EMBARGO: DEVELOPMENT OF NOVEL PEPTIDES FOR BLOCKING AGGREGATION AND CASPASE-3 CLEAVAGE OF TDP-43

by

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ABSTRACT

TAR DNA-binding protein 43 (TDP-43) is a protein that is thought to be involved in the pathology of amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). Several TDP-43 mutations have been found in sporadic and familial ALS and FTLD. In patients with these neurodegenerative diseases, TDP-43 forms insoluble inclusions in the nucleus and cytoplasm of neurons. Evidence shows that TDP-43 is abnormally cleaved by caspase-3 and that the truncated inclusions were toxic to cells, which may be the cause of neurodegenerative diseases. To explore novel treatments of neurodegenerative diseases, we identified regions in TDP-43 that are bound by TDP-43 itself or by caspase-3 using high density peptide array analysis. Based on the identification of the key regions, peptides that might be able to block the interactions were designed. We found our synthetic peptides could effectively inhibit the formation of TDP-43 protein aggregations in a concentration-dependent manner in Hela cells in which a mutated human TDP-43 gene was overexpressed. However, these peptides could not prevent cell death. These results suggest that TDP-43 aggregation is a consequence of the cell death process rather than a cause. In addition, the synthetic peptides that are able to block the binding of caspase-3 to TDP-43 in a cell-free assay were also effective in inhibiting the cleavage of TDP-43 in cultured neuronal cells after NMDA insults. Furthermore, application of our peptide was able to block NMDA-induced, caspase-3 dependent cell death. These results suggest new approaches to treatment of ALS and FTLD.
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CHAPTER 1: INTRODUCTION

1.1 General structure of TDP-43

The TAR DNA binding protein 43, also named TARDBP or TDP-43, is a 414 amino acid protein encoded by the TARDBP gene on chromosome 1. TDP-43 possesses two highly conserved RNA recognition motifs (RRM1 and RRM2) and a carboxy-terminal glycine-rich domain. RRM1 and RRM2 are required for binding double-stranded DNA, single-stranded DNA, RNA and regulation of alternative splicing (Ou, Wu et al. 1995; Buratti and Baralle 2001; Bose, Wang et al. 2008). The glycine-rich domain has been shown to regulate of exon 9 skipping of the human cystic fibrosis transmembrane conductase regulator (CFTR) exon 9 skipping during splicing (Wang, Wang et al. 2004). The N-terminal region contains a nuclear localization signal (NLS) located at aa residues 82-98 and a nuclear export signal (NES) located at aa residues 239-250, which are important for subcellular transport (Winton, Igaz et al. 2008). Three caspase cleavage sites have been identified in TDP-43 protein at aa 10, 86 and 216 (Zhang, Xu et al. 2008). In addition, previous studies reported the crystal structure of TDP RRM1 and RRM2 (Kuo, Doudeva et al. 2009), which is very useful for identification of the critical residues in TDP-43.

1.2 Known functions of TDP-43

TDP-43 is a highly conserved heterogeneous nuclear ribonucleoprotein (hnRNP) (Krecic
and Swanson 1999) which controls gene transcription, gene splicing and RNA stability through binding both DNA and RNA (Buratti and Baralle 2001). TDP-43 was originally identified as a transcriptional repressor of the human immunodeficiency virus type 1 (HIV-1) gene (Ou, Wu et al. 1995) and the mammalian gene SP-10 (Acharya, Govind et al. 2006). Additionally, TDP-43 has been shown to regulate exon splicing of several genes, including CFTR, the human apolipoprotein A-II (apoA-II) and the survival motor neuron (SMN) gene (Buratti, Dork et al. 2001; Wang, Wang et al. 2004; Mercado, Ayala et al. 2005). More recent findings indicate that TDP-43 downregulates cyclin-dependent kinase 6 (CDK6) expression levels in human cells (Ayala, Misteli et al. 2008). Another recent finding shows that TDP-43 contributes to stabilization of the human low-molecular-weight neurofilament (hNFL) mRNA (Strong, Volkening et al. 2007). Thus, the functions of TDP-43 are diverse but still incompletely understood.

1.3 TDP-43 and neurodegenerative diseases

A seminal report in 2006 indicated TDP-43 is the main protein component of the ubiquitinated inclusions observed in frontotemporal lobar degeneration (FTLD-U), frontotemporal lobar degeneration with motor neuron disease (FTLD-MND) and amyotrophic lateral sclerosis (ALS) (Neumann, Sampathu et al. 2006). Then more evidence confirmed this report in familial and sporadic cases of FTLD-TDP (Cairns, Neumann et al. 2007; Brandmeir, Geser et al. 2008). In addition, TDP-43 pathology can be found in many other disorders as an important but secondary histopathological feature
of disease, including Alzheimer’s disease (AD), Parkinson disease (PD) and Huntington disease (HD) (Amador-Ortiz, Lin et al. 2007; Higashi, Iseki et al. 2007; Schwab, Arai et al. 2008; Arai, Mackenzie et al. 2009).

1.4 TDP-43 and FTLD

FTLD is the second most common type of early-onset neurodegenerative dementia after Alzheimer’s disease below the age of 65 years old and accounts for 5%~10% of all patients with dementia and 10%~20% of early onset neurodegenerative disease patients (Haugarvoll, Wszolek et al. 2007; Rademakers, Baker et al. 2007). FTLD are clinically characterized by behavioral dysfunction, personality changes, language impairment and cognitive decline. In the early stage of FTLD, the patients do not show an amnestic syndrome, which is different from Alzheimer’s disease (Liscic, Storandt et al. 2007).

In FTLD patients, 25%-50% of all the cases carry the familiar form of this disease, indicating that FTLD has a strong genetic component (Stevens, van Duijn et al. 1998; Chow, Miller et al. 1999; Bird, Knopman et al. 2003; Rosso, Donker Kaat et al. 2003; Neary, Snowden et al. 2005). Recently, several genes have been linked to familial FTLD: microtubule associated protein tau (MAPT) gene on chromosome 17, chromatin modifying protein 2B (CHMP2B) gene on chromosome 3, valosin-containting protein (VCP) gene on chromosome 9 and the progranulin (PGRN) gene on chromosome 17.
Firstly, FTLD was reported to associate with mutations on the MAPT gene on chromosome 17q21, which encodes the microtubule-associated protein tau (MAPT, tau) (Wilhelmsen 1997; Hutton, Lendon et al. 1998; Poorkaj, Bird et al. 1998). In several familiar FTLD cases, abnormally hyperphosphorylated tau protein deposition was found in the brain. This significant finding proved that tau dysfunction could directly lead to neurodegeneration (Goedert 2005). Tau is a microtubule-binding protein and abundantly expressed in both the central and peripheral nervous system. Tau proteins interact with tubulin to stabilize microtubules and promote tubulin assembly into microtubules (Cleveland, Hwo et al. 1977). There have been over 40 MAPT mutations founded in exons 1, 9, 10, 11, 12 and 13 in FTLD patients (Reed, Wszolek et al. 2001; Hayashi, Toyoshima et al. 2002). MAPT mutations fall into two broad classes: missense or deletion mutations change the tau protein sequence and alter the ability of tau to bind to microtubules. That leads to the potential of tau to aggregate into filaments (Nacharaju, Lewis et al. 1999); other types of mutations disrupt the regulation of alternative splicing in exon 10. Six isoforms of tau are expressed in adult brain. Alternative splicing of tau exon 10 results in the three or four microtubule-binding repeats tau isoforms (3R-tau and 4R-tau). Mutations in exon 10 alter the 3R-tau/ 4R-tau ratio (Wszolek, Tsuboi et al. 2006).

FTLD is also linked to chromosome 3 in a single Danish FTLD pedigree (Brown, Ashworth et al. 1995). The gene responsible for disease in this family is CHMP2B
(Skibinski, Parkinson et al. 2005; van der Zee, Urwin et al. 2008). CHMP2B is part of the endosomal secretory complex required for transport (ESCRT) III. Mutations in CHMP2B gene alter normal endosomal trafficking and affect autophagic degradation of proteins, and then lead to neurodegeneration (Urwin, Ghazi-Noori et al. 2009).

In 2004, it was reported that the VCP gene is also associated with FTLD. Mutations in VCP cause Inclusion Body Myositis with Paget’s disease and frontotemporal dementia (IBMPFD) (Watts, Wymer et al. 2004). VCP is a member of the AAA-ATPase gene superfamily and associated with several cellular functions, including ubiquitin-proteasome dependent protein degradation, cell cycle control and membrane fusion. Therefore mutant VCP might induce neurodegeneration by several mechanisms.

However, in most FTLD families in which linkage was shown to the same region on chromosome 17q21, no demonstrable MAPT mutations have been identified. The composition of inclusions in patient brains was tau negative and ubiquitin positive (Rosso, Kamphorst et al. 2001). In 2006, two research groups described the association of mutations in the gene coding for PGRN located on the chromosome 17 with FTLD (Baker, Mackenzie et al. 2006; Cruts, Gijselinck et al. 2006).

Progranulin (PGRN), also named proepithelin or epithelin precursor, was firstly cloned from human leukocytes and identified as an epidermal growth factor (Shoyab, McDonald et al. 1990; Bhandari, Palfree et al. 1992). Human PGRN protein is predicted to be
composed of 593 amino acid residues. It is made of a signal peptide of 17 residues and 7.5 sequentially arranged granulin (GRN) or epithelin (EPI) motifs of 56-57 residues rich in cysteine (Shoyab, McDonald et al. 1990; Bateman and Bennett 1998). Each 6kDa GRN motifs contains 12 cysteine residues and was separated by short intervening spacer sequence (Bhandari and Bateman 1992; Plowman, Green et al. 1992). The PGRN protein has a molecular weight of 68kDa and glycosylation leads to formation of multiple higher molecular weight forms. The most common one is an 88kDa protein. Proteolytic processing of the full length PGRN protein give rise to seven distinct GRNs (GRN A-G) possessing different biological activity and regulatory function (He and Bateman 2003).

PGRN is a widely expressed multifunctional protein. Previous work on PGRN has focused on its role in embryonic development. As a growth factor for trophoectodermal cells, PGRN accelerates the onset of cavitation and blastocele expansion (Diaz-Cueto, Stein et al. 2000). Depletion of endogenously secreted PGRN dramatically delayed the formation of blastocysts in culture. PGRN is also expressed in the brain and spinal cord during late embryonic development. It has been prove PGRN is involved in the early sexual differentiation of brain (Suzuki, Yoshida et al. 1998).

PGRN is also actively involved in the process of wound healing. PGRN mRNA levels are upregulated shortly after transcutaneous puncture injury and remain high for at least 10 days post injury in dermal wounds, including keratinocytes, inflammatory cells, dermal
cells and endothelial cells (He, Ong et al. 2003). Keratinocytes consistently express PGRN, while fibroblasts and endothelial cells do not express PGRN in uninjured skin. Secreted PGRN protein activates inflammatory infiltration (mainly of neutrophils). Neutrophils migrate and adhere to the injured areas, then release inflammatory protein. PGRN and its smaller peptides GRN have opposite effects during these events. For example, PGRN suppresses inflammatory mechanism, while GRN B promotes this process. The conversion between PGRN and GRN is mediated by elastase and secretory leukocyte protease inhibitor (SLPI) (Zhu, Nathan et al. 2002).

Furthermore, PGRN exerts an important influence on tumor growth. This protein regulates tumorigenesis and is highly expressed in aggressive cancer cell lines and clinical specimens, including breast, ovarian, renal cancers as well as in gliomas (Liau, Lallone et al. 2000; He and Bateman 2003). Depletion of PGRN using antisense approaches greatly suppresses tumor growth (Zhang and Serrero 1998).

Recent interest in PGRN has been focused on its functions in the neurodegenerative diseases by the discovery that PGRN mutations cause FTLD with ubiquitin positive inclusions (FTLD-U). In FTLD patients, 5-10% were found to have mutations in PGRN. At least 68 different pathogenic PGRN mutations have been identified (http: www.molgen.ua.ac.be/FTDMutations/). Most of the mutations are predicted to cause premature termination of the coding sequence and then result in the degradation of
mutant mRNA species by nonsense mediated decay and consequently reduced PGRN level (Baker, Mackenzie et al. 2006; Cruts, Gijselinck et al. 2006). Some missense mutations were also identified and predicted to produce non-functional PGRN protein (van der Zee, Le Ber et al. 2007). These proteins that fail to be transported are degraded along the secretory pathway (Shankaran, Capell et al. 2008). Therefore, it appears that PGRN mutations cause FTLD-U due to loss of functional PGRN rather than accumulation of mutant protein.

After the discovery of PGRN gene mutations in FTLD patients with ubiquitin-positive, tau- and alpha-synuclein-negative inclusions, TDP-43 was found to be the major protein composition of the inclusions in FTLD-U cases. TDP-43 normally is found in the nucleus, while pathological TDP-43 inclusions are normally hyperphosphorylated, ubiquitinated and cleaved to 35 and 25 kDa species. In some cases, truncated TDP-43 translocates to the cytoplasm (Arai, Hasegawa et al. 2006; Neumann, Sampathu et al. 2006; Davidson, Kelley et al. 2007).

FTLD-TDP patients can be divided into four main subtypes according to their clinical and pathological heterogeneities. Type 1 pathology is characterized by neuritis inclusions predominantly; type 2 pathology is characterized by cytoplasmic inclusions predominantly; type 3 pathology is delineated by intracellular inclusions predominantly and type 4 associated with VCP mutations (Neumann, Sampathu et al. 2006; Cairns,
Neumann et al. 2007). Although a TDP-43 negative subtype of FTLD-U has been found (Roeber, Mackenzie et al. 2008) and sporadic FTLD seems to have no association with pathological TDP-43 (Schumacher, Friedrich et al. 2009), TDP-43 is still the signature protein of FTLD-U and appears to play an important but unknown role in the pathological process.

1.5 TDP-43 and ALS

In addition to FTLD, TDP-43 proteinopathy is also associated with amyotrophic lateral sclerosis (ALS) (Wijesekera and Leigh 2009). ALS is the most common adult-onset progressive motor neuron disease (MND). The majority of ALS cases are sporadic. TDP-43 inclusions are found in patients with familial ALS and most of sporadic ALS cases. Another ALS related gene, superoxide dismutase gene (SOD1) mutations account for 10-20% of familial ALS and 1-2% of sporadic ALS (Rosen, Siddique et al. 1993). However, TDP-43 inclusions are not observed in SOD1 related ALS cases, suggesting a distinct disease mechanism in these patients (Mackenzie, Bigio et al. 2007; Tan, Eguchi et al. 2007). Recently, several missense mutations of TDP-43 in ALS familial and sporadic cases have been found. In ALS, ubiquitinated TDP-43 is absent from the nucleus and is redistributed to the cytoplasm. There it shows a skein-like or dense granular appearance of aggregation (Giordana, Piccinini et al. 2009). TDP inclusions can be found in the whole brain, but the most severely affected areas are the spinal cord, the basal ganglia, the motor cortex and the thalamus (Geser, Brandmeir et al. 2008). Moreover, some cases
of ALS or FTLD show features of both FTLD and ALS. TDP-43 pathology is the key histopathological feature of such overlap cases. It indicates that understanding the mechanism of TDP-43 mediated neurodegenerations is vital in the disease treatment.

1.6 Aims of the thesis

Although there is increasing amount of medical data, it is still unclear how TDP-43 is involved in the pathogenesis of neurodegenerative diseases with TDP-43 positive inclusions. Recent researches focus on TDP-43 aggregation and TDP-43 cleavage by caspase-3. Thus, inhibition of TDP-43 binding to itself and caspase-3 is useful to explore the TDP-43 pathogenic mechanisms.

High density peptide array is a powerful tool to study protein-protein interactions. We hypothesize that the high density peptide array technology is capable of identifying interaction domains.

Aim 1: Identify and validate peptide candidates that may block TDP-43 aggregation.

Aim 2: Identify and validate peptide candidates that block interaction between TDP-43 and caspase-3.
CHAPTER 2: IDENTIFY AND VALIDATE PEPTIDE CANDIDATES THAT MAY BLOCK TDP-43 AGGREGATION

2.1 Introduction

TDP-43 inclusions are a major pathological hallmark of ALS and FTLD-U. Some groups established cell models to recapitulate key features of TDP-43 pathology. For instance, a mutant TDP-43 lacking residues 187-192 was constructed by Nonaka and colleagues (Nonaka, Arai et al. 2009). When this mutant TDP-43 was expressed in SY5Y cells, it formed intranuclear dot-like inclusions. In addition, after proteasome inhibition MG132 treatment, cells transfected with mutant plasmid (∆187-192) showed ubiquitin positive and phosphorylation positive TDP-43 inclusions.

Inhibition of TDP-43 aggregation is considered to be major therapeutic avenue for ALS and FTLD-U. As for other neurodegenerative diseases, current tools include antibodies, molecular chaperones, chemical compounds and synthetic peptides. The strategy we used to design aggregation blockers was to identify and synthesize TDP-43 fragments that bind with full length TDP-43 protein. High density peptide array technology was used to find the binding regions.

The high density peptide array has emerged as a useful tool to map domain-mediated protein-protein interaction. The peptide array might have hundreds to thousands of
immobilized peptides. Several peptide synthesis procedures have been developed. The peptide technology used for our protein array is SPOT synthesis. SPOT synthesis was developed by Ronald Frank and co-workers in 1990 (Frank 1992). Generally, the optimal range of each peptide is between 6 and 18 amino acids. In the SPOT synthesis, peptides are synthesized by sequential spotting of activated amino acids and immobilized to cellulose membranes with chemical reagents. The immobilized peptides adopt a uniform orientation on the membrane which mimics their native and active states as they interact with other proteins.

Here we used high density peptide array technology to identify the peptides that might block TDP-43 interactions. Then we investigated whether the synthetic peptides could reduce the formation of TDP-43 aggregation in Hela cells and prevent cell death.
2.2 Methods

2.2.1 Protein array

The membranes and peptides were synthesized by the UBC Peptide Synthesis facility using a previously described protocol (Hilpert, Winkler et al. 2007). Derivatized cellulose-based membranes were obtained from Intavis AG (Köln, Germany). The peptides were built up from the C-terminus to the N-terminus. The C-terminal amino acid was attached to the cellulose membrane by an amide bond. Pentafluorophenyl ester (OPfp) preactivated amino acids were delivered to the corresponding positions on the membranes with 0.11µl per spot. Nonspot areas were blocked by acetylation. Then the membranes were Fmoc deprotected by washing four times with DMF for 30s, twice with 20% piperidine/DMF for 5 min, four times with DMF for 30s and twice with methanol. The spots were stained by treatment with a methanolic solution containing 0.02% bromophenol blue. After final washing with methanol and drying in the air stream of the fume hood, the membranes were ready for coupling of the next amino acid. Finally, the peptide chain protecting groups were cleaved by trifluoroacetic acid (TFA) treatment in the presence of 3% triisopropylsilane, 2% water and 1% phenol. The peptide scans were performed by synthesizing overlapping 12 or 14-mer peptides spanning the entire 414 amino acid sequence of TDP-43 with a frame shift of 2 amino acids per spot.

TDP-43 protein was purified from E. coil overexpressing the human TDP-43 gene. Membranes were blocked with 5% sucrose and 4% nonfat dry milk in TBST for 4h and
then incubated with TDP-43 protein (3-10µg/ml) or peptides overnight at 4°C. Membranes were washed three times for 15 min in TBST and then incubated with rabbit polyclonal TDP-43 antibody (1:5000; Protein Tech Group, Chicago, IL) overnight at 4°C. After washing three times for 15 min, membranes were incubated with donkey anti-goat conjugated with donkey anti-rabbit IgG conjugated with horseradish peroxidase (1: 5000; R&D Systems, Minneapolis, MN) for 3h at room temperature. Membranes were then washed three times for 15 min and protein interaction was visualized by enhanced chemiluminescence reaction assay (PerkinElmer Life Sciences) (Figure 2.1).

2.2.2 Cell-penetrating peptides synthesis

Cell-penetrating peptides consisting of the truncated TAT domain at the N-terminal were synthesized by GL biochem Ltd. (Shanghai) and purified by HPLC. The resulting peptides were more than 90% pure and verified by mass spectrometry. All the peptides were first dissolved in DMSO and further diluted in water.

2.2.3 Blocking assay of TDP-43 interaction with the membrane

After blocking, membranes were incubated with the mixture of TDP-43 protein (5µg/ml) and/or single synthetic peptides (100µg/ml) overnight at 4°C. Then the membranes were incubated with primary antibody, secondary antibody and washed with TBST. Protein interaction was visualized by enhanced chemiluminescence reaction assay (PerkinElmer Life Sciences).
2.2.4 Cell cultures

A human cell line: human cervical epithelia (Hela) cells, was obtained from the American Type Culture Collection. The cells were cultured following the instructions of the American Type Culture Collection in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY) and 1% antibiotics (Gibco-BRL, Grand Island, NY). Cultures were maintained at 37 °C in a humidified incubator (NuAir, Plymouth, MN) with 5% CO₂.

2.2.5 Transfection

The plasmids were transfected into the Hela cells using the Lipofectamine 2000 system (Invitrogen, Carlsbad, CA). The cells were plated and cultured in DMEM containing 10% fetal bovine serum 24 hours prior to the transfection experiment. For 24 well plate, 2µl Lipofectamine 2000 and 0.8µg DNA were diluted and mixed separately in 50µl Opti-MEM I (Invitrogen, Carlsbad, CA) Reduced Serum Medium per well. After 5 minutes of incubation, the diluted Lipofectamine and DNA were combined, mixed gently and incubated for 20 minutes at room temperature. Then 100µl of the mixture was added into the cell culture medium per well. After 5 hours of incubation, the transfection mixture was replaced with 500µl fresh DMEM containing 10% fetal bovine serum. The cells were returned to the incubator for an additional 48 hours.
2.2.6 Assay of TDP-43 self-interaction in vitro

After incubating the cells with the transfection mixture for 5 hours, the medium was replaced with fresh DMEM (10% fetal bovine serum) with 2.5-20µM synthetic peptides. After 24 hours, the medium was replaced with fresh peptides.

2.2.7 Immunocytochemistry

The cells were plated and cultured on poly-D-lysine (Sigma, Saint Louis, MO) coated cover slips at a density of 2.5 x 10^4 cells per well in 24 well plate. After transfection, the cells were washed with pre-warmed PBS (pH 7.4) and fixed with pre-warmed 4% Paraformaldehyde (PFA; Sigma, Saint Louis, MO) containing 4% sucrose (Sigma, Saint Louis, MO) in PBS for 10 minutes at room temperature. Then the cells were rinsed with PBS 3 times and permeabilized with 0.1% Triton X-100 (Sigma, Saint Louis, MO) in PBS for 2-3 minutes at room temperature. After washing with PBS 3 times, the reaction was blocked with 10% Bovine serum albumin (BSA; Invitrogen, Carlsbad, CA) in PBS for 1 hour at room temperature. Then the cells were incubated with primary antibody against TDP-43 (1:150; ProteinTech, Chicago, IL) overnight at 4°C. After washing with PBS, the cells were incubated with anti-rabbit antibody conjugated with Alexa 488 (Invitrogen, Carlsbad, CA) for 1 hour at room temperature and then washed with PBS 3 times. Cell nuclei were labeled with DAPI (1:10000; Invitrogen, Carlsbad, CA) for 2-3 minutes at room temperature. After washing with PBS, the coverslips were mounted on glass slides with antifade reagent (Invitrogen, Carlsbad, CA). Images were obtained with
2.2.8 MTT assay for cell viability

The viability of cultured cells was assessed using the MTT assay. MTT (3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide) (Sigma, Saint Louis, MO) was dissolved in PBS to reach stock concentration of 5mg/ml. Cells were seeded at a concentration of $2.5 \times 10^4$ in 24 well plates. After treatments, the culture medium was replaced with 200 µl fresh DMEM containing 0.5mg/ml MTT each well. After the cells were incubated at 37°C for 4 hours, 400µl lysis buffer at pH 4.8 containing 50% (vol/vol) N, N-dimethylformamide (Sigma, Saint Louis, MO), 200mg/ml SDS (BioRad, Hercules, CA) and 0.4% (vol/vol) glacial acetic acid (Fisher Scientific, PA) was added to culture medium. Then the cells were incubated overnight in a 37°C humidified 5% CO$_2$ incubator. The optical density of each sample was measured at 590 nm absorbance with a reference filter of 750nm using the “uQuant” microplate spectrophotometer (Bio-Tek Instruments, USA).

2.2.9 TUNEL assay for cell viability

The viability of cultured cells was assessed using deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) assay. TUNEL staining was performed using an in situ
cell death detection kit (Roche Applied Science, IN). Cells were seeded on poly-D-lysine coated cover slips at a density of $2.5 \times 10^4$ cells per well in 24 well plate. After treatment, cells were fixed and stained with anti-TDP-43 (1:150; ProteinTech, Chicago, IL), anti-GFP (1:1000; Invitrogen, Carlsbad, CA), Alexa 647 anti-rabbit (1:1000; Invitrogen, Carlsbad, CA) and Alexa 488 anti-chicken (1:1000; Invitrogen, Carlsbad, CA). The enzyme solution (terminal transferase) and label solution were mixed in a volume ratio of 1:9 to obtain the TUNEL reaction mixture. The cells were incubated with the 50μl TUNEL reaction mixture at 37°C for 1 hour and then washed three times with PBS. Then the cover slips were mounted on glass slides with antifade reagent (Invitrogen, Carlsbad, CA). Images were obtained with an Olympus Fluoview FV1000 Confocal scanning microscope.
2.3 Results

2.3.1 TDP-43 protein binds with TDP-43 derived peptides on the membrane

The protein array described in the methods section was used to determine the binding regions between the recombinant TDP-43 protein and the TDP peptide array membrane (Figure 2.1). On the peptide array membrane, positively interacting peptides generate dark spots, while non-interacting peptides leave blank spots. Each binding region has a series of spots. Because each successive spot contains twelve amino acids shifted by two amino acids, the common sequence of dark spots is the possible binding site (Figure 2.1). The non-specific binding sites were recognized by TDP-43 antibody (Figure 2.2A). Five separate binding regions were found on the TDP-43 membrane (Figure 2.2B). Probing using TDP-43 protein and membrane with overlapping TDP-43 peptide attached revealed several possible interaction domains in five binding areas. Based on these possible interaction domains, five peptide candidates were designed and synthesized. The peptides were derived from the sequence of full length TDP-43. The overlapping amino acids are the possible binding domains.

2.3.2 The synthetic peptides are able to block the interaction between recombinant TDP-43 protein and its peptide array membrane

The five peptides were tested for their ability to inhibit the binding between TDP-43 full length protein and the TDP-43 membrane (Figure 2.3). A scrambled peptide TC was designed as control. Peptide TC has the same length, net charge, and amount of
hydrophilic and hydrophobic amino acids as Peptide C. It has a random order and substitutions with the structure disrupting amino acids of original peptide. The results showed that a mixture of the five peptides blocked the interaction between TDP-43 and its membrane. However, single peptide A-E or peptide TC did not affect the interaction (Figure 2.4).

2.3.3 Mutated TDP-43 plasmid can form aggregation in Hela cell

In a variety of neurodegenerative diseases, TDP-43 aggregates in neuronal cytoplasm or nuclei. Previous papers have demonstrated that mutant TDP-43 lacking residues 187-192 resulted intracellular dot-like structure in SH-SY5Y cells (Nonaka, Arai et al. 2009). To investigate whether the expression of mutant TDP-43 (187-192) also leads to intracellular aggregation in Hela cells, immunohistochemical analysis was performed on cells transfected with mutated TDP-43 and GFP. Wild type TDP-43 and GFP were transfected into other cells as controls. 48 hours after transfection, the cells transfected with mutant TDP-43 and GFP which had been labeled positive with anti-TDP-43 and anti-GFP antibodies showed intracellular TDP aggregates (Figure 2.5). Endogenous TDP-43 was expressed in the nucleus (Figure 2.5A). Stronger nuclear staining of TDP-43 was detected in cells transfected with mutant TDP-43 (Figure 2.5B, C, D), as compared with non-transfected cells. Transfection with mutant TDP-43 (187-192) but not normal TDP-43 caused dot-like nuclear aggregation (Figure 2.5B).
2.3.4 Synthetic peptides can inhibit TDP-43 aggregation

To investigate whether the synthetic peptides could inhibit the nuclear aggregation, Peptide A, B, C, D, E and TC were added to the cell culture medium separately or together after transfection. TAT was attached to the N-terminal of all peptides. The TAT peptide (YGRKKRRQRRR) is derived from the transactivator of transcription of human immunodeficiency virus and allows attached peptides to penetrate cell membranes. The TAT peptide has been used to deliver large molecules and small particles across both the plasma membrane and the nuclear membrane (Nagahara, Vocero-Akbani et al. 1998).

When the transfected cells were treated with Peptide B or C, the number of inclusions decreased (Figure 2.5C). The other peptides were also effective. Compared to untreated cells, we observed a 52% reduction of inclusions with Peptide B (10μM), a 72% reduction of inclusions with Peptide C (10μM) and a 91% reduction of inclusions with a Peptide B and C mixture (10μM) (Figure 2.6A). In addition, the peptides blocked aggregation in concentration-dependent manner (Figure 2.6B). 0μM, 5μM, 10μM, 15μM and 20μM Peptide B and C mixture caused 0%, 18%, 26%, 66% and 87% reduction of inclusions. 0μM, 5μM, 10μM, 15μM and 20μM Peptide B caused 0%, 6%, 30%, 25% and 40% reduction of inclusions. 0μM, 5μM, 10μM, 15μM and 20μM Peptide C caused 0%, 3%, 29%, 43% and 61% reduction of inclusions (Figure 2.6B). No obvious change was noted with treatment using Peptides A, D, E or TC (Figure 2.6A). The results show that Peptide B and C can inhibit the aggregation efficiently.
2.3.5 The inhibition of aggregation cannot prevent cell death

To ask whether TDP-43 aggregation is the cause or a symptom impending cell death, we used MTT and TUNEL assays to examine cell viability. Even when TDP-43 aggregation was inhibited by Peptide B and C, there was no significant change in viability between untreated cells and peptides treated cells (Figure 2.7, 2.8). MTT assay showed 64.60% viable cells without peptide treatment, 69.51% viable cells treated with scrambled peptides and 71.40% viable cell treated with peptides B and C (Figure 2.7). TUNEL assay showed TDP-43 aggregation decreased to 48.86% after peptide treatment compared to untreated cells. In the cells without peptide treatment, treated with scrambled peptide and treated with peptides B and C, there were 21.05%, 22.22% and 22.50% of transfected cells showed TUNEL positive signals. In addition, almost all the cells with aggregation showed TUNEL positive signals (Figure 2.8, 2.9). The results indicate that TDP-43 aggregation was associated with cell death, but inhibition of TDP-43 aggregation could not rescue or prevent cell death. In addition, the peptides did not induce higher cell death rate than control in the untransfected cells. It showed the peptides were nontoxic.
2.4 Discussion

In this study, we have identified peptides that block TDP-43 aggregation. Previous studies have reported the crystal structure of TDP-43 RRM1 and RRM2 domains (PDB accession code: 3D2W, 2CQG) (Suzuki, Muto et al. 2005; Kuo, Doudeva et al. 2009). Three peptides (Peptide A, C, D) that we identified lie within these two domains. Using the program PyMOL to view the three-dimensional structure of the RRM1 and 2 domains, we can see that two peptides (Peptides C and D) are mostly found on the protein surface (Figure 2.10, 2.11). Peptide B is found in the depth of the protein.

We examined the effects of the potential TDP self-binding domain peptides on the formation of TDP-43 inclusions using a Hela cell model. First, we confirmed earlier studies in SY5Y cells showing that mutated TDP-43 (Δ187-192) can induce TDP-43 aggregation and cell death in Hela cells. Second, two of our five peptide candidates blocked the interaction between TDP-43 with itself on the membrane. Finally, the peptides blocked the aggregation of TDP-43 in a concentration-dependent manner in Hela cells, but did not prevent cell death. These data suggest TDP-43 aggregation is not the cause of cell death in our model, but is instead associated with the process of cell death.

Until now, the mechanism of TDP-43 cellular toxicity is still unclear. To answer this question will be vital for finding effective therapeutic strategies. TDP-43 toxicity could be caused through two mechanisms: gain of a toxic function or loss of an essential
function of TDP-43.

One of the most important pathological features of TDP-43 proteinopathies is cytoplasmic inclusion of TDP-43 aggregates. Moreover, Ubiquitinated and phosphorylated TDP-43 aggregates is reported in ALS and FTLD-U patients. In addition, pathological TDP-43 is cleaved to generate C-terminal fragments of 25 and 35 kDa. In ALS and FTD-U cases, TDP-43 inclusions in the cortex and hippocampus are comprised of C-terminal fragments, while the composition of inclusions in spinal cord motor neurons is primarily full length TDP-43 (Igaz, Kwong et al. 2008). As with other protein misfolding diseases, TDP-43 mediated toxicity possibly results from a toxic gain of function from the nuclear and cytoplasmic aggregation. Johnson and colleagues have established a yeast model with overexpressed full-length human TDP-43 or various TDP-43 truncation products (Johnson, McCaffery et al. 2008). They found that truncated TDP-43 plasmids which contain both the C-terminal and RRM2 formed aggregation. Only the aggregated TDP-43 induced toxicity to yeast cells. This suggested that TDP-43 misfolding and aggregation might be the cause of cell death in neurodegenerative diseases. Another group confirmed this conclusion in human cell models (Zhang, Xu et al. 2009). The 25 kDa C-terminal fragment of TDP-43, which is corresponding to the caspase-3 cleavage product, was overexpressed in HEK293 and differentiated M17 neuroblastoma cells. The 25 kDa fragment of TDP-43 formed cytoplasmic inclusions, induced cell toxicity and didn’t disturb endogenous TDP-43 functions. These evidences
imply the toxicity of TDP-43 aggregation.

However, it is important to note that, although the inclusions are toxic, it still has another possibility that TDP-43 loss of function causes toxicity in TDP-43 proteinopathies. Toxicity might be caused by the aggregated TDP-43 being sequestered away from its proposed normal functions. TDP-43 acts as a transcription repressor and RNA splice regulator of a variety of genes, including CFTR, SMN, CDK6 and apoA-II (Buratti, Dork et al. 2001; Wang, Wang et al. 2004; Mercado, Ayala et al. 2005; Ayala, Misteli et al. 2008). Previous studies have shown that TDP-43 knockout reduces histone deacetylase 6 (HDAC6) expression in HEK293E cells, SH-SY5Y cells and Drosophila. HDAC6 is an important protein related with autophagy-mediated degradation of misfolded proteins. TDP-43 silencing reduces aggregation formation but increases the proteotoxicity. Thus loss of TDP-43 function causes HDAC6 downregulation and then impairment in toxic protein turnover (Fiesel, Voigt et al. 2009). Moreover, TDP-43 knockout in Drosophila caused deficient locomotive behaviors, reduced life spans and a decreased number of synaptic terminals (Feiguin, Godena et al. 2009). In addition, loss of TDP-43 by RNAi in differentiated Neuro-2a cells suppressed Rho family GTPases, inhibited neurite outgrowth and induced cell death (Iguchi, Katsuno et al. 2009). Taken together, these data demonstrate TDP-43 loss of function is detrimental.

Here we show that the mutant TDP-43 can induce nuclear aggregation and then cause
toxicity in Hela cells. The toxicity could be a gain of function or a loss of function. However, our data showed prevention of TDP-43 aggregation by peptides could not reduce the toxicity. It suggests that TDP-43 aggregation is associated with cell death, but not the cause of cell toxicity. Thus, we propose that mutant TDP-43 may induce cell death via loss of function instead of gain of function. Barmada and colleagues recently established a TDP-43 proteinopathy model by transfecting mutant TDP-43 in primary rat cortical neurons (Barmada, Skibinski et al. 2010). They observed that mutant TDP-43 is mislocalized from nucleus to cytoplasm and induces cell death. However, there were no inclusions in neurons. We also got similar results in the previous data of our lab (Guo, Tapia et al. 2010). Knockdown of PGRN in mouse neuron induces TDP-43 translocalization from nuclei to cytoplasm and enhanced cell toxicity. But we did not observe either nuclear or cytoplasmic TDP-43 aggregation. These data implied that mislocalization of TDP-43, but not TDP-43 aggregation, is necessary for neurodegeneration pathogenesis. This is also strong evidence supporting TDP-43 aggregation is a consequence but not necessary cause of neurodegenerative diseases.

Overall, these findings seem to solidify the loss of function for TDP-43 and indicate the possible mechanism for TDP-43 pathology in FTD and ALS. Future study will be focus on our primary culture neurons and TDP-43 mutant transgenic mouse. Several groups have reported that TDP-43 mutant transgenic mouse develop TDP-43 nuclear and cytoplasmic inclusions in neurons as observed in ALS and FTLD-U patients.
(Wegorzewska, Bell et al. 2009; Wils, Kleinberger et al. 2010). Thus administration of peptides in a transgenic mouse model will be better for our future studies of TDP-43 related toxicity.
Figure 2.1. Schematic process of high density peptide array technology. The peptides are built up from C-terminus to N-terminus on derivatized cellulose-based membranes (Intavis AG; Köln, Germany). The C-terminal amino acid was attached to the cellulose membrane with an amide bond. Each peptide chain consists of 10 AA with a frame shift of 2 AA between subsequent spots. The interaction sites between membrane and protein were recognized by antibody staining.
Figure 2.2. Identification of peptide candidates that may block TDP-43 binding to itself using the high density peptide array. TDP-43 protein associates with TDP-43 membrane on specific regions. A, antibody only; B, Membrane with overlapping TDP-43 peptides attached was incubated with TDP-43 protein (three repeats). Five peptide candidates (Peptide A-E) were designed according to the binding regions.
Figure 2.3. Identify the peptides candidates that may block TDP-43 aggregation.
Figure 2.4. Validated peptide candidates that may block TDP-43 binding using high density peptide array. Peptides inhibit the binding between TDP-43 protein and membrane. Membranes were incubated with a, TDP-43 protein; b, TDP-43 protein and peptides; c, TDP-43 protein and scrambled peptides. These data showed that these peptides could inhibit the binding of TDP-43 and membrane.
Figure 2.5. Expression of mutated TDP-43 (deletion of 187-192 AA) results in the formation of inclusions. Immunofluorescence detection of TDP-43 in Hela cells with anti-TDP-43 antibody (left panel, green), nuclear staining by Hoechst (middle panel, blue) and merged image (right panel). A: untransfected Hela cells; B: cells 48h post transfection with mutated TDP-43; C and D: cells 48h post transfection with mutated TDP-43, followed by addition of peptides (10µM) (C) or scrambled peptides (10µM) (D) to culture medium. The peptides could inhibit aggregation of TDP-43 in Hela cells.
Figure 2.6. Synthetic peptides can inhibit TDP-43 aggregation. A: Peptide B, C and B+C can inhibit aggregation more efficiently than other three peptide candidates.

B: Peptide B, C and B+C can inhibit aggregation in a concentration-dependent manner (*, p<0.05; **, p<0.01, one-way ANOVA).
Figure 2.7. Identification of cell viability by MTT assay in Hela cell. Cells were transfected with wild type or mutated TDP-43 plasmids. Peptide B and C cannot prevent cell death. (*, p<0.05; **, p<0.01, one-way ANOVA)
Figure 2.8. Identification of cell death by TUNEL assay in Hela cell. Cells were transfected with GFP and mutated TDP-43 plasmid. Peptides B and C can inhibit aggregation, but not cell death. In addition, the very low percentage of cell death in untransfected cells suggests the peptides are not toxic to cells (*, p<0.05; **, p<0.01, one-way ANOVA).
A  Wild type TDP-43

Total cell

Transfected cell
Cell death (6.08%)

Untransfected Cell
Cell death (3.14%)

Cells with aggregation
Cell death (81.10%)

Cells without aggregation

B  Δ187-192

Total cell

Transfected cell
Cell death (21.05%)

Untransfected Cell
Cell death (2.28%)

Cells with aggregation
Cell death (92.57%)

Cells without aggregation
Figure 2.9. Schematic diagram of TUNEL assay in Hela cell. A, Cells transfected with wild type TDP-43; B, Cells transfected with mutated TDP-43 (Δ187-192); C, Cells transfected with mutated TDP-43 and treated with scrambled peptides; D, Cells transfected with mutated TDP-43 and treated with peptides B and C.
Figure 2.10. Crystal structure of TDP-43 RRM1 region. A. Peptide A (in green) is mostly buried in the protein interior. Peptide C (in blue) is mostly exposed on the surface of three dimensional structures; B. This view is rotated 90 degrees to A; C. This view is rotated 180 degrees to A.
Figure 2.11. Crystal structure of TDP-43 RRM2 region. A. Peptide D (in blue) is mostly exposed on the protein surface of TDP-43; B. This view is rotated 90 degrees to A.
CHAPTER 3: IDENTIFY AND VALIDATE PEPTIDE CANDIDATES THAT BLOCK INTERACTION BETWEEN TDP-43 AND CASPASE-3 IN NEURONAL CULTURES

3.1 Introduction

Apoptosis is a process of programmed cell death characterized by volume reduction, cell surface blebbing, chromatin condensation, DNA degradation and formation of apoptotic bodies. It is mediated by a series of cysteine proteases that cleave their substrates. Caspase-3 is one of the important mediators of apoptosis and it recognizes an accessible DEVD/DXXD sequence. In addition, the caspase family of proteases has non death related cellular functions in the nervous system. Caspase-3 has been shown to promote cell differentiation by cleavage-directed activation of signaling pathways (Fernando, Brunette et al. 2005). Moreover, caspase-3 has been shown to regulate synaptic plasticity, because many important proteins involved in neuronal plasticity are caspase-3 substrates (Chan and Mattson 1999).

The pathological mechanisms associated with TDP-43 translocation and deposition are currently unclear. TDP-43 is normally localized in the nucleus; however, it is deposited cytoplasmically in neurons of FTLD-U and ALS patients. Immunoblot studies of pathological tissue have also demonstrated that cytoplasmic TDP-43 deposition is associated with the accumulation of C-terminal fragments (25kDa). The putative
caspase-3 cleavage sites in TDP-43 have been suggested to lead to the formation of aggregates and toxicity (Zhang, Xu et al. 2009). Here, we used synthetic peptides to block the cleavage of TDP-43 and then explore the effects of this on pathological mechanism involving TDP-43.
3.2 Methods

3.2.1 Protein array

Please refer to Chapter Two Methods section for the complete procedures. The peptide scans were performed by synthesizing overlapping 12 or 14-mer peptides spanning the entire 277 amino acids of caspase-3 with a frame shift of 2 amino acids per spot.

3.2.2 Cell-penetrating peptides synthesis

Cell-penetrating peptides consisting of the truncated TAT domain at the N-terminal were synthesized by GL biochem Ltd. (Shanghai) and purified by HPLC. The resulting peptides were more than 90% pure and verified by mass spectrometry. All peptides were first dissolved in DMSO and further diluted in water before use.

3.2.3 Blocking assay of TDP-43 interaction with caspase-3 membrane

Please refer to Chapter Two Methods section for the complete procedures.

3.2.4 Mouse primary cortical neuronal culture

Mouse primary cortical neuronal cultures were prepared from the embryos of E15 timed pregnant CD1 mouse. All animal research was conducted according to the guidelines of Institutional Animal Care and Use Committee (IACUC). After the pregnant mouse was anesthetized and sacrificed, embryos were transferred to cold dissection buffer containing Hanks Balanced solutions (Gibco-BRL, Grand Island, NY) and 10mM HEPES (Sigma,
Saint Louis, MO), pH 7.4 and osmolarity 310-320 mOsm. After the whole brains were removed from embryos, the meninges were gently peeled from the individual cortical lobes. The cortices were separated and transferred to 0.25% trypsin (Gibco-BRL, Grand Island, NY) for 10-20 minutes at 37°C. Then the cortical tissues were washed twice with DMEM (Gibco-BRL, Grand Island, NY) containing 10% Fetal Bovine Serum (Gibco-BRL, Grand Island, NY) and 1% penicillin/streptomycin (Gibco-BRL, Grand Island, NY) to remove trypsin. After washing, the cortical tissues triturated with 10mL pipette and centrifuged at 1400rpm for 3 minutes. The supernatant was discarded and the cell pellet was resuspended with 5-10ml plating neurobasal medium containing Neurobasal (Gibco-BRL, Grand Island, NY), 2% B27 supplement with AO (Gibco-BRL, Grand Island, NY), 2mM L-glutamine (Sigma, Saint Louis, MO), 25μM glutamic acid (Sigma, Saint Louis, MO), 10mM β-mercaptoethanol (Gibco-BRL, Grand Island, NY) and 1% penicillin/streptomycin (Sigma, Saint Louis, MO).

Neuronal cells were plated and cultured on poly-D-lysine coated tissue culture dishes at a density of 8 x 10^5 cells per well in 6 well plates. Cultures were maintained at 37 °C in a humidified incubator with 5% CO₂. Total medium was changed into fresh maintaining medium containing Neurobasal, 2% B-27 supplement with AO, 2mM L-glutamine, 1% penicillin/streptomycin. Then half of the volume of medium was replaced with fresh maintenance medium every 3 or 4 days.
3.2.5 Cytotoxicity treatment with N-methyl-D-aspartic acid (NMDA)

Mature cortical neurons of 10 days in vitro (DIV) were used for experiments. NMDA and Glycine (Sigma, Saint Louis, MO) were dissolved in double distilled water to 50mM and 20mM, respectively. The solutions were filtered through a 0.45μm membrane filter (Millipore, Billerica, MA). Then NMDA and Glycine were added to the culture medium at 1:1000 to reach final concentrations of 50μM and 20μM, respectively. After incubation at 37°C for 1h, NMDA and Glycine were removed by changing the medium. Control neurons (without NMDA/Glycine treatment) were cultured under the same conditions as experimental samples. 200μM Benzyloxycarbonyl-Val–Ala–Asp fluoromethylketone (Z-VAD-fmk), a caspase inhibitor, (R&D Systems, Minneapolis, MN) was added to culture medium 1 hour before NMDA treatment.

3.2.6 Peptide treatment in cultured cells

After NMDA treatment, the synthetic peptides were added to the culture medium at concentrations of 2.5μM, 5μM and 10μM. The cells were returned to the incubator for an additional 24 hours before processing.

3.2.7 Protein extraction

Cells were washed with cold PBS three times and then lysed with 2x sample buffer on ice. The 2x sample buffer was made with: 62.5mM Tris-HCl (pH 6.8 at 25°C), 25% Glycerol, 2% sodium dodecyl sulfate (SDS), 0.01% Bromophenol blue and 5% β-mercaptoethanol.
For the 6 well plates, 100-150 μl sample buffer was used for each well. Then the cell lysate was boiled at 100°C for 5-10 minutes to denature proteins in the sample and finally stored at -80°C.

3.2.8 Dc protein assay

Protein concentrations of cell lysate were determined using a Dc protein assay protocol (BioRad) protocol. Bovine serum albumin (BSA) was dissolving in the sample buffer at concentrations of 0.3125mg/ml, 0.625mg/ml, 1.25mg/ml, 2.5mg/ml or 5mg/ml as a linear standard curve. The cell lysate was incubated with reagents in the 96-well plate according to the manufacturer’s instructions. Then the optical density of each sample was measured at 570nm absorbance using a “uQuant” microplate spectrophotometer (Bio-Tek Instruments, USA).

3.2.9 Western immunoblotting

40μg of protein for each sample was analyzed with 10% SDS-polyacrylamide resolving gels and 5% stacking gels using a Bio-Rad Gel electrophoresis system (Bio-Rad, Hercules, CA). PageRulerTM Plus Prestained Protein ladder (Fermentas, CA) was used to determine protein molecular weight. The Bio-Rad Wet Transfer system (Bio-Rad, Hercules, CA) was then applied to transfer proteins from gels to nitrocellulose membranes (Bio-Rad, Hercules, CA) by running at 100 volts for 2 hours in a cold room
(4°C). The membrane was blocked with 5% non-fat milk in Tris buffered saline tween 20 buffer (TBST) for 1 hour and then probed with primary antibodies diluted in 3% BSA/TBST at 4°C overnight. The primary antibodies used included: rabbit polyclonal Actin antibody (1:1000, Cell Signaling, Danvers, MA); rabbit polyclonal TDP-43 antibody (1:1000; ProteinTech, Chicago, IL); rabbit polyclonal caspase-3 antibody (1:1000; Cell Signaling, Danvers, MA); and rabbit polyclonal PARP antibody (1:1000; Cell Signaling, Danvers, MA). Then the membrane was washed three times with TBST for 15min and incubated with HRP-conjugated goat anti- rabbit secondary antibody (1:1000; Cell Signaling, Danvers, MA) for 1 hour at room temperature. The membrane was then washed three times for 15 min and results were visualized using an enhanced chemiluminescence reaction assay (PerkinElmer Life Sciences). The software, Image J (NIH), was used to calculate and normalize the intensity of protein against actin control for each sample.

3.2.10 Lactate dehydrogenase (LDH) assay for cell viability

The viability of cultured cells was assessed using the Lactate dehydrogenase (LDH) assay (In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based; Sigma, Saint Louis, MO). The culture medium was incubated with reagents for 1 hour in the 96-well plate according to the manufacturer’s instructions. The optical density of each sample was measured at 490 nm absorbance with a reference filter of 750nm using the “uQuant” microplate spectrophotometer.
3.3 Results

3.3.1 TDP-43 protein binds with caspase-3 on the membrane

The protein array was used to identify the binding regions between TDP-43 and caspase-3. Four binding regions were found on the caspase-3 membrane (Figure 3.1). Probing using TDP-43 protein and membrane with overlapping caspase-3 peptide attached revealed several possible interaction domains. According to these possible interaction domains, four peptide candidates were designed and synthesized (CAS 1-4). The peptides were derived from the sequence of full length caspase-3. Thus we thought it! possible that they could interfere with the interaction between TDP-43 and caspase-3 by acting as decoys for the TDP-43-caspase binding. Scrambled peptides were designed as controls.

3.3.2 The synthetic peptides could block the interaction between TDP-43 protein and caspase-3 membrane

Four peptides were tested for their ability to inhibit the binding between the TDP-43 protein and the caspase-3 membrane (Figure 3.2). The results showed that the mixture of all four peptides (CAS 1-4) blocked the interaction (Figure 3.3); while no single peptide or scrambled peptide did affect the interaction.

3.3.3 The peptides prevent TDP-43 but not PARP cleavage.

To investigate whether the synthetic peptides could inhibit the interaction between
TDP-43 and caspase-3 in brain cells, we used NMDA treated neurons as our model. Excitotoxic neuronal damage caused by overactivation of NMDA receptors is thought to be a major cause of neuronal loss in many acute and chronic pathologic conditions (Lipton and Rosenberg 1994). Activation of caspase-3 has been implicated in NMDA-induced neuronal apoptosis (Okamoto, Li et al. 2002). In our model, caspase-3 was activated after one hour NMDA (50μM) and Glycine (10μM) treatment in primary cultured neurons (DIV 10) (Figure 3.4A). Full length TDP-43 was cleaved to 35kDa fragment. We did not observe 25kDa fragment of TDP-43. After treatment with the synthetic peptides (5μM), TDP-43 cleavage was reduced (Figure 3.4B). After NMDA treatment, 22.0% of full length TDP-43 was cleaved to 35kDa via the activation of caspase-3. CAS 1, CAS 2, CAS 4 and the CAS 1-4 mixture decreased the percentage of cleaved TDP-43 to 5.0%, 8.9%, 7.2% and 9.0%. CAS 3 and the scrambled peptide did not reduce cell death significantly (Figure 3.5). In addition, the synthetic peptides did not block the cleavage of Poly ADP-ribose polymerase (PARP) by caspase-3 (Figure 3.4C and 3.6). As expected, Z-VAD reduced the cleavage of both TDP-43 (93.6%) and PARP (34.7%) by caspase-3 but did not block the activation of caspase-3.

3.3.4 The peptides inhibited the cleavage of TDP-43 in concentration-dependent manner.

TDP-43 cleavage was reduced in a concentration-dependent manner when the CAS 1-4 mixture was added to the culture medium after one hour NMDA treatment. 25.1% of full
length TDP-43 was cleaved to 35kDa. 11.7%, 7.5% and 2.8% of full length TDP-43 was cleaved when the cells were treated with progressively higher concentrations (2.5μM, 5μM and 10μM) of the CAS 1-4 mixture, while scrambled peptide did not reduce TDP-43 cleavage of all (Figure 3.7).

3.3.5 The peptides rescued neurons from cell death.

To ask whether blocking TDP-43 cleavage could rescue cell death, we used the LDH assay to examine cell viability. Compared with control samples, the CAS 1-4 peptide mixture, CAS1, and CAS2, decreased cell death efficiently. 5μM and 10μM of the CAS 1-4 mixture caused a 25.6% and 52.7% reduction of cell death respectively. 5μM and 10μM of CAS 1 caused 78.8% and 88.8% reduction of cell death. 5μM and 10μM CAS 2 caused 51.1% and 77.4% reduction of cell death (Figure 3.8). However, administration of CAS 3, CAS 4 or scrambled peptide showed increased cell death. This may have been due to peptide toxicity.
3.4 Discussion

In this study, we first identified peptides that potentially block the interaction between TDP-43 and caspase-3. Then we tested the effects of the peptides on the membrane and in neuronal in vitro models. To see whether they could interfere with binding and cleavage of TDP-43 by caspase-3, we found that the synthetic peptides could block the interaction between TDP-43 and caspase-3 on the membrane. Second, the peptides reduced the cleavage of TDP-43, but not PARP, by activated caspase-3, suggesting the peptides specifically bind with TDP-43 and block the interaction between TDP-43 and caspase-3. Finally, the peptides reduced NMDA induced cell death. This suggests the cleavage of TDP-43 is a prerequisite for the neurotoxicity associated with NMDA treatment.

Caspase-mediated apoptotic cell death is a signature feature of many neurodegenerative diseases, including as Alzheimer’s disease, Parkinson’s disease and FTLD (Friedlander 2003). Many researches have focused on developing caspase inhibitors as therapeutic agents (Yuan, Lipinski et al. 2003; Emamaullee, Davis et al. 2010). For instance, overexpression of caspase-3 inhibitory proteins, such as neuronal apoptosis inhibitory protein (NAIP) and X chromosome-linked inhibitor of apoptosis protein (XIAP), have been shown to reduce ischemia-induced apoptosis of hippocampal neurons (Xu, Crocker et al. 1997; Katz, Lotocki et al. 2001). In addition, peptide inhibitors, such as Z-VAD-fluoromethylketone (FMK), have also been shown to reduce apoptosis (Noorden 2001). Though pan caspase inhibitors can arrest apoptosis, they may also create
unanticipated side effects, such as promoting autophagic cell death (Yu, Alva et al. 2004). Since pharmacological suppression of caspase-3 is not an appropriate therapeutic method for FTLD-U and ALS, selective inhibition of the interaction between TDP-43 and caspase-3 may possibly represent a novel therapeutic strategy.

Kanthasamy and colleagues developed a peptide inhibitor targeting the caspase-3 cleavage site of PKCdelta which is related to Parkinson’s disease (Kanthasamy, Anantharam et al. 2006). The peptide inhibitor specifically blocked the cleavage of PKCdelta rather than other caspase-3 substrates and protected cells from neuronal cell death induced by MPP⁺ and 6-OHDA.

Cleavage of TDP-43 to 35 and 25kDa fragments is one of the hallmarks of ALS and FTLD-U. There are three potential caspase-3 cleavage consensus sites within the TDP-43 sequence (Zhang, Xu et al. 2007). This was confirmed in Hela and H4 cells. In our data, we found the similar results in primary culture neurons. TDP-43 was cleaved to 35kDa by activated caspase-3, thereby recapitulating some features of TDP-43 pathology in FTD and ALS. However, we did not see the 25kDa fragment of TDP-43. Previous data in our lab also showed only one cleavage fragment of TDP-43 (35kDa) in NMDA treated neurons (Guo, Tapia et al. 2010).

The caspase-cleavage product of TDP-43 (25kDa) but not full length TDP-43 has been
shown to cause cellular toxicity in cell cultures. One group used a yeast model transformed with TDP-43 fragments and found that a TDP-43 fragment containing both C terminal and RRM2 (similar to 25kDa TDP-43) caused toxicity in cells (Johnson, McCaffery et al. 2008). Zhang and colleagues also demonstrated caspase-3 cleavage is a prerequisite for TDP-43 translocation. In their studies, H4 cells transfected with wild type TDP-43 showed increased 35kDa fragment with staurosporine treatment and cytoplasmic translocation. When caspase cleavage sites were mutated, TDP-43 was resistant to cleavage and stayed in the nucleus with staurosporine treatment (Zhang, Xu et al. 2007). In another paper, HEK293 cells were transfected with 35 and 25kDa TDP-43 fragments corresponding to the caspase-3 cleavage products. Both the 35 and 25kDa TDP-43 fragments formed phosphorylated inclusions in the cells and the 25kDa TDP-43 fragment caused cell toxicity. In addition, inhibition of TDP-43 phosphorylation did not prevent the formation of inclusions (Zhang, Xu et al. 2009). These data strongly suggest that cleavage plays an important role in TDP-43 redistribution from nucleus to cytoplasm, hyperphosphorylation, and cell toxicity. Therefore, TDP-43 cleavage is a possible pharmacological target to prevent neurodegenerative diseases.

TDP-43 has multiple functions in transcriptional repression, pre-mRNA splicing and translational regulation. Caspase-3 has been found to be necessary for normal brain development and apoptosis. So inhibition all the activities of TDP-43 or caspase-3 may disrupt their normal functions in brain. Therefore, the interaction between TDP-43 and
caspase-3 may be an attractive neuroprotective therapeutic target. Our peptides were able to suppress TDP-43 cleavage by activated caspase-3 and prevent NMDA induced cell death.

The question is whether these peptides block the cleavage of TDP-43 specifically. To investigate this question, we used PARP as control. PARP is a well established caspase-3 substrate. Our peptides did not prevent PARP cleavage by activated caspase-3. This suggests these peptides can block TDP-43 cleavage specifically. In conclusion, the peptides’ ability to block interaction between TDP-43 and caspase-3 is a novel target for development of a neuroprotective therapeutic strategy for FTLD and ALS.
Figure 3.1. Identification of peptide candidates that may block interaction between TDP-43 and caspase-3 using the high density peptide array. TDP-43 protein associates with caspase-3 membrane on specific regions. A, antibody only; B, Membrane with overlapping caspase-3 peptides attached was incubated with TDP-43 protein (three repeats). Four peptide candidates (Peptide CAS1-4) were designed according to the binding regions.
Caspase-3

Figure 3.2. Identify the peptides candidates that may block interaction between TDP-43 and caspase-3.
**Figure 3.3.** Validated peptide candidates that may block interaction between TDP-43 and caspase-3 using the high density peptide array. Peptides inhibit the binding between TDP-43 protein and caspase-3 membrane. Membranes were incubated with A, TDP-43 protein; B, TDP-43 protein and peptide CAS 1-4 mixture; C, TDP-43 protein and scrambled peptide. These data showed that these peptides could inhibit the binding of TDP-43 and caspase-3.
**Figure 3.4.** The peptides prevented TDP-43 but not PARP cleavage induced by NMDA treatment. A, Caspase-3 was activated with NMDA treatment; B, TDP-43 was cleaved to 35kDa fragment with NMDA treatment. Peptide CAS1, CAS2 or CAS1-4 mixture reduced TDP-43 cleavage sharply; C, PARP was cleaved with NMDA treatment. Peptides did not prevent the cleavage; D, Actin was used as control.
Figure 3.5. TDP-43 was cleaved by NMDA induced caspase-3 activation. CAS 1, CAS 2, CAS 4 and mixture can inhibit TDP-43 cleavage more efficiently than CAS 3 and scrambled peptide (*, p<0.05; **, p<0.01, one-way ANOVA).
Figure 3.6. PARP was cleaved by NMDA induced caspase-3 activation. The peptides did not prevent caspase-3 mediated PARP cleavage (*, p<0.05; **, p<0.01, one-way ANOVA).
Figure 3.7. The peptides prevented TDP-43 cleavage in a concentration-dependent manner (*, p<0.05; **, p<0.01, one-way ANOVA).
Figure 3.8. Identification of cell death by LDH assay. CAS 1, CAS 2 and CAS 1-4 mixture rescue cell death more efficiently than other peptides. Cell death percentage was normalized to NMDA treated neurons (*, p<0.05; **, p<0.01, one-way ANOVA).
CHAPTER 4: CONCLUSIONS AND FUTURE PROSPECTS

As described in previous chapters, we used a high density peptide array technology to obtain insight into the mechanisms and treatment targets of FTLD and ALS. The results are summarized as follows:

1.

1) Using high density peptide array technology, interaction sites between TDP-43 and itself were identified.

2) Mutated TDP-43 (Δ187-192) can induce TDP-43 aggregation in Hela cells.

3) According to the interaction sites, the peptides were designed and blocked the aggregation of TDP-43 in concentration-dependent manner, but did not prevent cell death.

4) Our results suggest TDP-43 aggregation is not the cause of cell death in our model, but is instead associated with the process of cell death.

2.

1) Using high density peptide array technology, interaction sites between TDP-43 and caspase-3 were identified.

2) NMDA treatment in mouse cortical neurons induced TDP-43 cleavage by caspase-3 and cell death.
3) Peptides were designed according to the interaction sites, and blocked TDP-43 cleavage and rescued NMDA induced neuronal cell death.

We also used the high density peptide array technology to find interaction sites between TDP-43 and progranulin (PGRN). One of the major causes of FTLD was recently shown to be mutations in PGRN gene. PGRN has wide-ranging functions in the peripheral and central nervous system. The biological functions of PGRN are also mediated by protein-protein interactions. For example, PGRN binds with secretory leukocyte protease inhibitor (SLPI) and inhibits the elastase-mediated proteolysis of PGRN (Zhu, Nathan et al. 2002). The links between PGRN and TDP-43 remains unclear at this point. However, it was demonstrated that reduction of PGRN expression enhanced TDP-43 cleavage in Hela and H4 cells (Zhang, Xu et al. 2007) and translocation in mouse neurons (Guo, Tapia et al. 2010). So we hypothesize that PGRN somehow interacts with TDP-43, directly or indirectly, and mediates TDP-43 pathology.

We did not observe interaction between PGRN and TDP-43 by co-immunoprecipitation (Co-IP) (data not shown). However, using high density peptide array technology, interaction sites were found between TDP-43 and PGRN (data not shown). This may be because the protein used for high density peptide array was denatured or unfolded and then lead to nonspecific binding sites. Although this technology is a powerful tool for identification of protein-protein interaction, it still has some disadvantages.
For future studies, the peptides will be tested in vivo. Recently, several transgenic mice overexpressing mutant forms of human TDP-43 were generated, such as TDP-43 A315T and M337V (Wegorzewska, Bell et al. 2009; Stallings, Puttaparthi et al. 2010). The mouse models showed cortical and spinal motor neuron degeneration and accumulation of TDP-43 nuclear and cytoplasmic aggregation. In addition, the TDP-43 protein was shown to be cleaved to 35 and 25kDa fragments as in ALS and FTLD patients. Our caspase and TDP peptides were found to block TDP-43 cleavage in primary cultured mouse neurons. It will be very important to determine whether these peptides have the same functions in the transgenic mouse and if so, whether they are neuroprotective in that model. In addition, although the TDP-TDP aggregation blocking peptides were unable to rescue cell death, they do block aggregation caused by overexpressed mutated TDP-43. Applying the peptides in transgenic mouse may also decrease cell aggregation and help to reveal other aspects of TDP-43 pathology.
BIBLIOGRAPHY


Buratti, E., A. Brindisi, et al. (2005). "TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail: an important region for the inhibition of cystic fibrosis transmembrane conductance regulator exon 9..."


Davidson, Y., T. Kelley, et al. (2007). "Ubiquitinated pathological lesions in frontotemporal lobar degeneration contain the TAR DNA-binding protein,


with opposing activities on epithelial cell growth." _J Biol Chem_ **267**: 13073-13078.


