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Overexpression of U1 snRNA induces decrease of U1 spliceosome function associated with Alzheimer’s disease

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ABSTRACT
We recently reported that presenilin-1 (PS1) induced an increase of U1 snRNA expression accompanied with the change of amyloid precursor protein expression, β-amyloid level and cell death. In the present study, our data showed that both overexpression and knockdown of U1 snRNA could cause the loss in the function of U1 snRNA and resulted in PCPA as well as the same downstream phenomena including the expression changes of genes specific to AD, tau hyperphosphorylation on the site of Thr212, the decrease of acetylated α-tubulin, the reduction of cell viability and upregulation of RIPK1, RIPK3 and caspase8. These findings not only helped researchers better understand the functions of U1 snRNA, but also paved the way to reveal the mechanisms of AD from a different point of view and may find a new therapeutic target for the disease.

Introduction
U1 small nuclear RNA (snRNA) is one of the most (about 10^6 copies per cell) abundant noncoding RNA in human cells. In human cells, the 164 nt U1 snRNA is synthesized in cell nucleus, and then retrieved other core spliceosome components in the cytoplasm, finally the U1 snRNA-protein complex returns to the nucleus (Guiro & O’Reilly, 2015). The snRNA-protein complex function in pre-messenger RNAs (pre-mRNAs) splicing events. In addition, studies over the past 20 years showed that U1 snRNA has diverse biological functions (Guiro & O’Reilly, 2015), particularly in the protection of pre-mRNAs from premature cleavage and polyadenylation (PCPA) at their 3′ poly-A sites (Berg et al., 2012).

Alzheimer’s disease (AD) is a chronic neurodegenerative disease. Although the main constituents of pathologic lesions of AD have been discovered (e.g. accumulation of Amyloid-beta (Aβ) proteins, hyperphosphorylated tau proteins and neuronal death), the pathogenesis of AD and the underlying mechanisms are still unclear. In recent several reports, RNA processing abnormalities were identified in several neurodegenerative diseases, including AD, amyotrophic lateral sclerosis (ALS) and possibly frontotemporal dementia (FTD) in a number of studies (Bossers et al., 2010; Mills & Janitz, 2012; Tsuji et al., 2013). Further study indicated that the accumulation of both U1 snRNA and U1 small nuclear ribonucleoproteins (snRNP) in AD brains has been detected (Bai et al., 2013; Hales et al., 2014). U1 snRNA aggregation was present in 100% of familial AD cases resulting from presenilin 1 (PS1) and amyloid precursor protein (APP) gene mutations (Hales et al., 2014). Recently, we discovered that the PS1 protein, encoded by PSEN1 gene, increased the expression level of U1 snRNA and then enhanced the expression level of Aβ40 level, which could cause AD-related tau cytoskeletal pathology and cell apoptosis (Cheng, Du, Shang, Zhang, & Zhang, 2017). However, the relationship between U1 snRNA overexpression and U1 snRNP loss of function and others key constituents of pathologic lesions in AD remain unknown.

In this study, we designed experiments using functional knockdown of U1 snRNA and the NR3C1 mini-gene to investigate the relationship between U1 snRNA overexpression and RNA processing abnormalities in SH-SY5Y cells and others constituents of pathologic lesions of AD (e.g. Aβ, hyperphosphorylated tau and cell death). To better understand the mechanistic basis for spliceosomal alterations in AD, we tried to determine the functional consequences of functional knockdown of U1 snRNA in a model of cell SH-SY5Y. A hypothesis has been raised that the disruption of U1 snRNA function induces AD-like pathology. Accordingly, U1 snRNA functional knockdown in SH-SY5Y cells was performed, the level of AD risk gene was determined, and then the main constituents of pathologic lesions of AD were detected.

Methods
Functional knockdown of U1 snRNA
For the functional knockdown of U1 snRNA, U1 antisense moroholino oligonucleotide (AMO) and its control
moroholino oligonucleotide (CMO) were used, which is a 25-mer scrambled sequence 5'-CCCTTACCTCAGTTAC AATTATA-3' (Kaida et al., 2010). The 25-mer U1 AMO is 5'-GGTATCTCCCCTGCCAGGTAAGTAT-3', which is reverse complementary to the first 25 nucleotides in human U1 snRNA (RefSeq: NR_004430.2). Since U1 snRNA functions rely on 12 of its first 25 nucleotides, the knockdown of U1 snRNA is named functional knockdown of U1 snRNA (Catterall et al., 1978). To detect PCPA, we constructed the NR3C1 mini-gene consisting of exon 2, intron 2 and exon 3 from its 5' end to 3' end based on a previous study (Kaida et al., 2010). DNA regions of the NR3C1 mini-gene were amplified using total DNA from SH-SY5Y cells using specific primers (Kaida et al., 2010). 3' RACE was used to detect the isoforms of the NR3C1 mini-gene. The cDNA synthesis was performed using 40-nt primer (CTGATCTAGAGGTACC GGATCCTTTTTTTTTTTTTTTT) with 3'-Full RACE Core Set with PrimeScript™ RTase (Takara, Ohtsu, Japan) and the cDNA was amplified using the specific forward primer and the XbaKpnBa reverse primer. The detailed information of primers used in this study was provided in Supplementary 1.

**Cell experiments**

Cells were cultured and transfected by electroporation using a Nucleofector with control or overexpression vectors to U1 snRNA at 4 μg for 8 h as previously described (Cheng et al., 2017). The cells were replaced seven days before transfection with DMEM medium containing only 3% FBS and all-trans-retinoic acid (10 μM). For each experiment, SH-SY5Y cells were sampled for six times (about 2 x 10^6 cells per sample), then trypsinized, washed once with PBS and resuspended in 100 μL Nucleofector Solution (Lonza-Amaxa, German). Three samples in the treatment group were transfected with U1 AMO. The other three samples in the control group were transfected with CMO. Oligo transfection into each sample was performed by electroporation using a Lonza-Amaxa Nucleofector Pulser with the program setting A-023 and then cultured in 12-well plates with 1.9 ml RPMI-1640 medium at 37°C for 8 h.

**In situ hybridization and qPCR**

**In situ** hybridization of U1 snRNA in cell was performed using 25 μm biotin-labeled LNA probes with the sequence 5'-CCCTTACCTCAGTTAC AATTATA-3' (Exiqon, Denmark). For each sample, 10 nM probes were added into hybridization buffer (including 50% v/v formamide, 2X SSC, 50 mM sodium phosphate with pH 7 and 10% dextran sulfate). The 15 μl hybridization buffer was added to each sample (covered individually by a plastic cover slip for hybridization buffer) in a humidified chamber at 55°C for 12 h. After hybridization, cells were washed using 2X SSC, incubated in 0.1% Triton for half an hour at 4°C, and then washed using 2X SSC for three times. A fluorescent Alexa Fluor 594 (red) streptavidin conjugate (Yeasen, China) was added to the cells to stain biotin-labeled LNA probes. Following staining, the cells were washed in 4X SSC with 0.1% Triton three times at 4°C, and washed in 2X SSC, 1X SSC and PBS subsequently at room temperature. Cellular nuclei in all samples were stained by DAPI (blue).

Total RNA was extracted from animal tissue or cultured cells using RNAiso plus (TaKaRa, Ohtsu, Japan). cDNA was synthesized using Mir-X™ miRNA First-Strand Synthesis Kit (Clontech, Palo Alto, CA, USA) following the manufacturer’s instructions. For each sample, 200 ng of cDNA product with specific primers (see Supplementary 1) was used for qPCR amplification. The qPCR was performed using SYBR® Premix Ex Taq™ II (TaKaRa, Ohtsu, Japan). All the genes were quantified relatively using U6 snRNA as internal control.

**ELISA, Western blot and immunofluorescence**

After cultured for 8 h, the cells were harvested and washed using PBS and lysed in lysis buffer on ice, and further centrifuged at 4°C for 15 min at 12,000 rpm. The cells were mixed with protein loading buffer and boiled at 100°C for 15 min. The ELISA assay was performed following the manufacturer’s procedure (Invitrogen, Carlsbad, CA, USA). The Western blot protocol was modified based on a previous study (Wang, Gao, Li, Yang, & Zhang, 2016). For each sample, 30 μg proteins were subjected to electrophoresis in 10% SDS – PAGE gel, and then the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Subsequently, the PVDF membranes were blocked in tris-buffered saline (TBS) including 5% no-fat milk powder at room temperature, cells were washed with PBS three times, and then the proteins were blocked in tris-buffered saline (TBS) including 5% no-fat milk powder at room temperature for 2 h. And then, they were incubated with primary antibodies and diluted in blocking buffer overnight at 4°C. After washing four times with TBST for 5 min each time and once with TBS for 10 min, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 40 min.

After transfected with U1 AMO or CMO, about 2 x 10^5 cells were seeded into each well of 6-well culture plates for 12 h. Following 30 min fixation in 4% paraformaldehyde at room temperature, cells were washed with PBS three times, incubated with 0.5% Triton X-100 and blocked with 2% BSA at 4°C for 2 h. Subsequently, the cells were incubated with human α-tubulin primary antibody at room temperature for 2 h. After washing with PBS three times, they were incubated with the Alexa 488 (green) conjugated goat anti-mouse IgG secondary antibody. Thereafter, cellular nuclei were stained by DAPI (blue). The detailed information of all antibodies used in the study was provided in Supplementary 1.

**Data and statistical analysis**

All data were presented as mean ± SD. The difference between two groups was tested by t-test using p values .05. Statistical computation and plotting were performed using the software R v2.15.3 with the Bioconductor packages (Gao, Ou, & Xiao, 2014).

**Results**

**Confirmation loss of U1 function**

First of all, 3’ RACE was performed to determine the U1 spliceosome function in the U1 snRNA overexpression cells.
PCPA was observed in U1 snRNA overexpression cells, however very little was seen in control cells, which could be due to destabilization of U1 spliceosome binding upon pre-mRNA inhibition (Figure 1(A)). This finding suggests that there is a loss of U1 function in U1 snRNA overexpression cells (Kaida et al., 2010).

To assess U1 snRNA loss of function in a cell model, we performed U1 snRNA functional knockdown in SY-SY5Y cells and sought to determine possible effects on cell. After SH-SY5Y cells were transfected with U1 AMO for 8 h, qPCR was performed to validate the knockdown of U1 snRNA. The data showed a dose-dependent decrease of U1 snRNA in U1 knockdown samples, indicating that U1 snRNA had successfully been knocked down in SH-SY5Y cells (Figure 1(B)). Using mini gene, PCPA was also observed in the cells which were transfected with 4 nmol U1 AMO (Figure 1(C)). In situ hybridization images (Figure 1(D)) showed that 4 nmol U1 AMO caused an almost complete loss in the observation of U1 snRNA transcripts. Therefore, 4 nmol U1 AMO was used in all the subsequent transfection experiments. Overexpression using U1-containing plasmids or functional knockdown with 4 nmol U1 AMO almost caused the loss in the pre-mRNAs splicing functions of U1 snRNA in SH-SY5Y cells.

**Functional knockdown of U1 snRNA regulates AD-specific genes**

In our previous study (Cheng et al., 2017), we proved that overexpression of U1 snRNA up-regulated APP and Aβ, which were commonly used as AD markers (Cheng et al., 2017). In this study, the qPCR experiment results showed that functional knockdown of U1 snRNA upregulated APP (Figure 2(A)) and the ELISA experiment results showed that...
Functional knockdown of U1 snRNA up-regulated endogenous Aβ40 in SH-SY5Y cells (Figure 2(B)). Furthermore, we proved that all of three isoforms (APP695, APP751 and APP770) in human APP (Rockenstein et al., 1995) were up-regulated by functional knockdown of U1 snRNA (Figure S1).

In the present study, we also proved that functional knockdown of U1 snRNA upregulated two main AD risk genes clu and apoe in SH-SY5Y cells (Figure 3(A,B)), which was consistent with the results using overexpression of U1 snRNA in the previous study (Cheng et al., 2017). Among the total 11 AD risk genes (Bertram, Lill, & Tanzi, 2010; Logue et al., 2011), 8 of them (PICALM, BIN1, GAB2, ABCA7, CD2AP, CD33 and HLA-DRB5) showed consistent changes, and 1 (MS4A6E) showed inconsistent changes in the expression levels (Figure 3(C)). In addition, the expression levels of two AD risk genes (SORL1 and EPHA1) did not change significantly due to functional knockdown of U1 snRNA (Figure 3(C)). In contrast, α-synuclein as a marker for Parkinson’s disease (PD) and TARDBP as a marker for Frontotemporal Lobar Degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) were measured, and the data showed that there were no changes in their expression levels (Figure 3(D)). These results suggested that both overexpression and functional knockdown of U1 snRNA induced tau hyperphosphorylation.

Another previous study demonstrated a strong decrease of acetylated α-tubulin expression and microtubule stability in AD (Hempen & Brion, 1996). In our previous study, we used double immunofluorescence staining to perform the partial co-localization of α-tubulin with the phosphorylated tau at Thr212 in SH-SY5Y cells. The results confirmed that α-tubulin was closely associated with tau immunoreactivity and also showed that U1 snRNA overexpression downregulated α-tubulin. In the study, we detected a significant decrease in the mRNA levels of α-tubulin caused by functional knockdown of U1 snRNA (Figure 4(C)). After SH-SY5Y cells were transfected with 4 nmol U1 AMO for 36 h, the morphological analysis showed that the staining intensity of bright fluorescent particles in U1 knockdown cells was visibly faded, suggesting that there was a damage of the

Functional knockdown of U1 snRNA caused AD-like cellular effects

Previous studies demonstrated that tau, as one of the major microtubule-associated proteins (MAPs), became hyperphosphorylated in nerve cells from AD brains due to an imbalance of multiple protein kinases and phosphatases (Ballard et al., 2011; Gunning, Ghoshdastider, Whitaker, Popp, & Robinson, 2015). The associations of the Alzheimer-hyperphosphorylated tau (AD P-tau) with the high molecular weight MAPs (HMW-MAPs) MAP1 and MAP2 resulted in an inhibition of MAP-promoted microtubule assembly (Alonso, Grundke-Iqbal, Li, & Iqbal, 1997).

In this study, we used Western blotting to detect changes on the phosphorylation level of tau induced by functional knockdown of U1 snRNA (Figure 4(A)). The results showed that the phosphorylation level on the site of Thr212 was increased without changes of tau protein expression level (Figure 4(B)), which was consistent with the results by overexpression of U1 snRNA in our previous study. This suggested that both overexpression and functional knockdown of U1 snRNA induced tau hyperphosphorylation.

Figure 3. Enhancement AD risk gene transcripts by U1 function deficiency. (A,B) U1 snRNA deficiency enhances the level of (A) clu and (B) apoe in SH-SY5Y cells transfected with 4 nmol U1 AMO for 8 h. The control group was transfected with CMO. (C) The mRNA level of AD risk genes were significantly altered in U1 functional knockdown cells. (D) No statistical difference of α-synuclein and TARDBP mRNA levels between controls and U1 functional knockdown cells. Data are presented as mean ± SD. *p < .05; **p < .01 as compared with that of the Con group.
microtubules in these cells (Figure 4(D)). This result was consistent with that from a previous study (Hempen & Brion, 1996).

A recent study proved that necroptosis, as a programmed form of necrosis, was executed by the mixed lineage kinase domain-like (MLKL) protein, which was triggered by receptor-interactive protein kinases 1 (RIPK1) and 3 (RIPK3) (Caccamo et al., 2017). Another study indicated that RIPK1 and RIPK3 were up-regulated by caspases 8 (Green, Oberst, Dillon, Weinlich, & Salvesen, 2011). In this study, the expression levels of RIPK1, RIPK3 and caspase8 were proved to be increased caused by functional knockdown of U1 snRNA using qPCR (Figure 5(A)). Cell viability was measured using MTT assays. The results showed that the viability of SH-SY5Y cells was significantly decreased by functional knockdown of U1 snRNA (Figure 5(B)). This suggested that U1 snRNA knockdown could result in cell death through RIPK-dependent necroptosis.

Discussion

In the study, we designed experiments using functional knockdown of U1 snRNA and the NR3C1 mini-gene to

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**Figure 4.** U1 snRNA deficiency to be associated with AD-related tau cytoskeletal pathology. (A,B) Western analyses on p-tau levels showed that there was a statistical difference in Thr212 phosphorylated site, however, there was no significant difference of the total tau levels. (C) qPCR analysis of α-tubulin transcripts was performed in the SH-SYSY cells upon U1 functional knockdown. (D) The morphological evaluation of acetylated α-tubulin was performed by immunofluorescence in U1 snRNA deficiency cells. Data are presented as mean ± SD. **p < .01; as compared with that of the Con group.

**Figure 5.** Death in U1 functional knockdown cells, and APP up-regulation upon splicing inhibition. (A) qPCR analysis of RIPK1, RIPK3 and caspase 8 transcripts was performed in the SH-SYSY cells upon U1 snRNA functional knockdown. (B) Effects of U1 snRNA deficiency were assessed by MTT assay on cell viability measured. Data are presented as mean ± SD. **p < .01 as compared with that of the Con group.
investigate the relationship between U1 snRNA expression levels, pre-mRNAs splicing, RNA processing abnormalities in AD and other possible constituents of pathologic lesions (e.g. PCPA). The main finding of the study is that both overexpression and knockdown of U1 snRNA caused the loss in the function of U1 snRNA and resulted in PCPA as well as the same downstream phenomena in SH-SY5Y cells. We hope these results could provide a novel clue for future studies in the function of U1 snRNA and in the pathology of AD or other neurodegenerative diseases.

Interestingly, our experimental results showed that both overexpression and knockdown of U1 snRNA caused the loss in the function of U1 snRNA and resulted in PCPA in SH-SY5Y cells. They could also cause AD-like pathology based on such downstream phenomena: (1) causing expression changes of genes specific to AD; (2) inducing tau hyperphosphorylation on the site of Thr212; (3) decreasing acetylated α-tubulin; (4) reducing cell viability; (5) upregulating RIPK1, RIPK3 and caspase8. Although we did not perform high-throughput sequencing of differentially expressed transcripts in the experiment, and there were no sufficient data to fully assess potential changes in telescripting in both the cell and animal models (Berg et al., 2012; Kaida et al., 2010), the detection of mRNA sequencing data revealed more different poly(A)-containing mRNAs reads in the 3' end of transcripts among U1 snRNA overexpression cells/U1 snRNA functional knockdown cells and control cells. Accordingly, these findings suggest that the partial loss of U1 spliceosome function in nerve cells results in enhancing incompletely spliced mRNAs in addition to altered RNA splicing. It may further highlight the importance of the U1 snRNA dysregulation to cause neurodegeneration in AD.

Our data also showed that U1 snRNA deficiency caused the increase of Aβ, hyperphosphorylated tau at Thr212 and necroptosis in SH-SY5Y cells. In the disease state of AD, the accumulation of Aβ and hyperphosphorylated tau at Thr212 could lead to: (1) neuronal death in brain; (2) a reduced affinity for microtubules and ineffective microtubule polymerization; (3) loss of microtubule rails; (4) tau insolubility, its self-aggregation and deposition in affected neurons (Alonso et al., 2008; Alonso Adel, Mederlyova, Novak, Grundke-Iqbal, & Iqbal, 2004; Beharry et al., 2014; Braak, Braak, & Mandelkow, 1994; Braak & Del Trecidi, 2015; Duyckaerts, Delatour, & Potier, 2009; Hitomi et al., 2004; Ryoo et al., 2007, 2008). Furthermore, we also demonstrate that there is a decrease of acetylated α-tubulin in U1 functional knockdown cells using immunofluorescence microscopy with fluorophores. It is consistent with the outcomes of a previous study, in which a strong decrease of acetylated α-tubulin in AD (Hempen & Brion, 1996). Therefore, the hyperphosphorylated tau at Thr212 induced by the U1 functional knockdown provided a possible pathway to trigger neurodegeneration in AD, in which disintegration of microtubule molecules could be caused by U1 dysfunction. Further evaluation of other tau phosphorylation sites in U1 functional knockdown cells still needs to be performed, since Aβ accumulation is known to act upstream of tau cytoskeletal pathology (Huang & Mucke, 2012; Querfurth & Laferla, 2010).

In summary, these results suggest that mammalian cells require U1 snRNA with expression levels in a restricted range for their normal biological processes and the loss in the regulation of U1 snRNA expression levels could cause diseases. Although it is easy to understand PCPA caused by knockdown of U1 snRNA, the mechanisms of PCPA caused by overexpression of U1 snRNA are still unclear. One hypothesis is that the overexpression of U1 snRNA induces a feedback to down-regulate U1 snRNA or U1 snRNP and finally result in PCPA. Another hypothesis is that the bound U1 snRNA on the mRNA strand requires a proper linear density for its functions and the space between U1 snRNAs determines alternative splicing types. Low density of U1 snRNA due to knockdown directly causes PCPA, while high density of U1 snRNA due to overexpression is prone to other alternative splicing types, so exon 2 and exon 3 in the NR3C1 mini-gene cannot splice together and then cause PCPA.

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Disclosure statement

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