Effects of U1 Small Nuclear Ribonucleoprotein Inhibition on the Expression of Genes Involved in Alzheimer’s Disease

Wenbo Zhu,† Xuefei Wei,† Yanyang Wang, Jingjing Li, Lu Peng, Kui Zhang,* and Bing Bai*

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ABSTRACT: Deposition and dysfunction of U1 small nuclear ribonucleoprotein (snRNP) have been revealed in Alzheimer’s disease (AD), but whether U1 is involved in the amyloid precursor protein (APP) and Tau pathways remains unclear. Here, we investigate this by inhibiting the U1 components in cultured cells and examining the expression changes of AD-related genes to these two canonic pathways. We find that knockdown of U1-70K and U1C increases the protein expressions of APP and GSK-3β while reduces that of Nicastrin in a dose-dependent manner. Knockdown of U1A shows no effects on the expression of these proteins. The real-time PCR results show that the mRNA expression levels of APP, Nicastrin and GSK-3β are unchanged, decreased, and increased, respectively. In addition, U1-70K knockdown suppresses Tau phosphorylation and causes altered splicing of Tau exon 10. This study suggests that the effect of U1 snRNP knockdown is component-specific and more likely involved in APP deregulation in AD.

1. INTRODUCTION

We have found U1 small nuclear ribonucleoprotein (snRNP) alteration and RNA splicing deficiency in Alzheimer’s disease.1 U1 snRNP components (U1-70K, U1A, and snRNA) are depleted from nuclei and form cytoplasmic neurofibrillary tangle-like structures in neurons. Meanwhile, the pre-RNA premature cleavage and polyadenylation due to the insufficient amount of U1 was also detected in AD brains. It is notable that U1 proteopathy is widely present in AD brains and occurs early during the dementia progression.

AD is hallmarked by extracellular amyloid plaques and the intracellular neurofibrillary tangles in the brain,2 and their core components (Aβ and Tau) are key players in AD pathogenesis. Aβ is generated from APP (amyloid precursor protein) cleaved by β- and γ-secretases sequentially. The microtubule-associated protein Tau is hyperphosphorylated by GSK-3β and other potential kinases.3 The currently prevalent AD hypothesis posits that Aβ is the initiating factor, while Tau mediates this devastating disease.4 Whether U1 snRNP dysfunction is involved in APP and Tau regulations remains unclear.

Indeed, deregulation of RNA regulating proteins causes neurodegeneration.5 It is well known that TDP-43 proteopathy is a feature in frontotemporal lobar degeneration and amyotrophic lateral sclerosis, and its mutations are found in familial cases.6 Also, mutations in the gene SMN (survival motor neuron) causes degeneration of motor neurons in the spinal muscular atrophy.6 In addition, alterations in the U2 snRNA genes lead to ataxia in mice with extensive neurodegeneration in the cerebellum.6 Besides, the mutant hnRNP2B1 and hnRNP1 elicit neurodegenerative pheno-types in drosophila.7 Therefore, it is possible that U1 snRNP might similarly contribute to AD.

Actually, inhibition of U1 snRNP has been shown to increase the protein expression level of APP and Aβ.1a The chemical RNA splicing inhibitor Isoginkgetin also regulates the intracellular traffic of APP.10 In order to provide a better understanding of the involvement of U1 snRNP in the mechanism of AD, we here investigate whether the deregulation of U1 snRNP regulates protein and RNA expressions of genes involved in the canonical Aβ and Tau pathways.

2. MATERIALS AND METHODS

2.1. Cell Culture and Transfections. HEK293T cells were used in this study. The transfection of siRNAs was done with Lipofectamine RNAiMAX (13778-150, Life Technologies). The siRNAs were purchased from GenePharma (Shanghai, China), including: TGATAGGTCCGTA-CACCTC, ATTCTTTTGATAGGTCCGT, CGCTCGTGTTCGTACTCGA, and TGCTGTCGCCGCTCAATCT for U1-70K; CUGCAGUGGAAGGAAACACAA for U1C; GCUUUAAGAUCACGCA-
GAA for U1A; and UGGUUACAGUGUCAUA as the nontargeting control. The transfection of siRNAs was usually begun during cell subculture, and later, transfection of Tau441 plasmid (synthesized at GenScript, Nanjing, China) was done after 24 h.

2.2. Western Blot Analysis. Cells were lysed in the culture well with 2% SDS in 50 mM Tris buffer (pH 7.4) with 1X protease inhibitor cocktail (11697498001, Roche) to collect all nuclear U1 snRNP proteins. After protein concentration quantification by the bicinchoninic acid (BCA) assay, samples were separated by SDS-PAGE and then electrotransferred to a nitrocellulose membrane (0.2 μm pore size; 162-0112, Bio-Rad) followed by 5% nonfat milk block for about 15 min. The primary antibody in 3% BSA in 1X TBST was added to the membrane for 2 h at room temperature. After washing, the membrane was incubated with the HRP-conjugated secondary antibody in 2.5% milk. The signals were developed by the SuperSignal West Pico (34087, ThermoFisher Scientific) on films. Antibodies included: anti-APP (Y188, 1565-1, Epitomics), anti-U1-70K (ab51266, Abcam), anti-U1A (sc-376027, Santa Cruz Biotechnology), anti-U1C (sc-374428, Santa Cruz Biotechnology), anti-pTau (AT8, ThermoFisher Scientific), anti-total Tau (ab32057, Abcam), and anti-β-tubulin (ab6046, Abcam). The density of the blot bands were quantified by the freely available software Image J (https://imagej.nih.gov/ij/).

2.3. Tau Alternative Splicing Reporter and RNA Splicing Inhibition. The Tau exon 10 alternative splicing reporter plasmid was courteously provided by the group of Dr. Jane Y. Wu. The HEK293T cells were first transfected with U1-70K, U1A, or U1C siRNAs for 24 h. At this time point, protein expressions of these genes were usually suppressed by more than 70% in preliminary experiments. In the chemical RNA splicing inhibitor treatment, HEK293T cells were first treated by 33 μM Isoginkgetin (416154, Merck Millipore) for 12 h. After the U1 knockdown or the chemical inhibition, the Tau minigene construct prepared in lipofectamine 2000 (ThermoFisher Scientific) was added to the medium for 24 h before the enhanced green fluorescent protein (EGFP) expression was examined and the cells were harvested for qPCR.

2.4. Quantification of mRNA Levels by Reverse Transcription and Real-Time PCR. The total RNAs were extracted from the harvested cells with the RNeasy mini kit (QIAGEN) and synthesized into the first DNA strand by SuperScript III reverse transcriptase (ThermoFisher Scientific).

In the regular reverse transcription PCR, the primers used were U1-C (GGCAGTGGAAGGAAACACAAAG and CTGGTGAAGCAGAACAGGTAATA), U1-A (GCAGGGTTTCCCTTTATGA and TTGCAACAGCAGCAATTTGA), and Tau (TCCGACTCCACGCAAGT and TCAGGTCAACTGGTTTGTAGAC). The

![Figure 1. Effects of U1 knockdown on the protein expression of APP, Nicastrin, and GSK-3β. (A) Western blot to demonstrate the expression of these proteins under U1 knockdown. Antibodies used: APP, Y188. (B–D) Quantitative and correlation analyses of the protein expression levels of APP, Nicastrin, and GSK-3β under the knockdown of U1-70K, U1A, and U1C. Overexpression and knockdown were performed in HEK293T cells cultured in 24-well plates. Blank: no siRNA were added; control: scrambled siRNA. 1–4: four different siRNAs that target distinct sequences of U1-70K mRNA. Doses used for the titration: 10, 3, and 1 pmol, respectively, in which the mixture of four siRNAs was used for the U1-70K knockdown. Ctl: control. Three repeats of each treatments were used in a separate experiment for each protein in which siRNA 1 was used for U1-70K knockdown. Image J was used for quantification of the intensity of the Western blot bands. The dose effect data in (C) were from the quantitation results of blots in (A). Student’s t test was used for statistical analysis. The asterisk (*) indicates p < 0.05, and “N.S.” means insignificant.

![Image J](https://imagej.nih.gov/ij/)
3. RESULTS

3.1. Effect of U1 snRNP Knockdown on the Expression of AD-Related Proteins. The protein expressions of U1-70K, U1A, and U1C were all markedly inhibited after knockdown (Figure 1A). All four individual U1-70K siRNAs reduced its protein expression levels substantially and consistently increased protein expressions of APP and GSK-3β, while reducing the expression of Nicastrin. The knockdown of U1C caused a similar effect, but the U1A knockdown induced no obvious change. It was notable that the effect on the expression of APP, Nicastrin, and GSK-3β was dose-dependent in the U1-70K knockdown.

Quantitative analysis of the blots showed that the suppression of U1-70K protein expression reached about 70% in which the increase or decrease of APP, Nicastrin, and GSK-3β was 150, 70, and 120% approximately (Figure 1B). In the results of dose-dependent U1-70K knockdown, the protein expression levels of APP, Nicastrin, and GSK-3β were highly correlated with the levels of U1-70K, indicating a close regulation of U1 snRNP on the expression of these proteins (Figure 1C). Further quantitative analyses showed that U1C induced significant changes of the APP, Nicastrin, and GSK-3β protein levels, while U1A exerted no effects on any of them (Figure 1D).

3.2. The mRNA Expression Levels of APP, Nicastrin, and GSK-3β in U1-70K Knockdown. To determine whether the regulation of U1-70K on APP, Nicastrin, and GSK-3β was through transcription, we measured their mRNA levels by real-time PCR. The results showed that the mRNA level of U1-70K was reduced by about 90%, confirming the efficiency of knockdown (Figure 2). The data also showed that there were no significant changes in the APP mRNA levels during the U1-70K knockdown. However, the mRNA level of Nicastrin decreased and that of GSK-3β increased, respectively, consistent with their protein expression changes (Figure 1A).

3.3. The Effect of U1-70K Knockdown on the Phosphorylation of Microtubule-Associated Protein Tau (MAPT). In AD brains, U1-70K redistributes from the nuclei to the cytoplasm and forms tangle-like structures overlaying with the Tau neurofibrillary tangle in neurons.1a These Tau tangles are usually hyperphosphorylated, which might potentially contribute to the accelerated aggregation of Tau. To determine whether there is a possible relationship between these two events, we expressed Tau in the HEK293T cells, and meanwhile, knocked down U1-70K. However, the phosphorylation of Tau was not enhanced but reduced instead (Figure 3).

3.4. Effect of U1-70K Knockdown on Tau Alternative Splicing. Altered splicing of Tau exon 10 is involved in several neurodegenerative disorders and is sufficient to cause neuronal death and dementia.12 It has been reported that U1 and other U snRNPs can regulate the Tau exon 10 splicing.11 We therefore investigated whether the inhibition of the U1 protein components might regulate the alternative splicing of Tau exon 10 using a minigene reporter.11

In this cleverly designed minigene, an additional nucleotide is constructed to the end of exon 10, followed by exon 11 and an EGFP sequence that expresses a fluorescent green protein.
Therefore, when exon 10 is included in the splicing product, there will be a reading frame shift due to this additional nucleotide, resulting in no expression of EGFP. By looking at the expression change of green fluorescent signals, a splicing alteration event could be determined (Figure 4A).

We first used the established RNA splicing chemical Isoginkgetin as a positive control to see the effectiveness of this minigene reporter. Indeed, in the Isoginkgetin-treated cells, more green fluorescence was observed, indicating the validity of the experimental system (Figure 4A). We then knocked down U1-70K, U1C, and U1A and transfected these cells with this reporter plasmid and examined the EGFP expression change. However, there is no obvious difference in the expression of green fluorescence in the U1 knockdown cells and the control cells (Figure 4C).

To confirm these results, we collected these cells and examined their mRNA and protein expression by reverse transcription PCR and Western blotting in which both the mRNA and protein levels of U1-70K, U1C, and U1A were reduced after knockdown as expected (Figure 4D). Further analysis of the Tau minigene plasmid revealed the exclusion of exon 10 under the Isoginkgetin treatment, but this exclusion was not seen and no significant changes of exon 10 occurred in the knockdowns of these three U1 components, consistent with the previous EGFP expression results.

4. DISCUSSION

In this study, we examined the effect of U1 snRNP functional deficit on the expression of major proteins that are involved in the AD pathogenesis. We found that the knockdown of U1-70K and U1C increased protein expressions of APP and GSK-3β while reduced that of Nicastrin. However, these effects were not seen in the U1A knockdown. Further quantitative analyses reveal that the increasing or decreasing effects of U1-70K knockdown might be through transcriptional regulation on Nicastrin and GSK-3β, but not on the APP. GSK-3β is one of the major kinases that phosphorylate Tau, and it was increased during the U1-70K knockdown. However, the phosphorylation of Tau was not increased accordingly but decreased instead when U1-70K was knocked down. This suggests that the aggregation and functional impairment of U1 snRNP is less likely involved in the hyperphosphorylation of Tau in AD brains. Lastly, knockdown of U1-70K, U1C, or U1A exerted no effects on the alternative splicing of exon 10 of Tau.

The similar effect of U1C to that of U1-70K on APP and other AD-related proteins might be attributed to the direct regulation between them. The APP-increasing effect of U1-70K is not through the transcriptional enhancement but more likely due to the altered protein trafficking of APP, causing its intracellular accumulation. This is consistent with the observation of increased smearing of APP on the Western blot, a possible result from the altered glycosylation during deregulated ER-to-Golgi traffic. It is not known why similar effects were not seen in the knockdown of U1A, possibly because U1A might have distinct functions in the U1 snRNP complex. It is notable that the phenotypes and the molecular alterations are different in genetically manipulated U1A and U1-70K experimental models.

Knockdown of U1-70K and U1C inhibited the protein expression of Nicastrin. As Nicastrin is a component of the γ-secretase, its loss-of-function causes unbalanced Aβ production and thus leads to AD development. Therefore, U1 snRNP...
impairment in AD brains might contribute to the pathogenesis of this devastating disease.

The lack of accelerating effect of U1-70K knockdown on the phosphorylation of Tau is consistent with the fact that U1-70K depletion from the neuronal nucleus and the Tau hyperphosphorylation are not always coexistent in a neuron in AD brains. It is notable that we have only looked at Ser202 and Thr205, which are established hyperphosphorylated sites of Tau in AD. Whether the knockdown of U1-70K has an effect on phosphorylation of other important sites (such as Tyr18 and Thr231) might be an interesting question that requires further investigation.

In addition, the lack of effect of U1-70K, U1C, and U1A knockdown on Tau exon 10 splicing is consistent with the fact that there is no altered Tau exon 10 splicing in tangle-bearing neurons of the AD brains.

In summary, results of this study suggest the deregulation of U1 snRNP in AD might be more likely involved in the APP deregulation but not in Tau pathology, providing a better understanding of U1 dysfunction in the etiology of this devastating disease.

# AUTHOR INFORMATION

## Corresponding Authors

Kui Zhang — Department of Laboratory Medicine, Nanjing Drum Tower Hospital Clinical College of Nanjing Medical University, Nanjing, Jiangsu 210008, China; Department of Laboratory Medicine, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, China; Email: 13505151066@163.com

Bing Bai — Department of Laboratory Medicine, Nanjing Drum Tower Hospital, Department of Nuclear Medicine, Nanjing Drum Tower Hospital, and Center for Precision Medicine, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, China; Email: bb00004@outlook.com

## Authors

Wenbo Zhu — Department of Laboratory Medicine, Nanjing Drum Tower Hospital Clinical College of Nanjing Medical University, Nanjing, Jiangsu 210008, China; Department of Laboratory Medicine, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, China

Xuefei Wei — Department of Nuclear Medicine, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, China

Yanyang Wang — Department of Nuclear Medicine, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, China

Jingjing Li — Center for Precision Medicine, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, China

Lu Peng — Department of Laboratory Medicine, Affiliated Nanjing Brain Hospital, Nanjing Medical University, Nanjing, Jiangsu 210008, China

Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acsomega.0c03568

## Author Contributions

W.Z. and X.W. contributed equally.

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