textsuperscript{25}. This interesting MATR3 physiology shall be discussed further later on. Our result does not give us insight to the pathology of MATR3, because there is no observed pattern in the variation from WT to mutation. Further considerations needs to be made to improve the assay to distinguish pathology from normal phenotype.
Figure 10. Compound 8J ameliorates MATR3 and TDP-43 induced cytotoxicity. Our FACS experiment indicates that there is a significant increase in viability (Q4) in WT TDP-43 + WT MATR3 when treated with C8J. WT TDP-43 + WT MATR3 viable cell population have increased from 39% to 47% (Q4) when cells were exposed to 5µM C8J 24 hrs.
Previously, we have shown that C8J reverted the MATR3 induced TDP-43 inclusions back to the nucleus. To assess this effect on cytotoxicity, HT22 cells were plated and transfected with either WT TDP-43 WT MATR3 (double wild type condition) or TDP-43 A315T MATR3 S85C (double mutant condition), and subsequently treated with 5µM C8J (24 hrs). Our result indicates that there is a significant increase in viability in WT TDP-43 + WT MATR3 when treated with C8J (WT TDP-43 + WT MATR3 viable cell population have increased from 39% to 47% when cells were exposed to 5µM C8J 24 hrs), which suggests that the TDP-43 and MATR3 cytoplasmic inclusions that were previously observed have a net cytotoxic effect. Together with our previous in vitro result on C8J, this result also suggests that C8J enhances the survivability of cells by restoring TDP-43 back to the nucleus. For the double mutant TDP-43 A315T + MATR S85C, C8J was less effective at reducing cytotoxicity (TDP-43 A315T + WT MATR3 S85C viable cell population only increased from 37% to 38% when cells were exposed to 5µM C8J 24 hrs). Although the increase in viability is small, the result is still statistically significant (p<0.01) as over 20,000 cells were sampled from each FACS analysis.
DISCUSSION

Many reports have suggested that mutations in RNA-binding proteins either directly or indirectly contribute to ALS pathogenesis\textsuperscript{16,26}. Mutations in TDP-43 and FUS, proteins involved in pre-RNA splicing, transport and translation, led to the theory that aberrant RNA processing is behind the pathogenesis of ALS. There is increasing evidence indicating that aberrant protein aggregation in neurodegenerative disease proceeds through the stress granule pathway\textsuperscript{10}. Subsequent studies have verified that RNA binding proteins that are known to cause familial ALS, such as TDP43, are found sequestered into stress granules\textsuperscript{16}. Under oxidative stress or starvation, TDP-43 is translocated to the cytoplasm and accumulates into stress granules via prion-like mechanisms\textsuperscript{27}. And when stress is resolved, stress granules disintegrate with the help of disaggregases and chaperones, which allows TDP-43 to relocate back into the nucleus where it proceeds with its normal function\textsuperscript{27}. It is still unclear how the stress granule pathway is related to the mechanism by which mutant TDP-43 causes ALS. Nevertheless, discovery of modifiers of TDP-43 such as ataxin 2 (ATXN2), has given great insights into TDP-43 proteopathy\textsuperscript{28}. In our experiments, we investigated MATR3, another potentially crucial modifier of TDP-43, and demonstrated that MATR3 colocalizes with TDP-43. Our experiments showed that a mutation in MATR3 associated with familial ALS enhances the formation of cytoplasmic TDP-43 inclusions in response to stress. S85C mutation in MATR3 \textit{in vitro} induces, larger and more numerous TDP-43 and MATR3 positive inclusions compared to the wild type. The hallmark manifestation of ALS pathology is “larger, round and Lewy body-like inclusions\textsuperscript{7},” which is what we observed with the
mutant MATR3.

The immunocytochemistry has revealed that MATR3 inclusions are directly adjacent to stress granule or eIF3α inclusions but are not part of stress granule complexes. Subsequent immunocytochemistry revealed that MATR3 is not a part of p-bodies, as it is not stained by DDX6 (a major component of p-bodies involved in RNA degradation). The precise nature of MATR3 granules is currently a mystery. Our results suggest that MATR3 granules share TDP-43 with stress granules, and we hypothesize that while not a part of stress granule, MATR3 is closely associated with RNA metabolism via the stress granule pathway by relaying crucial components such as TDP-43. Another crucial player in RNA metabolism is autophagy. One next step for future research into MATR3 is to see if MATR3 granules are autophagosomes. Further research might require MATR3 immunoprecipitation (IP) and mass spectrometry. However, a method of separating RNA granules is difficult to devise, and cell lysate IP produces irrelevant data with a very low signal to noise ratio. Nevertheless, identifying key components of MATR3 granules would give us insights on how MATR3 modifies TDP-43 pathology.

Many reports imply that localization of cytoplasmic aggregates as stress granules is a crucial step in ALS pathogenesis. A compound that relocates TDP-43 and thereby mitigates prolonged stress granule mediated translational repression is proposed as a possible therapeutic for ALS. Our results show that C8J is able to disaggregate and relocate TDP-43 and MATR3 positive inclusions in vitro. Although the mechanism of action of C8J is still unknown, the results obtained with FACS indicate a significant
increase in viability when HT22 cells over-expressed with WT TDP-43 + WT MATR3 were exposed to C8J. C8J was not as effective on the double mutant cells (TDP-43 A315T + MATR3 S85C). Clinical findings have shown that TDP-43 positive inclusion from over-expression of WT TDP-43 is a hallmark of sporadic ALS\textsuperscript{6,7}, which suggests C8J as a potential therapeutic compound for sporadic ALS.

Our FACS experiment shows that over-expression of MATR3 greatly increases cytotoxicity, and expression of TDP-43 increases HT22 cell viability by 5-6 fold. Interestingly, in our previous experiment, we found that MATR3-expressed HT22 cells showed a more stretched-out morphology compared with other conditions, which suggests that MATR3 could induce differentiation of HT22 cells. Furthermore, He et al. report that differentiated HT22 gains glutamate receptors, which renders differentiated HT22 more susceptible to excitotoxicity\textsuperscript{29}. We hypothesize that MATR3 induces HT22 cells to differentiate and gain glutamate receptors, and, consequently, increase cytotoxicity by rendering HT22 cells more susceptible to excitotoxicity. Further
experiments are needed to test this hypothesis. To our disappointment, we did not see a significant difference in viability between mutated MATR3 and wild type. Since most neurodegenerative diseases begin innocuously with mild symptoms and progress gradually, we suspect that a better model needs to be devised for toxicity studies. Perhaps long-term expression of MATR3 is required to see a more dramatic effect on neuron viability. For our future cytotoxicity studies, we can design lentivirus that carries MATR3 and TDP-43 genes and create stable long-term expression lines. This might give us a more accurate model of neurodegeneration and more fully capture the effect of MATR3 mutation on cell viability and pathology.

MATR3 and its biochemistry have studied extensively. MATR3 has gone mostly unnoticed in neurological research, until the discovery by Johnson et al. that MATR3 related mutations are found in patients with familial ALS\textsuperscript{11}. It is known to be involved in RNA processing\textsuperscript{12}. Interestingly, it has been found as a component in paraspeckles that play an important role in retention of hyper-edited RNA\textsuperscript{13,30}, which regulates Ca\textsuperscript{2+} influx and acts as a regulator for many important cellular responses, such as post-translational protein modifications\textsuperscript{31} as well as cell death in response to glutamate-activated receptors\textsuperscript{24}. Furthermore, MATR3 is component of argonaute-containing complex that silences mRNA and regulate miRNA metabolism\textsuperscript{14}. Many \textit{in vivo} studies show that there is an increase in ALS mice of muscle and motor neuron specific miRNA compared with healthy mice\textsuperscript{32}. MATR3 biochemistry seems to connect to neurodegenerative diseases in several ways. Identifying the pathological connections between MATR3 and TDP-43 physiology will provide us with greater understanding of ALS pathology.
REFERENCES


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CURRICULUM VITAE

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Education

B.S., Chemistry
University of Massachusetts Amherst – Amherst, MA
Year of Graduation: 2012
Cumulated GPA: 3.847


Laboratory Skills
- Chromatography: Column, HPLC, gas
- Spectroscopy: 1D-HNMR, IR, UV-vis, MS
- Organic/Organometallic synthetic works
- DNA/RNA extraction
- Polymerase chain reaction
- Western blotting
- FACS
- MACS cell isolation
- Tissue/cell culturing

Relevant Experiences

Student Researcher
Fall 2008 – Spring 2012
Department of Chemistry, University of Massachusetts Amherst
Research focuses on nanoparticle facilitated drug delivery and gene therapy project, designing nanoparticles which can deliver drugs/proteins/DNA into cells through a charge-mediated endocytosis process. Extensive work was conducted on photolabile nanoparticles that transport DNA molecules into living cells, which can be regulated by light. The effectiveness of the nanoparticle is investigated by experiments involving different lines of cancer cells. The
efficiency of DNA release followed by nuclear internalization is observed upon UV irradiation.
Projects are centered on Gold nanoparticles (Au NPs) which possess great potential as vector for drug delivery. Tunable monolayer properties, stability, low inherent toxicity, functional versatility are all the characteristics of Au NPs that provide a plethora of options for the bottom-up approach to designing drug delivery systems. The ease of functionalization of Au NPs enables their adaptations to various strategic tasks. One approach involves hydrophobic drugs binding non-covalently to Au-NPs. Once the drug is loaded to Au-NPs, the versatility of Au NPs monolayer provides a tunable endogenous release mechanism.

One of the studies concerns with nanoimprinting, and designing biocompatible surfaces to modulate cell functions for future applications in tissue engineering and regenerative medicine.
Other involved projects include: 1.) designing new cationic aryl anime functionalized ligands which can controllably bind to protein allowing manipulation of protein expression. 2.) Organic synthesis of riboflavin for photovoltaic purposes. 3.) the effect of specially functionalized Au-NPs on cellular uptake and cytotoxicity.

Research Student

MRI Research, Beth Israel Deaconess Medical Center, Harvard Medical School
Magnetic Resonance Imaging is considered as the forefront of imaging diagnostics because of its ability to produce soft tissue contrast. Yet, the transition from basic to clinical molecular imaging is inhibited by its inadequate signal to noise ratio, and short lived T1 states. Thus, our focus was on the basic science and application development for hyperpolarized magnetic resonance imaging. Hyperpolarization enhances the sensitivity of MRI by a factor of 10,000 or more. This enhanced sensitivity enables real-time pathway-specific metabolic imaging of substrate molecules such as pyruvate and lactate; both are crucial metabolites of cancer tissues. Therefore, developing techniques for metabolic imaging of cancer may yield new tools for diagnosis and treatment monitoring. Additionally, novel techniques were explored for preparation of hyperpolarized contrast media based on parahydrogen-induced polarization to enhance the efficiency of Hyperpolarization. Quantum-mechanical approaches to extending the lifetime of the hyperpolarized signal enhancement were also studied.

Research Technician

Department of Medical Oncology, Dana-Farber Cancer Institute
Investigating the mechanism of sensitivity and resistance of multiple myeloma (MM). My work involved profiling and processing biospecimen samples from newly diagnosed MM patients.
Research Student  
*Summer 2014 – May 2015*

*Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine*

Currently studying the role of TDP43 and MATR3 nucleic acid binding proteins in the pathogenesis of amyotrophic lateral sclerosis (ALS). TDP43 and MATR3 have similar pathology in neurodegenerative disorders, so we are interested in their exact connection. I’m transfecting neuroblasts with these nucleic acid binding proteins conjugated to fluorescence proteins. Using fluorescence microscopy, we can visualize how these proteins interact in cells and induce stress granules.

Tutor  
*Fall 2010 – Spring 2011*

*Learning Resource Center, University of Massachusetts Amherst*

Our goal is to assist our peers in achieving academic success. Challenges in this field involve communicating complex knowledge across to our clients with a broad range of interests. Subjects of interest included Chemistry, Math, and Physics.

Supplemental Instruction Leader (SI leader)  
*Fall 2011 – Spring 2012*

*Learning Resource Center, University of Massachusetts Amherst*

Supplemental Instructions are informal peer facilitated review seminars meeting regularly for a particular course and sections. We focus is on the classes which gives the students the most difficulties such as organic chemistry and physical chemistry. Our goal is to help students succeed in these classes, and encourage them to explore different studying strategies. SI leaders have the experience of leading a large group of students in discussions of a specific subject. SI leaders are also trained to effectively communicate to a large and intellectually diverse population.

Volunteer Work with Patient Health Care  
*Spring 2011 – Present*

*Beth Israel Deaconess Medical Center, Harvard Medical School*

Volunteer Services offer opportunities in direct patient care. Specifically, I have been volunteering in GI recovery, post anesthesia care unit (PACU) and Emergency Department. Experiences include escorting recovering patients on wheelchair, transferring/relocating patients on stretchers, cleaning and maintaining a hygienic environment for patients and employees, and assisting hospital staffs as needed to all patients’ benefit.

Outreach Van Project  
*Summer 2014*

Boston University School of Medicine

Our mission is to fulfill unmet needs of the medically underserved population in Greater Boston area. We travel to East Boston weekly and provide basic necessities such as dry food, clothing, toiletries, and warm foods to the underserved communities. A majority of our clients are Central American
immigrants, and largely women and children who speak solely Spanish. Many of our other clients suffer from addictions and mental illnesses, and we aim to encourage them to seek proper treatments.

Abstracts and presentations

Peer reviewed publications